Diabetologia (1997) 40: 496–505

Diabetologia
© Springer-Verlag 1997

Originals

Alteration of Na,K-ATPase isoenzymes in diabetic cardiomyopathy: effect of dietary supplementation with fish oil (n-3 fatty acids) in rats

A. Gerbi[1], O. Barbey[2], D. Raccah[1], T. Coste[1], I. Jamme[3], A. Nouvelot[3], L. Ouafik[4], S. Lévy[2], P. Vague[1], J.-M. Maixent[2]

[1] Laboratoire de Diabetologie, Marseille, France
[2] Laboratoire de Recherches Cardiologiques, Faculté de Médecine, I.F.R. Jean Roche, Marseille, France
[3] Laboratoire de Neuroscience, CNRS URA 1829, Caen, France
[4] Laboratoire de Neuroendocrinologie Experimentale, INSERM U297, Faculté de Médecine, I.F.R. Jean Roche, Marseille, France

Summary Diabetic cardiomyopathy has been associated with a decrease in Na,K-ATPase activity and expression, as well as alterations in membrane lipid composition. The aim of this study was twofold; 1) to document in rats the effect of streptozotocin-induced diabetes on myocardial Na,K-ATPase and fatty acids, and 2) to evaluate the potential effect of a dietary supplementation with fish oil (n-3 fatty acids) on the streptozotocin-induced changes. Assays were performed in purified cardiac plasma membranes to determine Na,K-ATPase activity, expression of the different α- and β-subunits of Na,K-ATPase, and the fatty acid content of total phospholipids. Relative abundance of the mRNAs encoding the α1, α2 and β1 isoforms was studied by Northern blot analysis. Results demonstrated that diabetes significantly decreased activities of α1 and α2 isoforms and mRNA levels of α2 and β1 isoforms, and, at the protein level, increased α1-isoforms and decreased both α2- and β1-isoforms. Changes in fatty acid content of the membrane were consistent with inhibition of desaturase. Fish-oil supplementation produced an increase in membrane incorporation of eicosapentaenoic acid. It also increased the level of β1-isoforms and restored the activity of the α2-isoenzyme without significant changes in the level of α1- and α2-isoforms. Northern blot analysis showed no effect of fish oil supplementation. Experimental diabetes and prevention by the fish oil rich (n-3 fatty acids) diet induced specific effects on the activity and expression of α and β Na,K-ATPase subunit isoforms. These studies suggest that fish oil therapy may be effective in preventing some of the adverse consequences of diabetes. [Diabetologia (1997) 40: 496–505]

Keywords Diabetes mellitus, fish oil, Na,K-ATPase, isoform, mRNA, fatty acid.

Diabetes mellitus enhances the risk of morbidity and mortality from cardiovascular disease. Clinical and laboratory studies suggest that diabetic cardiac disease is related to the development of secondary cardiomyopathy independently of coronary artery disease [1, 2]. Diabetic cardiomyopathy has been associated with changes in enzymatic activities in the cardiac sarcolemma, with decreased Na,K-ATPase activity having been observed in the hearts of animals with experimentally induced diabetes [3–9]. The basis of this decrease in Na,K-ATPase activity is unclear.

Na,K-ATPase is a membrane-bound enzyme consisting of two subunits (α and β) and a large lipid core [10]. Since these subunits exist as multiple isoforms (α1, α2, α3, β1, β2) [11], diabetic impairment of Na,K-ATPase activity could be due to altered enzyme kinetics and/or altered subunit expression [7, 9]. The activity of membrane-bound enzymes may also be influenced by membrane environmental factors [12, 13] such as lipid content [14]. Alterations of membrane environment have been observed in various diseases [15–18]. There is also evidence to suggest

Received: 24 June 1996 and in revised form: 7 January 1997

Corresponding author: Dr. J.-M. Maixent, Laboratoire de Recherches Cardiologiques, Faculté de Médecine, I.F.R. Jean Roche, Bd Pierre Dramard, F-13015 Marseille, France

Abbreviations: STZ, Streptozotocin; EPA, eicosapentaenoic acid; DHA, docosahexenoic acid; PBS, phosphate buffered saline; SFA, saturated fatty acid; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids.

that membrane environment may have some effect on insulin sensitivity [19], insulin-stimulated glucose uptake [20], and Na,K-ATPase activity [18, 21].

Membrane environment can also be modified by diet [11–13] and a number of therapeutic diets are used [22]. Consumption of (n-3) polyunsaturated fatty acids, (e. g. fish oil concentrates) has been reported to lower the risk of cardiovascular disease [23, 24]. Beneficial effects of fish oil (n-3 fatty acids) supplementation include prevention of digitalis toxicity [25], correction of impaired heart performance in diabetic rats [26], and prevention of fatal ventricular fibrillation induced by ischaemia [27, 28].

In previous work, we demonstrated that the properties of Na,K-ATPase isoenzymes in the rat brain could be modified by administration of dietary fatty acids [12] and, in particular, by fish oil supplementation [13]. These effects have been attributed to changes in the fatty acid content of plasma membranes.

The aims of this study were twofold. We first documented the effect of streptozotocin (STZ)-induced diabetes on fatty acid composition and Na,K-ATPase activity in cardiac muscle membranes. Ouabain affinity and subunit expression were also assessed. We then evaluated the potential effect of a fish oil rich (n-3 fatty acids) diet on these STZ-induced changes.

