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Effect of streptozotocin-induced diabetes on rat liver Na^+/K^+-ATPase

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Na^+/K^+-ATPase during diabetes may be regulated by synthesis of its α and β subunits and by changes in membrane fluidity and lipid composition. As these mechanisms were unknown in liver, we studied in rats the effect of streptozotocin-induced diabetes on liver Na^+/K^+-ATPase. We then evaluated whether fish oil treatment prevented the diabetes-induced changes. Diabetes mellitus induced an increased Na^+/K^+-ATPase activity and an enhanced expression of the β_1 subunit; there was no change in the amount of the α_1 and β_3 isoenzymes. Biphasic ouabain inhibition curves were obtained for diabetic groups indicating the presence of low and high affinity sites. No $\alpha_{2\ and}$ α_3 isoenzymes could be detected. Diabetes mellitus led to a decrease in membrane fluidity and a change in membrane lipid composition. The diabetes-induced changes are not prevented by fish oil treatment. The results suggest that the increase of Na^+/K^+-ATPase activity can be associated with the enhanced expression of the β_1 subunit in the diabetic state, but cannot be attributed to changes in membrane fluidity as typically this enzyme will increase in response to an enhancement of membrane fluidity. The presence of a high-affinity site for ouabain ($IC_{50} = 10^{-7}$ M) could be explained by the presence of $(\alpha\beta)_2$ diprotomeric structure of Na^+/K^+-ATPase or an as yet unknown α subunit isoform that may exist in diabetes mellitus. These stimulations might be related, in part, to the modification of fatty acid content during diabetes.

Keywords: Na^+/K^+-ATPase; isoforms; diabetes mellitus; dietary fish oil.

Na^+/K^+-ATPase is a ubiquitous plasma membrane-bound enzyme complex that plays a fundamental role in cellular function. The Na^+/K^+-ATPase is composed of two subunits, a catalytic α subunit and a glycosylated β subunit. At present, four different α-polypeptides (α_1, α_2, α_3 and α_4) and three distinct β isoforms (β_1, β_2 and β_3) have been identified in mammalian cells [1]. The α subunit contains the binding sites for the cations, ATP and the inhibitor, ouabain [2–4]. The β subunit is essential for the normal activity of the enzyme [5,6]. It has been postulated to be involved in translocation of α subunits across the endoplasmic reticulum, to stabilize and regulate conformation of α subunits and catalytic activity and to participate in mitogen activation of sodium pump function required for animal cell proliferation [6]. Rat hepatocytes have been shown to express the α_1, β_1 and β_3 isoforms in normal rat [7,8]. Hepatic Na^+/K^+-ATPase is known to be responsible for the Na^+ gradient and consequently for the bile acids/Na^+ secondary active transport across the plasma membrane [9].

Na^+/K^+-ATPase has been implicated in the development of complications and adaptive changes in diabetes [10,11]. In experimental diabetes, changes in Na^+/K^+-ATPase activity have been reported in the heart, peripheral nerve, kidney and intestine [12–19]. The magnitude and direction of the changes depend on the duration of diabetes and the organ involved. The physiological significance of these changes is not clear, although experimental diabetes is known to result in adaptive changes in the liver. A defect in liver microsomal fatty acid desaturation has been reported [20] and these changes may have some effect on microsomal membrane-bound enzymes. Diabetic impairment of Na^+/K^+-ATPase activity could be due to altered enzyme kinetics and/or altered subunit expression [12]. As the molecular mechanisms regulating the hepatic Na^+/K^+-ATPase under diabetes were unknown, we have studied the effects of streptozotocin-induced diabetes, a model of insulin deficiency, on Na^+/K^+-ATPase.

The activity of Na^+/K^+-ATPase may also be influenced by dietary components, such as fish oil supplements [14,15,21]. Fish oils contain the polyunsaturated fatty acids of the series *n*-3, the eicosapentaenoic (C20:5 *n*-3) and docosahexaenoic acids (C22:6 *n*-3). Eicosapentaenoic and docosahexaenoic acids can affect the fatty acid composition of the various membrane phospholipids and can modify the physical properties of cell membranes [21]. These fatty acids can have a potential beneficial effect in various diseases [22] and in diabetes [14]. The effect of fish oil treatment was therefore studied to determine whether this dietary treatment normalizes the changes that occur in Na^+/K^+-ATPase with drug-induced diabetes mellitus.

In this study, we documented in rats the effect of streptozotocin-induced diabetes on hepatic Na^+/K^+-ATPase enzyme activity and specific isoform expression. Ouabain affinity, membrane fluidity, and fatty acid content were also assessed. We then evaluated the effect of a fish oil (*n*-3 fatty acids) diet on the diabetes-induced changes.

MATERIALS AND METHODS

Animals

Five-week-old male Sprague–Dawley rats weighing approximately 200 g were randomly divided into four groups of six animals. In two groups, diabetes was induced by intravenous injection of streptozotocin (Sigma) at 60 $mg \cdot kg^{-1}$, diluted

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immediately before injection in citric acid buffer (10 mM, pH 5.5). One group of diabetic animals was fed standard rat chow diet supplemented with olive oil (Do) administered over 8 weeks at a daily dose of 0.5 $g \cdot kg^{-1}$ by gavage. The other group of diabetic animals (Dfo) was fed standard rat chow diet supplemented with (*n*-3) fatty acid-enriched fish oil concentrate (MaxEPA, Pierre Fabre Santé, Castres, France). This supplement is rich in eicosapentaenoic acids and docosahexenoic acids. Diabetic rats were not treated with insulin. The non-diabetic control groups were also fed the standard rat chow diet supplemented with olive oil (Co) or fish oil (Cfo). The rats were fed with olive oil or fish oil after induction of diabetes with streptozotocin. Olive oil was chosen as the placebo because it does not contain (*n*-3) fatty acids. Water was given *ad libitum* to all groups. All treatments adhered strictly to all institutional and national guidelines for animal use. Blood samples were collected regularly from the tip of the tail, and blood glucose was measured with a reagent strip (Reflolux®, Boehringer Mannheim). After 8 weeks, animals were killed by decapitation. On the day of killing, blood samples were taken for determination of plasma glucose by the peridochrome glucose method (Boehringer Mannheim).