Materials and methods

Animals. Four-week-old male Sprague-Dawley ($n = 30$) rats weighing approximately 200 g were randomly divided into three groups of 10. In two groups, diabetes was induced by intravenous injection of streptozotocin (STZ) at 60 mg/kg (STZ; Sigma, L'Isle d'Abeau, Chesne, France) diluted immediately before injection in citric acid buffer (0.01 mol/l, pH 5.5). In the control group only citric acid buffer was injected. One group of diabetic animals (DM) was fed the standard rat chow diet supplemented with (n-3) fatty acid-enriched fish oil concentrate (MaxEPA, Pierre Fabre Santé, Castres, France) administered over 8 weeks at a daily dose of 0.5 g/kg by gavage. This dose has previously been shown to affect cardiac function of n-3 fatty acid treated diabetic animals [26]. This supplement is rich in eicosapentaenoic acid (EPA, C20:5 (n-3)) and docosahexenoic acid (DHA, C22:6 (n-3)). The other group of diabetic animals (DO) was fed the standard rat chow diet supplemented with olive oil. Diabetic rats were not treated with insulin. The non-diabetic control group (CO) was also fed the standard rat chow diet supplemented with olive oil. 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 animal treatments adhered strictly to all institutional and national ethical guidelines.

Blood samples were collected regularly from the tip of the tail, and blood glucose was measured with a reagent strip (Reflolux, Boehringer Mannheim, Mannheim, Germany).

Eighteen (6 per group) of the 30 animals were killed by decapitation after 8 weeks. On the day of killing, blood samples were taken for determination of glucose, cholesterol, triglyceride, HDL-cholesterol, and insulin using the following standard plasma test kits (Boehringer Mannheim): peridochrome glucose method; chod PAP cholesterol method; GPO-PAP triglyceride method; HDL-cholesterol method; and INSIK 5 insulin method (Sorin Biomedica, Salugga, Italy) using a standard of rat insulin (Novo, Copenhagen, Denmark).

Tissue preparations. Hearts were rapidly removed, rinsed with ice-cold saline in less than 30 s, frozen in liquid nitrogen, and stored at –80 °C until use. Frozen pieces of ventricle (200 mg) were homogenized directly in 10 volumes of ice cold buffer containing 20 mmol/l sodium pyrophosphate, 0.1 mmol/l phenylmethane sulfonyl fluoride, 1 mmol/l EDTA, 250 mmol/l sucrose, 80 mmol/l KCl, and 20 mmol/l imidazol/HCl (pH 7.4 at 25 °C) with a polytron PT20 (15 s; setting 5) [29]. The homogenate was subfractionated by two sequential differential centrifugations at $7000 \times g$ for 15 min and $46000 \times g$ for 30 min using a TLA-100.3 rotor Beckman, in the Beckman TL-100 Tabletop ultracentrifuge (Gagny, France). The pellet was resuspended in 100 mmol/l NaCl, 250 mmol/l sucrose, and 30 mmol/l imidazol/HCl (pH 7.4 at 25°C) and stored frozen in liquid nitrogen. Protein yields were consistently 1.9 %, 2 % and 2 % for animals in the CO, DO, and DM groups, respectively.

Enzymology study. Na,K-ATPase activity was determined by coupled assays at 37 °C with or without ouabain as previously described [30]. Assays were carried out with either native vesicles or opened vesicles treated with unmasking agents. The vesicle orientation of our membrane preparations was defined by detergent treatment performed with three SDS concentrations (0.1, 0.2 and 0.3 mg SDS/mg protein for 30 min at 20 °C). This information was important since the Na,K-ATPase activity of native membranes depends on the proportion of permeable (leaky) vesicles in which ouabain and ATP have free access to their sites of action. SDS treatment revealed latent ouabain sensitive activity in impermeable right-side-out and inside-out vesicles with ATP sites on the intravesicular and extravesicular sides, respectively.

The relative proportion of α1 and α2 isoenzymes was inferred from ouabain affinities, as estimated from dose-response curves on permeabilized membranes with the highest Na,K-ATPase activity (0.1 mg SDS/mg protein) [31]. Curves were fit to experimental data by a non-linear regression model [30] using MKModel software (Biosoft, Cambridge, UK). The number of independent sites used to model the data was chosen according to the Schwartz criterion [32].

SDS-PAGE and Western blots. Three microsomal preparations representative of the enzymological study were diluted in 3 volumes of denaturation buffer containing 0.5 mol/l Tris-HCl pH 6.8, 0.1 % glycerol, 10 % SDS, and 1 % bromophenol blue supplemented with 1 % β-mercaptoethanol. Electrophoresis was carried out with a Miniprotean II Cell Apparatus by SDS-PAGE on 4–15 % gradient ready gels (Bio-Rad, Ivry sur Seine, France) for 90 min at 100 volts. Proteins were then transferred to nitrocellulose (Hybond, Amersham, Les Ulis, France) in a transfer buffer containing 192 mmol/l glycine, 24 mmol/l Tris, 0.1 % SDS, and 10 % methanol at 4 °C for 60 min at 200 mA constant current. After incubation in phosphate buffered saline (PBS) (80 mmol/l Na_2HPO_4, 20 mmol/l NaH_2PO_4, and 100 mmol/l NaCl, pH 7.5, supplemented with 3 % low-fat milk) over night at 4 °C to minimize non-specific binding. The resulting nitrocellulose blots were probed for 60 min at 37 °C with antibodies specific for the various Na,K-ATPase isoenzymes. A site-directed rabbit polyclonal antibody specific for rat α1 was provided by R. Mercer (Washington University, St. Louis, Mo., USA). An anti-rat α2 (McB2) monoclonal antibody was provided by K. Sweadner (Harvard Medical School, Charlestown, Ma., USA), and an anti-rat α3 antibody was purchased from UBI (Lake Placid, N. Y., USA).