Liver plasma membrane isolation

Livers were removed, rapidly rinsed with ice-cold physiological saline in less than 30 s, frozen in liquid nitrogen and stored at −80 °C until use. Frozen pieces of liver (300 mg) were homogenized directly in ice-cold buffer containing 8% saccharose, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA and 30 mM imidazol/HCl pH 7.4 at 25 °C with a polytron PT 10 (20 s, setting 5). The homogenate was subfractionated by three sequential differential centrifugations at 120 ***g*** for 5 min, 6800 ***g*** for 15 min and 48 380 ***g*** for 30 min using a JA.20 rotor in the Beckman J2.21 centrifuge (Beckman instruments, Gagny, France). The final pellet was resuspended in 8% saccharose and 30 mM imidazol/HCl pH 7.4 at 25 °C and stored at −80 °C until use. These preparations consisted of a membrane fraction highly enriched in Na^+/K^+-ATPase [23].

Enzyme activity measurements

Na^+/K^+-ATPase was determined using the coupled assay method as previously described [24]. The activity was measured in an ATP-regenerating medium by continuously recording NADH oxidation using a UNICAM 8700 spectrophotometer. Enzyme activities were measured at 37 °C. Each cell contained (final volume, 1 mL) 50 mM KCl, 20 mM $MgCl_2$, 500 mM NaCl, 10 mM phospho*enol*pyruvate, 20 mM ATP, 150 mM imidazol/HCl, pH 7.4, 2 mM NADH, 3.5 U pyruvate kinase and 5 U of lactate dehydrogenase. The enzymatic reaction was initiated by the addition of protein (10 μg). Enzyme activities are expressed as μmol $P_i \cdot h \cdot mg^{-1}$ of protein. Assays were performed in the presence and absence of 0.1–0.3 mg SDS per milligram of protein. This information was important as the Na^+/K^+-ATPase activity of native membranes depends on the proportion of permeable vesicles in which ouabain and ATP have free access to their sites of action. Protein content was determined by the method of Lowry *et al*. [25] using bovine serum albumin as a standard.

Dose–response curves were obtained by measuring the Na^+/K^+-ATPase activity in the presence of varying concentrations of ouabain, ranging from 10^{-9} to 3×10^{-4} M. Residual activity was calculated by comparing the activities in the presence and absence of ouabain after correcting for the ouabain-insensitive ATPase activity measured in the presence of 2 mM ouabain. The relative proportion of α_1 isoenzyme was inferred from ouabain affinities, as estimated from dose–response curves on permeabilized membranes with the highest Na^+/K^+-ATPase activity [26]. Curves were fitted to experimental data by a nonlinear regression model [24] using MKMODEL software (Biosoft, Cambridge, UK). The number of sites model used to fit the data was chosen according to the Schwarz criterion [27] and to the likelihood ratio chi-square test.

SDS/PAGE and Western Blots

Gel electrophoresis and Western blots were performed as previously described [14]. Polyclonal antibodies specific for rat α_1 (F), α_3 (TED) and β_3 (RNTβ_3) were provided by E. Feraille (Nephrology laboratory, Hôpital Cantonal, Geneva, Switzerland), by T. A. Pressley (Texas Tech Health Sciences Center, Lubbock, Texas, USA) and by K. Sweadner and E. Arystarkhova (Harvard Medical School, Charleston, MA, USA), respectively. An anti-(rat α_2) mAb (McB2) was provided by K. Sweadner. P. Martin-Vasallo (Universidad de la Laguna, Tenerife, Spain) provided polyclonal antibody specific for human β_1 and an anti-(rabbit β_1) mAb was purchased from UBI (Lake Placid, NY, USA). Membranes were incubated with peroxidase-conjugated anti-(rabbit IgG) Ig or anti-(mouse IgG) Ig (Amersham, les Ulis, France). Samples of lysate from rat brain and kidney used as controls were provided by T. A. Pressley (Texas Tech Health Sciences Center, Lubbock, Texas, USA). Samples from the four groups were always loaded on the same gel and transferred to the same blot for quantitative densitometry. At least three independent blots were analysed with reproducible results. The amount of protein in bands from the autoradiograms was quantitated with a scanning densitometer linked to a Macintosh computer using the National Institutes of Health Image Software (NIH, Bethesda, MD, USA) and expressed as arbitrary units.

RT-PCR

RNA extraction was performed according to the acid-guanidium thiocyanate/phenol/chloroform protocol [28]. Amounts of mRNA encoding rat and mouse Na^+/K^+-ATPase proteins were assayed using a quantitative multistandard RT-PCR method that takes advantage of both Na^+/K^+-ATPase (α_1 and β_1 isoforms) and β-actin sequence conservation between animal species [29]. This protocol allowed us to normalize the amounts of Na^+/K^+-ATPase mRNA with regard to the β-actin mRNA amount in each sample. Briefly, total RNA samples extracted from rat tissues were mixed with a constant amount of total RNA prepared from mouse liver, which contained both competitive rat β-actin and Na^+/K^+-ATPase sequences and thus acted as an multistandard source. All RNA preparations were incubated in the presence of RNase-free DNase (Promega). The mixture was reverse-transcribed using random hexamer primers. Separate PCRs for Na^+/K^+-ATPase α_1 isoform, β_1 isoform and for β-actin amplification were undertaken with oligonucleotide primers that hybridize with rat and mouse sequences with the same efficiency. For Na^+/K^+-ATPase α_1 isoform amplification, the direct primer, 'A-Icom dir' 5′-CCGAAGTGCTACAGAAGAG-GAACC-3′ and the reverse primer 'A-Icom rev' 5′-TGCT-GAGGGACCATGTTCTTGAAGG-3′ were used. For Na^+/K^+-ATPase β_1 isoform, the direct primer, 'β-1RS dir' 5′-CAT-CTTCATCGGGACCATCC-3′ and the reverse primer, 'β-1RS

rev' 5′-CCAATGTTCTCACCATACGCC-3′ were used. For β-actin amplification, the direct primer (ACT53) extended from nucleotide 2309 to nucleotide 2330 and the reverse primer (ACT33) from nucleotide 3089 to nucleotide 3063, according to the rat sequence [30]. Each amplification product was then distinguished by restriction site polymorphism: the mouse Na^+/K^+-ATPase α_1 isoform products were digested by *Bsp*1286 into two fragments (96 bp and 57 bp), while rat product remained uncut. A similar difference in pattern of restriction sites was used to differentiate Na^+/K^+-ATPase β_1 isoform products: a single *Bgl*II restriction site (producing 594-bp and 117-bp fragments) was present in mouse, but not in rat. Finally, a difference in *Pvu*II restriction sites used to distinguish β-actin products: a single *Pvu*II restriction site (producing 475-bp and 94-bp fragments) was present in rat, but not in mouse. Quantification of each amplification product was performed after electrophoresis and analysis of ethidium bromide-stained gels. In each rat sample, the ratio of actual moles of Na^+/K^+-ATPase mRNA (α_1 or β_1 isoform) versus β-actin mRNA (Na,K-r/β-r) is equal to R × c, where R is the ratio of mouse Na^+/K^+-ATPase product vs. rat Na^+/K^+-ATPase product, normalized with regard to β-actin, and c is the ratio of actual amounts of Na^+/K^+-ATPase mRNA vs. β-actin mRNA in the mouse multistandard liver preparation [29].

Membrane fluidity

The liver membranes were labelled with 1,6-diphenyl-1,3,5-hexatriene (Sigma), a fluorescent probe, by incubating equal volumes of a liver plasma membrane suspension containing 100 $\mu g \cdot mL^{-1}$ of protein in phosphate buffer (5 mM NaH_2PO_4, 5 mM KCl, 145 mM NaCl, pH 4.7) and 2 μM 1,6-diphenyl-1,3,5-hexatriene suspension in the same buffer. The 1,6-diphenyl-1,3,5-hexatriene suspension was prepared just before use by vigorous shaking from a 2 mM stock solution in dimethylformamide. Incubation lasted 30 min at 37 °C, with gentle stirring, in the dark. Fluorescence measurements were performed at 37 °C. Excitation and emission wavelengths were, respectively, 365 and 428 nm. Polarization (p) and anisotropy (r) measurements were carried out on a model SLM 4800 polarization spectrofluorometer as described previously [31].

Fatty acid composition

Membrane lipids were extracted with methanol and chloroform according to the method of Bligh and Dyer [32]. Fatty acid composition was determined after methylation with BF_3/methanol (Sigma) according to Ohta *et al*. [33]. The fatty acid methyl esters were quantified by gas chromatography (PerkinElmer AUTOSYSTEM XL) using a flame-ionization detector and a fused silica capillary (length, 60 m; internal diameter, 0.22 mm) column (BPX 70; SGE, Villeneuve St Georges, France). Assays were carried out with a programmed oven temperature rise of 1 $°C \cdot min^{-1}$ from 160 to 205 °C. Hydrogen was used as carrier gas under a pressure of 6×10^4 Pa. Fatty acids were identified by comparing their retention times on the column with respect to appropriate standards.

Statistical analysis

Statistical evaluation utilized an ANOVA procedure with Tukey test for multiple comparisons of normal distributions, and the Kruskal–Wallis ANOVA with Dunn's test for multiple comparisons of nonparametric distributions (SIGMASTAT Statistical Software). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Body weights and plasma glucose levels

The mean body weights were measured before intravenous streptozotocin injection and at the end of study (Table 1). After 8 weeks of streptozotocin-induced diabetes, body weight gain of diabetic rats [diabetic rats fed olive oils (Do) or fish oils (Dfo)] was greatly reduced compared with control rats [control rats fed olive oils (Co) or fish oils (Cfo)]. The plasma glucose levels of streptozotocin-induced diabetes rats were significantly elevated indicating that all rats treated with streptozotocin were diabetic. Daily supplementation with fish oil at 0.5 $g \cdot kg^{-1}$ had no effect on the hyperglycemia.