Dr. P. Martin-Vasallo (Universidad de la laguna, Tenerife, Spain) provided antibodies specific for human $\beta 1$ and $\beta 2$. Membranes were then washed four times with PBS supplemented with 0.1 % tween 20 and incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham, les Ulis, France) for 15 min at 37 °C. After repeating the washing step four times with PBS alone, antigen-antibody reactions were detected by chemiluminescence. Brain and kidney membranes were used as controls; brain for $\alpha 2$ and $\alpha 3$ isoenzymes and kidney for $\alpha 1$ isoenzyme. These blots were exposed to X-ray film (Hyperfilm ECL, Amersham, les Ulis, France) for various times to ensure that chemiluminescent signals were within the linear range of the film. Samples from the three 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 resulting blots were scanned with a AGFA ARCUS densitometer (Agfa-Gevaert AG, Morbel, Belgium) in transparency mode at a resolution of 150 pixels per inch (ppi). The scans were processed on a Macintosh II running the public domain software, Image, written by Wayne Rasband at the Bethesda, Md., USA.

Total phospholipid fatty acid composition of various membrane fractions. Fatty acids were analysed as methyl esters on a Varian model 3300 gas chromatograph (Varian, les Ulis, France) equipped with a flame ionization detector using a spirawax capillary column (25 m × 0.2 mm internal diameter). The temperature program was 150 to 210 °C at 1.5 °C/min. Peak areas from the resulting chromatogram were measured with a Merck model D 2000 integrator (Merck, Nogent, France). After extraction of free acids according to the method of Folch et al. [33], fatty acid methyl esters were prepared according to Hagenfeld [34]. Nonadecanoic acid (C19:0) was added to the mixture before methylation as an internal standard.

Northern blot analysis. Total RNA was prepared from the same frozen cardiac ventricles used for Western blot analysis using the acid isothiocyanate-phenol-chloroform procedure [35]. Northern blot analysis was performed essentially as described by Ouafik et al. [36]. Briefly, RNA (20 μg/lane) was resolved by electrophoresis on 1 % agarose formaldehyde denaturing gels. The denaturd RNAs were transferred to Hybond-N membrane, cross-linked by ultra-violet irradiation, hybridized to ^{32}P-labelled full length $\alpha 1$, $\alpha 2$ and $\beta 1$ cDNA (gift of P. Martin-Vasallo, Universidad de la laguna, Tenerife, Spain). Filters were prehybridized, hybridized, and washed as previously described [36]. To correct for the actual amount of RNA in each lane, blots were stripped and hybridized to S26 cDNA probes [37]. The quantification was performed using the National Institutes of Health Image 1.54 Software (NIH, Bethesda, Md., USA).

Statistical analysis

Differences between the three groups (CO, DO and DM) were evaluated by one-way analysis of variance (ANOVA) followed by multiple comparisons with Scheffé tests using Statview software. Values of p less than 0.05 were considered statistically significant.

Table 1. Effects of STZ-induced diabetes and dietary fish oil supplementation on plasma characteristics: glucose, insulin, triglycerides, cholesterol and HDL and on body weight and heart weight relationship

Groups	Control + olive oil	Diabetic + olive oil	Diabetic + fish oil
Glucose (mmol/l)	7.2 ± 0.5^a	34.4 ± 4.4^b	38.8 ± 3.9^b
Insulin (ng/ml)	2.6 ± 1.5^a	0.301 ± 0.06^b	0.363 ± 0.09^b
Triglyceride (mmol/l)	1.1 ± 0.3^a	15.0 ± 3.2^b	13.2 ± 3.3^b
Cholesterol (mmol/l)	2.1 ± 0.2^a	4.4 ± 0.3^b	5.3 ± 1.5^b
HDL-cholesterol (mmol/l)	1.4 ± 0.3	1.6 ± 0.3	1.2 ± 0.16
Body weight (g)	463 ± 35^a	221 ± 58^b	245 ± 61^b
Heart weight (g)	1.38 ± 0.1^a	0.74 ± 0.14^b	0.92 ± 0.13^b
Heart weight/ body weight $\times 10^{-3}$	3.0 ± 0.2^a	3.6 ± 0.4^b	3.9 ± 0.5^b

Values are means ± SD of six animals. Values in the same lane not bearing the same superscript letters were significantly different at $p < 0.05$

Results

General features of control and diabetic rats. Table 1 shows various indices of metabolic status measured after 8 weeks of STZ-induced diabetes in control animals (CO), diabetic animals fed olive oils (DO), or fish oils (DM). Both DO and DM animals presented severe hyperglycaemia, hypoinsulinaemia and hypercholesterolaemia relative to CO animals. In contrast, there were no differences in plasma HDL-cholesterol levels between control, diabetic, and fish oil treated diabetic rats. Daily supplementation with fish oil (n-3 fatty acids) at 0.5 g/kg had no effect on the plasma modifications induced by diabetes.