Na^+/K^+-ATPase activities

Figure 1 shows Na^+/K^+-ATPase activity in liver microsomal membranes from control (Co, Cfo) and diabetic rats (Do, Dfo). The enzyme activities were significantly increased by diabetes as compared to their respective control groups (Fig. 1). In streptozotocin-induced diabetic rats, the Na^+/K^+-ATPase activity increased by 89% and 60%. The changes were of similar relative magnitude in detergent-treated (data not shown) and in non-detergent-treated microsomal membranes. Dietary fish oil (*n*-3 fatty acids) treatment had no significant effect on Na^+/K^+-ATPase activity. The activity in Cfo and Dfo groups appeared higher than in Co and Do groups, respectively (Fig. 1), but the difference was not statistically significant.

Ouabain sensitivity

Isoforms of Na^+/K^+-ATPase in rat are known to differ dramatically in their sensitivity to inhibition by ouabain. Enzymes containing the α_2 and α_3 isoforms are about 1000 times more sensitive to glycosides than α_1. Na^+/K^+-ATPase activity in detergent-treated membranes was assayed at different concentrations of ouabain to estimate the proportion of isoenzymes present in rat liver. As shown in Fig. 2A, monophasic ouabain inhibition curves were obtained for control groups, Co and Cfo, with IC_{50} values of $5.4 \pm 0.09 \times 10^{-5}$ M and $7.7 \pm 0.1 \times 10^{-5}$ M, respectively. Diabetic groups with or

Table 1. Effects of streptozotocin-induced diabetes and dietary fish oil supplementation on body weight and plasma glucose levels. Co, control + olive oil; Cfo, control + fish oil; Do, diabetic + olive oil; and Dfo, diabetic + fish oil. Body weight was measured before intravenous injection of streptozotocin and olive or fish oil supplementation, and at the end of the study. Glycemia was measured at the end of the study. Values are mean ± SEM of six animals per group.

Groups	Body weight (g)		[Glucose] ($mmol \cdot L^{-1}$)
	Before	End	End
Co	213.7 ± 1.9	471.0 ± 11.9	9.2 ± 0.7
Cfo	210.4 ± 1.6	455.0 ± 10.0	7.5 ± 0.5
Do	207.9 ± 1.2	216.5 ± 11.5*	41.1 ± 1.1*
Dfo	210.7 ± 1.5	205.0 ± 11.2*	41.7 ± 1.1*

* Significant difference between diabetic and control groups ($P < 0.05$).

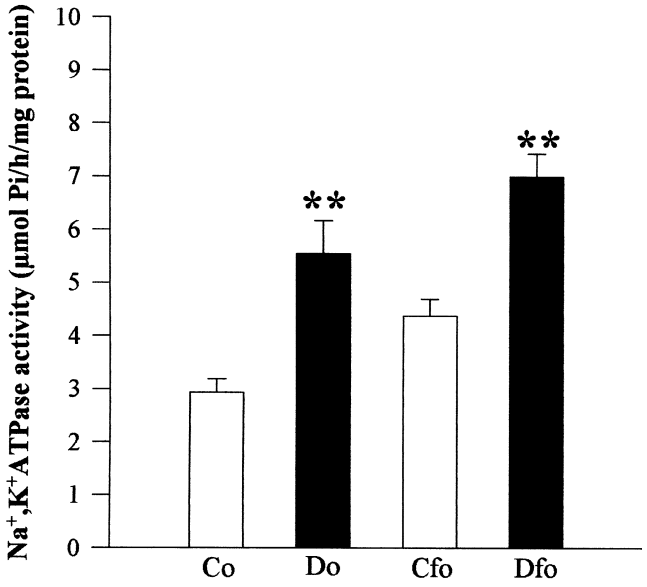

Fig. 1. Na^+/K^+-ATPase activities in liver membranes from control and streptozotocin-treated diabetic rats killed at 8 weeks after onset of diabetes. Co, control + olive oil; Cfo, control + fish oil; Do, diabetic + olive oil; Dfo, diabetic + fish oil. Na^+/K^+-ATPase was measured as the difference between ATPase activities in the absence and presence of 2×10^{-3} M ouabain. The data are presented as means ± SEM of activities performed in triplicate with six animals per group; ** indicates that Na^+/K^+-ATPase activity in diabetic groups (Do and Dfo) was significantly increased compared to their respective control groups ($P < 0.01$). The fish oil supplementation induced a nonsignificant increase in enzyme activity.

without fish oil treatment revealed dose–response curves that were biphasic and best modelled assuming two, rather than one, affinity constants according to the Schwarz criterion. Biphasic ouabain inhibition curves were obtained for diabetic groups (Fig. 2B), Do and Dfo, with IC_{50} of $1.3 \pm 0.04 \times 10^{-4}$ M, $3.9 \pm 0.11 \times 10^{-7}$ M and $8.6 \pm 0.17 \times 10^{-5}$ M, $1.6 \pm 0.03 \times 10^{-7}$ M, respectively.

Isoform expression

The immunodetection by Western blot analysis showed that the α_1, β_1 and β_3 subunits of Na^+/K^+-ATPase were expressed in all study groups. Reactivity of antibodies against rat brain and renal membranes was also examined to test their sensitivity and specificity. The polyclonal antibody against Na^+/K^+-ATPase α_1 subunit recognized bands (about 100 kDa) in the liver membranes (10 and 20 µg of protein were loaded). The β_1 subunit of Na^+/K^+-ATPase has been identified in most tissues, i.e. brain, heart, and kidney but its presence in liver has been difficult to establish [34,35] probably due to lower amounts of β_1 subunit relative to α_1 subunit [3,36,37]. Because polyclonal antibodies may recognize several isoforms, we next used an isoform-specific monoclonal against the β_1 subunit (UBI). Both polyclonal and mAb against β_1 subunit recognized bands of approximately 55 kDa (Fig. 3B) in the liver membranes (40 and 50 µg of protein were loaded). The rabbit polyclonal antibody against Na^+/K^+-ATPase β_3 subunit recognized bands of about 46 kDa (Fig. 4A) in the liver membranes (150 µg of protein were loaded). The immunodetection showed no significant effects of streptozotocin-induced diabetes on the abundance of α_1 (not shown) and β_3 isoforms (Fig. 4B), whereas the abundance of β_1 isoform showed significant increases in Do and Dfo, 3- and 3.5-fold respectively

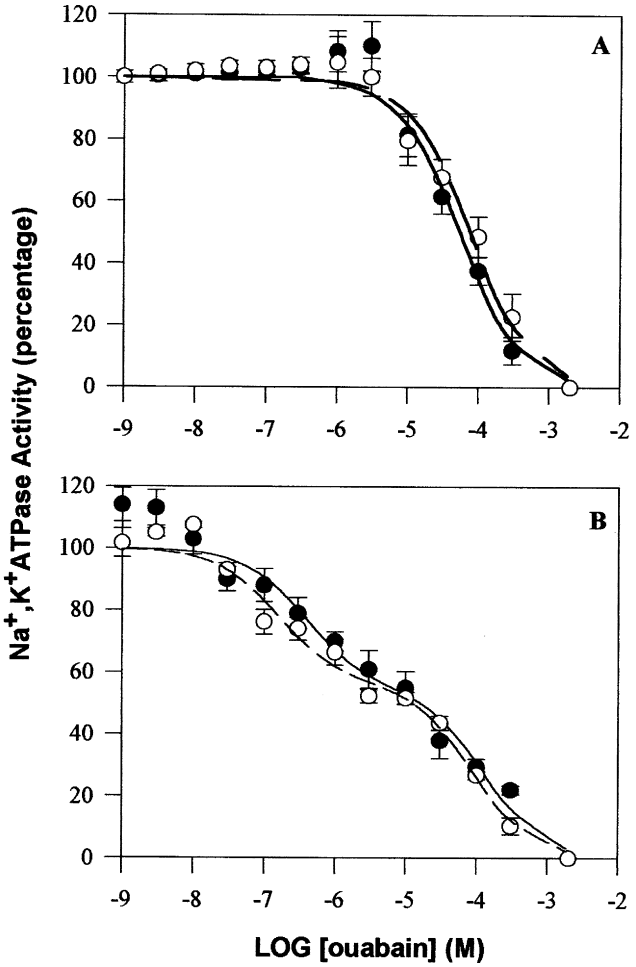

Fig. 2. Dose–response curves to ouabain of liver membrane Na^+/K^+-ATPase in control and diabetic rats with or without fish oil treatment. Different concentrations of ouabain, ranging from 10^{-9} to 2×10^{-3} M were analysed. (A) Two groups of six control animals (●, control + olive oil; ○, control + fish oil) and (B) two groups of six diabetic animals (●, diabetic + olive oil; ○, diabetic + fish oil). Na^+/K^+-ATPase activities are expressed as percentages. Each point is the mean ± SEM of six experiments. Data were analysed by a nonlinear regression model using MKMODEL software (Biosoft, Cambridge, UK). Solid and dotted lines represent the theoretical curves assuming a one- and two-site model fit, respectively. The number of sites model used to fit the data was chosen according to the Schwarz criterion. (B) The contribution of the low and high affinity Na^+/K^+-ATPase isoenzymes are 55 and 45% in Do group and 57 and 43% in Dfo group. Affinities for ouabain (IC_{50}) of the low and high affinity = $1.3 \pm 0.04 \times 10^{-4}$ M, $3.9 \pm 0.11 \times 10^{-7}$ M and $8.6 \pm 0.17 \times 10^{-5}$ M, $1.6 \pm 0.03 \times 10^{-7}$ M (Do and Dfo, respectively).

(Fig. 3C). Dietary fish oil treatment had no effect on the expression of α_1 (not shown) and β_3 isoforms (Fig. 4B), whereas the β_1 subunit levels in Cfo and Dfo groups appeared higher than in Co and Do groups, respectively (Fig. 3C) but the difference was not statistically significant. Immunoblots with α_2 and α_3 antibodies were performed from control and diabetic rats with or without fish oil treatment. The α_2 and α_3 isoforms were not detected in any group study, even when gels were loaded with large amounts (40 µg) of protein.

RT-PCR analysis

To determine whether the changes in Na^+/K^+-ATPase activity seen with diabetes were associated with changes in mRNA