The mean body and heart weights were decreased by diabetes as compared to control animals. The heart-to-body weight ratios were respectively 1.2 and 1.3 times greater in DO and DM animals.

Na,K-ATPase activities. Table 2 compares ouabain-insensitive (Mg-ATPase) and sensitive (Na,K-ATPase) ATPase activity in native microsomal vesicles from control animals and, diabetic animals fed olive or fish oils. Mg-ATPase and Na,K-ATPase activities were significantly lower in diabetic rats than control rats. Dietary fish oil (n-3 fatty acids) supplementation had no effect on Na,K-ATPase activity when measurements were made in native membranes. To reveal the full activity, we performed a detergent treatment at doses of 0.1, 0.2 and 0.3 mg SDS. This procedure demonstrated that the optimal dose was 0.1 mg SDS/mg protein (data not shown). When treated with SDS, Na,K-ATPase, but not Mg-ATPase, was significantly lower in diabetic rats than in control rats (Table 2). Furthermore, the Na,K-ATPase activity in DM rats appeared significantly more decreased than in DO rats (Table 2).

Table 2. Effects of STZ-induced diabetes and dietary fish oil supplementation on ouabain-insensitive, Mg-ATPase and Na,K-ATPase activities (native and SDS-treated), affinities for ouabain (IC_{50}) and contribution (%) of the high and low affinity Na,K-ATPase isoenzymes and on isoenzyme activities of cardiac membranes

Groups	Control + olive oil	Diabetic + olive oil	Diabetic + fish oil
ATPase activity (μmol Pi · h^{-1} · mg protein^{-1}) (u)			
Native membranes			
Mg-ATPase	204.3 ± 27.0^{a}	152.8 ± 33.0^{b}	145.9 ± 28.7^{b}
Na, K-ATPase	14.7 ± 2.7^{a}	9.9 ± 1^{b}	8.7 ± 1.9^{b}
SDS-treated membranes (0.1 mg SDS/mg protein)			
Mg-ATPase	137.2 ± 22.0	126.9 ± 12.7	105.9 ± 9.5
Na, K-ATPase	31.7 ± 6.4^{a}	23.1 ± 4.1^{b}	14.0 ± 3.3^{c}
Isoenzyme characteristics			
Low affinity (α1)			
Contribution (%)	87 ± 9^{a}	90 ± 10^{a}	66 ± 5^{b}
Activity (u)	27 ± 2.9^{a}	20.7 ± 2.3^{b}	9.3 ± 0.7^{c}
IC_{50} (mol/l)	$3.6 \pm 0.2 \times 10^{-4\,a}$	$4.4 \pm 3 \times 10^{-4\,a}$	$7.7 \pm 2 \times 10^{-5\,b}$
High affinity (α2)			
Contribution (%)	13 ± 2^{a}	10 ± 3^{a}	34 ± 5^{b}
Activity (u)	4 ± 0.6^{a}	2.3 ± 0.7^{b}	4.7 ± 0.7^{a}
IC_{50} (mol/l)	$4 \pm 1 \times 10^{-8\,a}$	$10 \pm 4 \times 10^{-8\,b}$	$8.1 \pm 0.3 \times 10^{-8\,b}$

Values are means ± SD of experiments done in triplicate with six animals per group. Data were analysed by the non-linear regression model (Materials and methods). Values in the same lane not bearing the same superscript letters were significantly different at $p < 0.05$

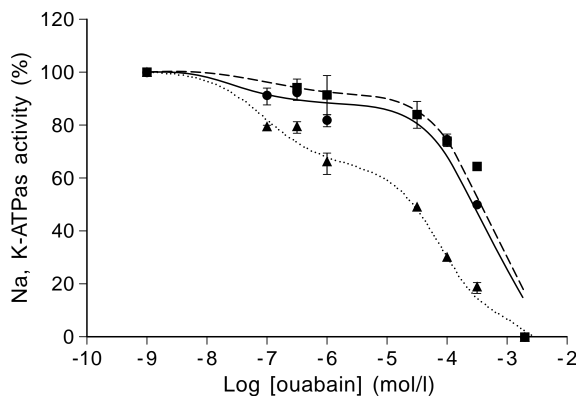

Fig. 1. Effect of diabetes and dietary fish oil supplementation on the ouabain inhibition of cardiac membrane Na,K-ATPase activity. Three groups of six animals were analysed (● control + olive; ■ diabetic + olive; ▲ diabetic + fish oil). Values are means ± SD; experiments done in triplicate. Data were analysed by a non-linear regression model (see Materials and methods). Lines represent the theorical curves assuming a two-site model fit. The computed affinities and percentages are reported in Table 2

Detergent treatment at different SDS concentrations also allowed us to determine the orientation of the vesicles and to confirm the absence of right-side-out vesicles in the three groups of membrane preparations (data not shown). The comparison of ouabain-sensitive and -insensitive activities in native and detergent-treated membranes allowed estimation of the relative proportions of leaky and inside-out vesicles in the different groups, i.e. 46 ± 3% (ratio of 14.7/31.7) and 54 ± 5%, respectively in CO; 43 ± 4% (ratio of 9.9/23.1) and 57 ± 5% respectively in DO; and 62 ± 3% (ratio of 8.7/14) and 38 ± 3% respectively in DM (Table 2). The proportion of leaky membranes was increased in the DM group as compared to that observed in the DO and CO groups.

Ouabain sensitivity. Isoforms of Na,K-ATPase in the rat are known to exhibit different affinities for ouabain [11]. Na,K-ATPase activity in detergent-treated membranes was assayed at different concentrations of ouabain to estimate the proportion of the two cardiac isoenzymes in the three study groups. Two isoenzymes were assumed to be present because the dose-response curves presented in Figure 1 were biphasic and best modelled assuming two, rather than one, affinity constants according to the Schwartz criterion (Materials and methods).

Diabetes induced no significant changes in the affinity and contribution of the low affinity ouabain binding sites (Table 2). In contrast the ouabain affinity for the high affinity sites in the DO group was significantly lower than in the CO group.

Fish oil (n-3 fatty acids) supplementation induced marked changes in the contribution of low and high affinity ouabain sites in diabetic rats. The contribution of high affinity sites increased from 1/10 to 1/3. Ouabain affinity for the low affinity sites in the DM group was significantly ($p < 0.05$) higher than in the DO group. No significant difference in ouabain affinity was observed for the high affinity sites.

Table 2 shows the contribution of the two isoenzymes in terms of activity expressed in μmol Pi · h^{-1} · mg of protein. Diabetes significantly decreased the activities of low and high affinity sites by 26 and 43%, respectively. Fish oil supplementation further

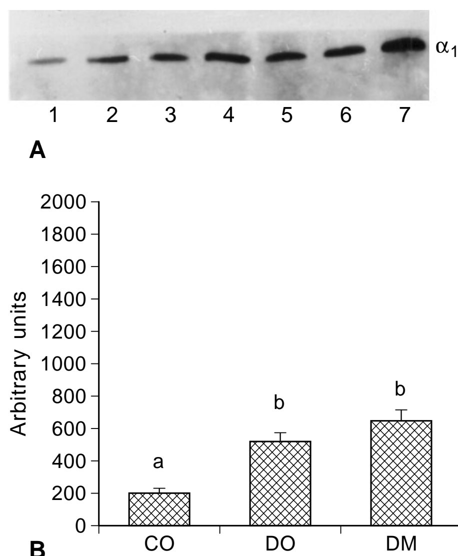

Fig. 2A, B. Effect of diabetes and dietary fish oil supplementation on expression of α1- isoform of Na,K-ATPase. **A** Samples of cardiac membranes from three rats representative of the enzymological study of the three groups: control + olive oil (CO), lanes 1 and 2, diabetic + olive oil (DO), lanes 3 and 4, diabetic + fish oil (DM), lanes 5 and 6 and kidney membranes, lane 7 were electrophoresed on 4–15 % polyacrylamide gradient gel, then blotted onto nitrocellulose, probed with isoform specific anti-rat α1 polyclonal antibodies and subunits detected by the enhanced chemiluminescence method. Two concentrations of protein were used: 10 μg for lanes 1, 3, 5, 7 and 20 μg for lanes 2, 4, 6. **B** Means values ± SD ($n = 3$) are plotted for α1-isoform. Bar not bearing the same superscript letters was significantly different at $p < 0.05$

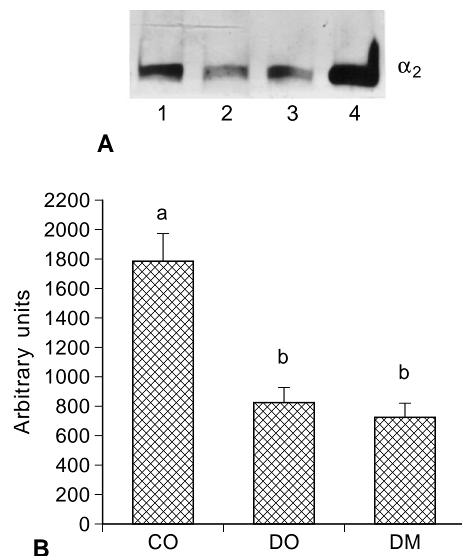

Fig. 3A, B. Effect of diabetes and dietary fish oil supplementation on expression of α2- isoform of Na,K-ATPase. Samples of cardiac membranes from three rats representative of the enzymological study of the three groups: control + olive oil (CO), lane 1, diabetic + olive oil (DO), lane 2, diabetic + fish oil (DM), lane 3 and brain membranes, lane 4 were electrophoresed on 4–15 % polyacrylamide gradient gel, then blotted onto nitrocellulose, probed with isoform specific McB2 (anti-rat α2) monoclonal antibodies and subunits detected by the enhanced chemiluminescence method. Two concentrations of protein were used: 45 μg for lanes 1, 2, 3 and 10 μg for lane 4. **B** Mean values ± SD ($n = 3$) are plotted for α2-isoform. Bar not bearing the same superscript letters was significantly different at $p < 0.05$

lowered low affinity site activity and increased high affinity site activity in SDS-treated vesicles similar to that of controls (Table 2).

Isoform expression. Immunodetection by Western blot analysis confirmed that the α1-, α2-, and β1 subunits of Na,K-ATPase were expressed in all three study groups. The α3- and β2-isoforms were not detected in any study group, even when gels were loaded with large amounts (45 μg) of protein (data not shown). Diabetes significantly increased the abundance of α1-isoforms regardless of the amounts of protein used (10 and 20 μg) (Fig. 2) and decreased the abundance of α2-and β1-isoforms (45 μg of protein amount) (Figs. 3 and 4). Fish oil supplementation prevented the diabetes-induced decrease in β1-isoform without any significant changes in levels of α1- and α2-isoforms in membranes. Moreover this treatment specifically increased β1-isoform expression to levels higher than those found in control vesicles (Fig. 4) (10 μg of protein).