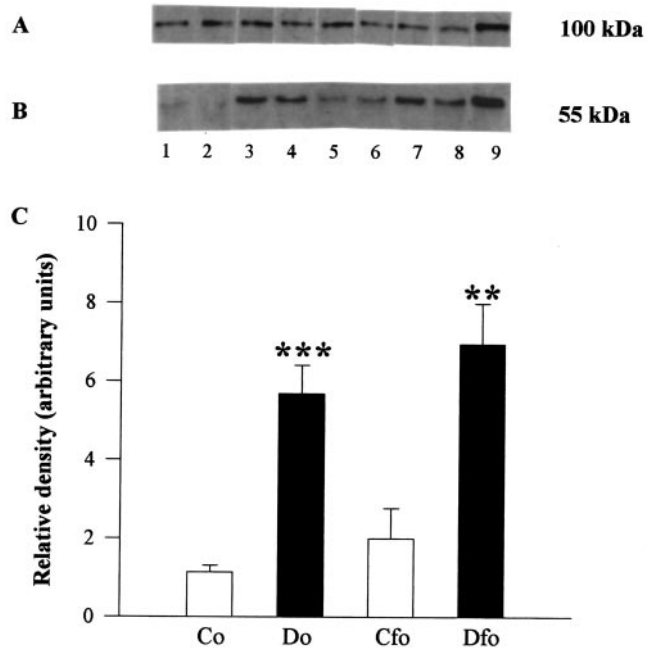

Fig. 3. Western blots of liver samples for α1 and β1 isoforms of Na$^+$/K$^+$-ATPase. Samples of liver membranes from six rats representative of the enzymological study of the four groups: control + olive oil (Co), lanes 1 and 2; diabetic + olive oil (Do), lanes 3 and 4; control + fish oil (Cfo), lanes 5 and 6; diabetic + fish oil (Dfo), lanes 7 and 8; and a sample of lysate from rat kidney, lane 9 were electrophoresed on 4–15% polyacrylamide gradient gel, then blotted onto nitrocellulose, probed with isoform specific anti-(rat α_1) polyclonal Ig (A) and anti-(rabbit β_1) mAb (B). The subunits were detected by the enhanced chemiluminescence method. Two concentrations of protein were used: 10 μg for lanes 1–8 (A), 40 μg for lanes 1–8 (B) and 10 μL for lane 9. (C) Mean values ± SEM ($n = 6$) are plotted for β_1 isoform. β_1 subunit expression is significantly higher in diabetic groups (Do and Dfo) than that in respective control groups. $^{**}P < 0.01$: Cfo vs. Dfo and $^{***}P < 0.001$: Co vs. Do.

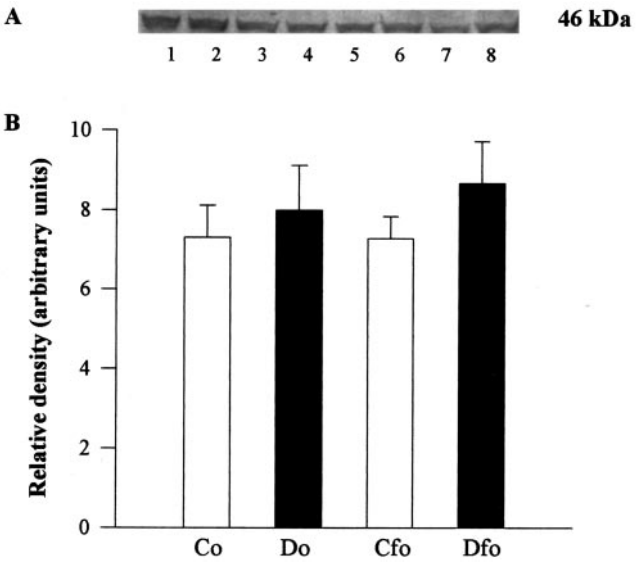

Fig. 4. Western blots of liver samples for the β$_3$ isoform of Na$^+$/K$^+$-ATPase. (A) Sample of liver membranes from six rats representative of the enzymological study of the four groups: control + olive oil (Co), lanes 1 and 5; control + fish oil (Cfo), lanes 2 and 6; diabetic + olive oil (Do), lanes 3 and 7; diabetic + fish oil (Dfo), lanes 4 and 8 were electrophoresed on 4–15% polyacrylamide gradient gel, then blotted onto cellulose, probed with rabbit polyclonal antibody RNTβ_3 and subunits detected by the enhanced chemiluminescence method. One concentration of protein was used: 150 μg for lanes 1–8. (B) Mean values ± SEM ($n = 6$) are plotted for β_3 isoform.

encoding the α and β subunits, RT-PCR was performed. Quantitation of the autoradiograms by densitometry revealed no significant changes in the amount of α_1 mRNA 8 weeks after the onset of diabetes, with or without fish oil treatment (data not shown). The amount of β_1 mRNA in diabetic rats (Do) and diabetic-treated rats (Dfo) was higher than in control rats (Co, Cfo) by 3- and 5.8-fold, respectively (Fig. 5). The amount of β_1 mRNA in Dfo group was 2.3-fold higher than that of Do group.

Table 2. Polarization and anisotropy measurements in liver membranes in control and streptozotocin-treated diabetic rats with or without fish oil supplementation. Co, control + olive oil; Cfo, control + fish oil; Do, diabetic + olive oil; and Dfo, diabetic + fish oil. Fluidity parameters were determined at 37 °C using 1,6-diphenyl-1,3,5-hexatriene as the probe. P, polarization; r, anisotropy. The data are means ± SEM of six animals.

Groups	P	r
Co	0.224 ± 0.006	0.162 ± 0.005
Cfo	0.230 ± 0.011	0.166 ± 0.004
Do	0.251 ± 0.004**	0.182 ± 0.003**
Dfo	0.245 ± 0.003	0.178 ± 0.003

** Significant difference between Do vs. Co ($P < 0.05$).

Membrane lipid fluidity

The fluidity parameters were determined at 37 °C using 1,6-diphenyl-1,3,5-hexatriene as the probe. The polarization (P) and anisotropy (r) values were higher in both diabetic groups