Fatty acid composition of cardiac membranes. The composition of fatty acids in total phospholipid from cardiac membranes was clearly different between diabetic (DO) and control (CO) rats (Table 3). Incorporation of two polyunsaturated fatty acids (PUFA), linoleic acid [C18:2(n-6)] and C20:3(n-6), was significantly ($p < 0.05$) increased in the membranes from diabetic rats. Since no difference in the total PUFA content of the (n-6) series was observed, the marked parallel decrease (–53 %) in arachidonic acid [C20:4(n-6)] compensated for the marked increase (+ 40 %) in linoleic acid and in C20:3(n-6). The explanation may be an impaired utilization of linoleate and of C20:3(n-6) resulting from inhibition of Δ6- and Δ5-desaturase activity, respectively in the diabetic membranes. The opposite effects on membrane content of two monounsaturated fatty acids (MUFA) from two different series, (n-9) and (n-7), the increase in oleic acid [C18:1(n-9)], and the decrease in C18:1(n-7) could also have resulted from diminished Δ6-desaturase activity.

The fatty acid content of the (n-3) series and the total amount of PUFA, saturated fatty acid (SFA), and MUFA, as well as the (n-6/n-3) molar ratio, were not affected by diabetes.

Table 3 (columns DO and DM) also shows the effects of fish oil supplementation on the fatty acid content of membranes from diabetic rats. The content of C20:5(n-3) (EPA) increased twofold without modification of PUFA, MUFA, and SFA contents or the

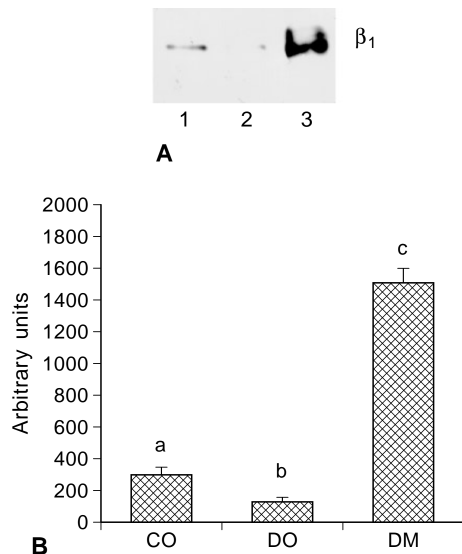

Fig. 4A, B. Effect of diabetes and dietary fish oil supplementation on expression of β1- isoform of Na,K-ATPase. **A** Samples of cardiac membranes from three rats representative of the enzymological study of the three groups: control + olive oil (CO), lane 1, diabetic + olive oil (DO), lane 2, diabetic + fish oil (DM), lane 3 were electrophoresed on 4–15 % polyacrylamide gradient gel, then blotted onto nitrocellulose, probed with isoform specific anti-human *β*1 polyclonal antibodies and subunits detected by the enhanced chemiluminescence method. One concentration of protein was used: 10 μg for lanes 1, 2, 3. **B** Mean values ± SD ($n = 3$) are plotted for β1-isoform. Bar not bearing the same superscript letters was significantly different at $p < 0.05$

Table 3. Effects of STZ-induced diabetes and dietary fish oil supplementation on total phospholipid fatty acid composition of cardiac membranes

	Control + olive oil	Diabetic + olive oil	Diabetic + fish oil
16 : 0	13.1 ± 0.09	10.53 ± 0.2	11.20 ± 0.06
16 : 1 (n-7)	0.17 ± 0.01[a]	ND	0.04 ± 0.06[b]
18 : 0	25.13 ± 0.08	22.25 ± 0.71	23 ± 0.16
18 : 1 (n-9)	2.34 ± 0.03[a]	3.47 ± 0.32[b]	3.38 ± 0.05[b]
18 : 1 (n-7)	2.67 ± 0.01[a]	1.3 ± 0.15[b]	1.71 ± 0.03[b]
18 : 2 (n-6)	27.61 ± 0.32[a]	45.76 ± 1.49[b]	43.67 ± 0.02[b]
18 : 3 (n-3)	0.18 ± 0.01	0.23 ± 0.14	0.14 ± 0.01
20 : 0	0.24 ± 0.05	0.10 ± 0.14	0.28 ± 0.01
20 : 1 (n-9)	0.12 ± 0.05[a]	0.15 ± 0.22[ab]	0.35 ± 0.06[b]
20 : 2 (n-6)	0.26 ± 0.03	0.10 ± 0.15	0.25 ± 0.02
20 : 3 (n-9)	0.19 ± 0.01	0.35 ± 0.24	0.18 ± 0.01
20 : 3 (n-6)	0.40 ± 0.01[a]	1 ± 0.05[b]	0.75 ± 0.05[b]
20 : 4 (n-6)	21.24 ± 0.22[a]	10 ± 0.15[b]	8.74 ± 0.02[b]
20 : 5 (n-3)	0.29 ± 0.08[a]	0.27 ± 0.01[a]	0.57 ± 0.03[b]
22 : 0	0.69 ± 0.08[a]	ND	0.87 ± 0.12[a]
22 : 4 (n-6)	0.51 ± 0.12	0.47 ± 0.07	0.36 ± 0.04
22 : 5 (n-6)	0.27 ± 0.02	0.11 ± 0.16	0.25 ± 0.01
22 : 6 (n-3)	1.93 ± 0.3	1.94 ± 0.06	1.72 ± 0.73
PUFA	52.88 ± 1.11	60.25 ± 2.52	56.65 ± 0.88
MUFA	5.3 ± 0.09	4.92 ± 0.69	5.48 ± 0.2
SFA	39.55 ± 0.3	32.88 ± 1.05	35.35 ± 0.35
(n-6)	50.29 ± 0.72	57.45 ± 2.07	54.03 ± 0.11
(n-3)	2.4 ± 0.38	2.45 ± 0.21	2.44 ± 0.76
(n-6)/(n-3)	20.95 ± 1.9	23.44 ± 2.5	22.1 ± 2.2