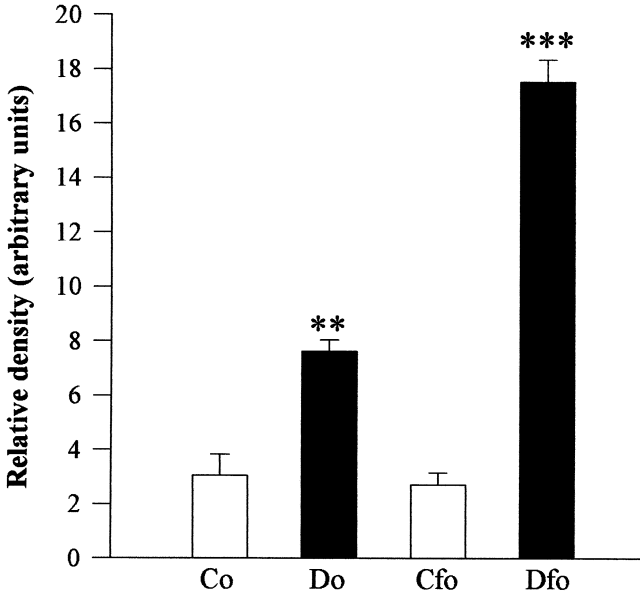

Fig. 5. Densitometric analysis in Co (control + olive oil), Cfo (control + fish oil), Do (diabetic + olive oil), and Dfo (diabetic + fish oil). Means ± SEM ($n = 6$) are shown. β_1 subunit mRNA amount is significantly higher in diabetic groups than that in respective control groups ($^{**}P < 0.01$: Co vs. Do; $^{***}P < 0.001$: Cfo vs. Dfo) and in Dfo than Do group ($^{***}P < 0.001$).

Table 3. Effects of streptozotocin-induced diabetes and fish oil supplementation on fatty acid composition of liver membranes. Co, control + olive oil; Cfo, control + fish oil; Do, diabetic + olive oil; and Dfo, diabetic + fish oil. Values represent the relative amounts, expressed as a percentage of the total identified fatty acids by weight. Values are means ± SEM of six animals. Values in the same lane not bearing the same superscript letters were significantly different at $P < 0.05$. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acids.

	Co	Cfo	Do	Dfo
16:1 (*n*-7)	1.52 ± 0.27[a]	0.87 ± 0.18[b]	0.34 ± 0.05[c]	0.64 ± 0.06[b]
18:0	17.8 ± 2.02	16.4 ± 0.39	19.6 ± 0.07	18.3 ± 0.33
18:1 (*n*-9)	13.1 ± 0.90[a]	9.29 ± 1.07[b]	7.39 ± 0.27[b]	8.84 ± 1.19[b]
18:2 (*n*-6)	15.3 ± 1.45[a]	19.9 ± 0.23[b]	23.0 ± 0.46[c]	20.8 ± 0.52[b]
18:3 (*n*-3)	0.60 ± 0.10	0.40 ± 0.04	0.43 ± 0.04	0.44 ± 0.03
20:1 (*n*-9)	0.78 ± 0.09[a]	0.68 ± 0.07[a]	0.36 ± 0.04[b]	0.59 ± 0.11[a]
20:3 (*n*-6)	1.41 ± 0.05	1.54 ± 0.03	1.33 ± 0.22	1.32 ± 0.12
20:4 (*n*-6)	17.7 ± 1.61[a]	19.0 ± 1.04[a]	8.78 ± 0.26[b]	9.33 ± 0.72[b]
20:5 (*n*-3)	1.34 ± 0.34[a]	1.75 ± 0.18[a]	0.59 ± 0.07[b]	1.17 ± 0.18[a]
22:5 (*n*-3)	2.29 ± 0.28	2.10 ± 0.13	1.11 ± 0.07	1.20 ± 0.07
22:6 (*n*-3)	7.72 ± 0.54[a]	7.75 ± 0.36[a]	16.8 ± 0.37[b]	15.6 ± 0.73[b]
Σ SFA	17.8 ± 2.02	16.4 ± 0.39	19.6 ± 0.07	18.3 ± 0.33
Σ MUFA	15.4 ± 1.16[a]	10.8 ± 1.27[b]	8.08 ± 0.27[b]	10.1 ± 1.21[b]
Σ PUFA	44.6 ± 4.25	53.6 ± 1.24	52.3 ± 0.97	50.2 ± 1.30

than in control groups (Table 2), indicating decreased fluidity of the membranes from diabetic groups. These results were significant only in the Do group relative to the Co group ($P = 0.01$).

Fatty acid composition of liver membranes

The composition of fatty acids in purified membranes from liver from rats having streptozotocin-induced diabetes with or without fish oil treatment was determined and compared with that of the nondiabetic groups (Table 3).

The effect of diabetes (Co vs. Do) in rats was a significant decrease in palmitoleic [16:1 (*n*-7)], oleic [18:1 (*n*-9)], arachidonic [20:4 (*n*-6)] and eicosapentaenoic [C20:5 (*n*-3)] acids, whereas the percentage of linoleic [C18:2 (*n*-6)] and docosahexenoic [C22:6 (*n*-3)] acids was increased. This effect was not significant on the level of saturated fatty acid, or polyunsaturated fatty acid amounts, but the total amount of monounsaturated fatty acids decreased significantly.

The main effect of fish oil supplements (Co vs. Cfo) in rats was a significant decrease in [16:1 (*n*-7)] and [18:1 (*n*-9)] fatty acids, whereas the percentage of [C18:2 (*n*-6)] fatty acid was significantly increased. This specific effect was not significant for the level of saturated fatty acid and polyunsaturated fatty acid amounts, but the total amount of monounsaturated fatty acids decreased significantly.

In diabetic animals (Do vs. Dfo), the [C18:2 (*n*-6)] fatty acid levels decreased after fish oil supplementation. There was no significant change in the percentages of oleic, arachidonic and docosahexenoic fatty acids, whereas the palmitoleic and eicosapentaenoic fatty acid levels increased significantly.

DISCUSSION

The purpose of this study was to examine the effect of streptozotocin-induced diabetes on liver Na^+/K^+-ATPase and to determine whether dietary fish oil treatment prevented the changes occurring during diabetes.

This study has shown that diabetes increased the Na^+/K^+-ATPase activity in liver membranes. This increased Na^+/K^+-ATPase activity could be due to changes in membrane lipid fluidity and/or protein content. Liver Na^+/K^+-ATPase activity has been reported to be sensitive to membrane fluidity [38]. This enzyme will increase activity in response to enhanced membrane fluidity, but this is not the case in our study. Polarization and anisotropy measurements on membranes from diabetic groups indicate that their lipid structure decreased in fluidity. The changes in membrane fluidity therefore cannot be responsible for the increase in Na^+/K^+-ATPase activity. An active Na^+/K^+-ATPase consists of two subunits, and rat hepatocytes express α_1, β_1 and β_3 isoforms [7,8]. The increased relative abundance of the β_1 subunit was associated with stable α_1 and β_3 isoenzyme expression. This study demonstrated that the main mechanism for increased Na^+/K^+-ATPase activity can be attributed to the β_1 subunit. Our results suggest that the liver β_1 isoenzyme is the rate-limiting subunit in the enzyme activity or that an overabundance of α_1 subunit exists in the liver. Consistent with this hypothesis, others have shown that the content of α mRNAs exceeds that of β mRNAs in hepatic tissue [34,36,39,40]. Furthermore, in response to a variety of stimuli that enhance Na^+/K^+-ATPase biosynthesis, the magnitude of the associated increments in β mRNA abundance often greatly exceed the relative increments in α mRNA abundance [41]. Lu and Leffert [42] reported that during partial hepatectomy, the β_1 isoform mRNA levels, initially undetectable, increase about eightfold, whereas α_1 isoform mRNA remains essentially unchanged. An increase (about threefold) in enzyme activity is also observed. Koch and Leffert [43] showed that the increased Na^+/K^+-ATPase mRNA β_1 isoform abundance preceded hepatic epithelial cell proliferation.

Only one isoform (α_1) of Na^+/K^+-ATPase has been detected previously in rat hepatocytes [7]. The present study shows the presence of the α_1 isoform in both control and diabetic liver membranes, as determined by immunoblot analysis. The observed ouabain inhibition curves indicated that only one isoform (presumably α_1) was present in both control groups, whereas two reactivities, possibly corresponding to two isozymes, were present in both diabetic groups. Isoforms of Na^+/K^+-ATPase in the rat are known to exhibit different affinities for ouabain. In the absence of α_2 and α_3 isoenzymes, this second site of high affinity for ouabain found in the diabetic groups could represent either a specific conformational state of α_1 or the presence of a novel isoenzyme. The possibility that $\alpha_1\beta_3$ represents the component of high affinity for ouabain is unlikely as the other β subunits do not influence the kinetics of the enzyme toward the cardiotonic steroids [1]. Moreover, the α_4 subunit has never been found outside the rat testis [44]. The biphasic sensitivity curves can also be explained by the possibility of an $(\alpha\beta)_2$ diprotomeric structure of Na^+/K^+-ATPase with two interacting ouabain sites of different affinity [45–47]. Therefore, the highly ouabain-sensitive Na^+/K^+-ATPase encountered probably corresponds to a novel α isoenzyme or an $(\alpha\beta)_2$ diprotomeric enzyme that may exist in diabetes mellitus. Future study will be required to explain this finding.

The physiological relevance of the increased Na^+/K^+-ATPase activity seen in diabetes in the liver, kidneys and small intestine has been related to Na^+-dependent solute transport [48]. The increased Na^+/K^+-ATPase activity and β_1 isoform abundance may be an adaptation to increased metabolic requirements related to Na^+ transport. In kidney

and nerve, changes in Na^+/K^+-ATPase activity seem to be related to changes in sorbitol and *myo*-inositol metabolism and hyperglycemia-induced activation of protein kinase C [17,49]. Wald *et al*. [18] described enhanced Na^+/K^+-ATPase activity in rat proximal tubule to reabsorb glucose via the Na^+-dependent glucose cotransporter.

During diabetes, Na^+/K^+-ATPase activity has been demonstrated to be particularly sensitive to the membrane fatty acid environment [50,51]. A defect in fatty acid desaturation is one of the complications associated with experimental diabetes [52,53]. The decrease in arachidonic acid [C20:4 (*n*-6)] and the increase in docosahexaenoic acid [C22:5 (*n*-3)] in the liver lipids of the diabetic rats have been previously reported [54]. These changes suggest an inhibition of Δ6 and Δ5 desaturase activity. Although the specific means by which membrane polyunsaturated fatty acids modulate Na^+/K^+-ATPase activity are not well understood, several mechanisms have been suggested. They include fatty acid-dependent effects on the conformational properties of the protein complex, or on enzyme abundance [55] by changes in the thickness of the phospholipid bilayer [56].

The purpose of this study was also to assess the ability of fish oil treatment to prevent diabetes-induced changes. The potential effects of fish oil could be to prevent the onset or to delay the progression of the disease as was reported in our previous study [15]. However, in the present investigation, we did not find that fish oil delayed the progression of diabetes.

In conclusion, we have shown that the modulation of hepatic Na^+/K^+-ATPase enzyme activity in diabetes is dependent on a specific upregulation of β_1 subunit abundance. These results confirm and extend the role of β_1 subunit in pump function [57]. The increase in enzyme activity might be related, in part, to the modification of fatty acid content during diabetes.

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