Values represent the relative amounts, expressed as a percentage of the total identified fatty acids by weight. Values are means ± SD of six animals. Values in the same lane not bearing the same superscript letters were significantly different at $p < 0.05$ ND, Not detectable

(n-6/n-3) molar ratio. The amount of eicosanoid acids (C20:4 and C20:5) remained lower in both diabetic groups than in controls. Despite the fish oil (n-3 fatty acids) supplementation, the content of C22:6(n-3) (DHA) was not increased in the DM as compared to the DO group.

Northern blot analysis. To determine whether the changes in Na,K-ATPase activity and α1, α2 and β1 abundance seen with diabetes and fish oil supplementation were associated with changes in mRNA encoding these subunits, Northern blots of total RNA were probed with ^{32}P-labelled cDNA α1, α2 and *β*1. Quantitation of the autoradiograms by densitometry revealed no significant changes in the amount of α1 mRNA 8 weeks after the onset of diabetes with or without fish oil treatment (Fig. 5). The ratio of α2 and β1 to S26 (as an invariant control for gene regulation) in diabetic rats and diabetic-treated rats was lower than in control rats by 3.3-, 3.3-, 3.7- and 2.9-fold respectively. A 2-month fish oil supplementation did not prevent the diabetes-induced α2 and *β*1 mRNA decreases (Fig. 6 and 7).

Discussion

In this study, we measured diabetes-induced abnormalities in Na,K-ATPase activity, isoenzyme expression, and fatty acid content in heart membranes. We also assessed the preventive effects of dietary fish oil supplements (n-3 fatty acids, MaxEPA) on these abnormalities. Na,K-ATPase activity in cardiac membranes of adult rats decreased with diabetes, as did the relative abundance of the α2 and *β*1 isoforms (both mRNA and protein levels). In contrast, the abundance of the α1 isoforms increased in diabetic animals. The fact that α1-isoenzyme expression does not fully account for the decrease in enzyme activity suggests that alterations in the membrane environment may also play a role. Fish oil supplementation changed the fatty acid content of myocardial membranes and prevented the diabetes-induced altered activity and/or restored the activity of the α2 isoenzyme without changing its expression. Fish oil supplementation did not modify the native membrane Na,K-ATPase activity. However, alterations of membrane environment by SDS treatment showed a further decrease in the activity of the α1 isoenzyme without changing its expression. Changes in the fatty acid content of the cardiac

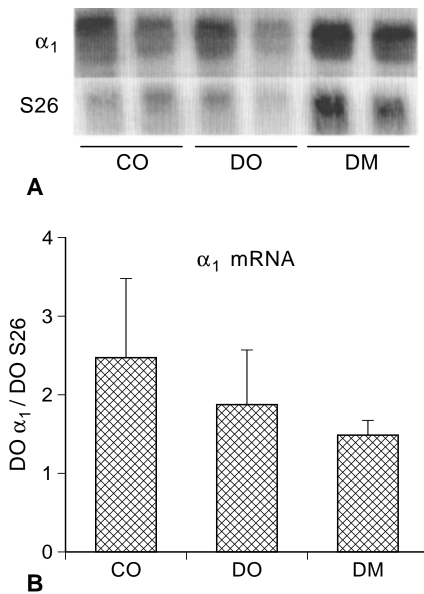

Fig. 5A, B. Levels of cardiac expression of α1 mRNA isoforms of Na,K-ATPase in CO, control + olive oil, DO, diabetic + olive oil and DM diabetic + fish oil. **A** Autoradiogram of Northern blot containing 20 µg of total RNA per slot. The blots were hybridized with ^{32}P-labelled α1 cDNA and S26 cDNA (an invariant control for gene regulation). **B** Densitometric analysis. The ratio of these two RNA, means ± SD (*n* = 3) are shown

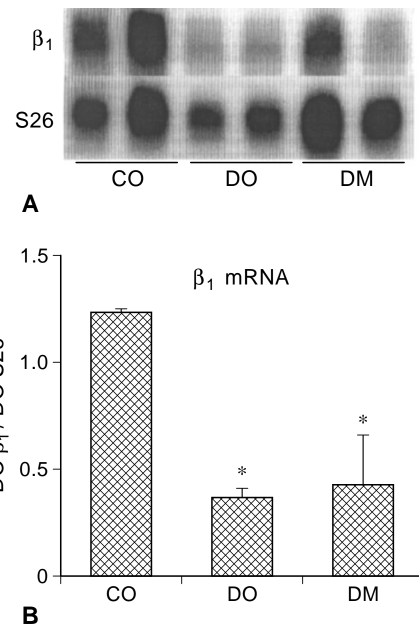

Fig. 7A, B. Levels of cardiac expression of *β*1 mRNA isoforms of Na,K-ATPase in CO, control + olive oil, DO, diabetic + olive oil and DM, diabetic + fish oil. **A** Autoradiogram of Northern blot containing 20 µg of total RNA per slot. The blots were hybridized with ^{32}P-labelled *β*1 cDNA and S26 cDNA (an invariant control for gene regulation). **B** Densitometric analysis. The ratio of these two RNA, means ± SD (*n* = 3) are shown $*p < 0.05$

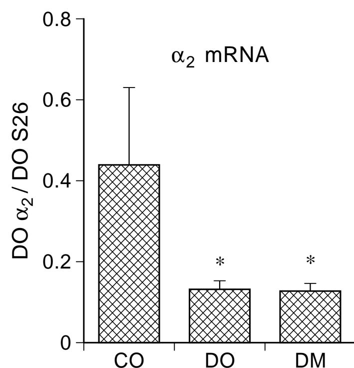

Fig. 6. Densitometric analysis of diabetes and dietary fish oil supplementation on expression of α2 mRNA isoforms of Na,K-ATPase. The ratio of α2 mRNA and S26 rRNA, means ± SD (*n* = 3) are shown $*p < 0.05$

membranes could be partly responsible for these observations.

We chose to study enzymatic activity, protein and mRNA expression, and fatty acid content in the membrane of diabetic rat hearts for several reasons. The first is that although diabetes has been shown to be associated with an impairment in essential fatty acid metabolism [38–40], accurate information about changes in isoenzyme activity, subunit expression and the relative importance of the lipid environment in diabetes-induced sarcolemmal alterations is lacking. Second, previous dietary experiments demonstrated the dependence of the activity and ouabain affinity of isoenzymes on membrane fatty acid content [12, 13], which influences membrane structure and function in brain. The third reason for our study is the clinical relevance of the experimental model and its utility in the evaluation of dietary influence. The impaired systolic and diastolic function observed in diabetic rats [41–43] is similar to the one used to prove the presence of cardiomyopathy in diabetic patients. We used a dose of MaxEPA (0.5 g · kg^{-1} · day) that affects cardiac function of n-3 fatty acid treated diabetic animals [26] and corresponds to a daily dosage of 90 mg EPA, equivalent to 6.3 g in a human with a body weight of 70 kg. Most clinical trials have been conducted using varying daily doses of fish oil from 1.5 to 6.7 g EPA [44]. A limitation is that the dose calculations comparing the rat and human doses are not very accurate since they are based on body weight. Indeed, lipid metabolism is more closely related to surface area than to weight.

The diabetic rats gained much less body weight than control rats. Heart weight and heart- to body-weight ratios were also significantly lower in diabetic rats. Without documentation of myocyte enlargement, histological data, and isozyme shifts, however, we cannot infer the presence of a cardiac hypertrophy in the diabetic rats. Furthermore, these changes in ratios, although significant, are too limited (+20%),

and they could simply result from a large body weight loss. It follows that cardiac hypertrophy may or may not explain the observed changes in Na,K-ATPase.

In this study, we found that diabetes induced a significant decrease of 33 and 26 % in Na,K-ATPase and Mg-ATPase activities, respectively. The reduction in Na,K-ATPase activity was consistent with the results of a previous study using the same experimental model with a different enzymatic assay, i. e. measurement of K-stimulated pNPPase, which is a partial reaction of the Na,K-ATPase enzymatic cycle [9]. The observation of a decreased Mg-ATPase activity in native vesicles could be related to the sensitivity of enzyme activity to lipid environments. The fact that detergent treatment with SDS diminished the reduction in Mg-ATPase activity only indicates that this activity is sensitive to membrane and/or protein-lipid interaction Foussard-Gilbert et al. [45] demonstrated the importance of changes induced in the membrane protein environment by detergent treatment. SDS treatment at multiple concentrations from 0.1 to 0.3 mg SDS/mg protein also allowed us to conclude that diabetes did not affect the relative proportion of the different vesicles in these membrane preparations.

To correlate the reduced Na,K-ATPase activity to isoenzyme activity, we plotted ouabain dose-response curves. We observed that the Na,K-ATPase activity of the α1-isoenzyme decreased by 26 % while that of the α2-isoenzyme decreased by 43 % (Table 2). The fact that both isoenzymes were affected could be due to an alteration in enzyme kinetics, protein subunit expression, or both. To answer this question, we assayed the pattern of isoenzyme expression and analysed the fatty acid content of cardiac membranes in parallel as previously described [12, 13].

Altered lipid environment and protein expression were the most likely explanations for the decrease in Na,K-ATPase activity observed in cardiac membranes in the present study. The two isoenzymes appear to be specifically affected by these two parameters. Ng et al. [9] studied changes caused by STZ-induced diabetes on expression of cardiac Na,K-ATPase subunit isoforms and reported that α2 and β1 protein expression decreased while α1 protein expression remained unchanged. The Northern blot analysis confirms these results on protein expression. The decrease of α2 protein expression was consistent with the lower levels of isoenzyme activity and density that were detected. In contrast, our findings indicate that α1 protein abundance increased. This increase did not correlate with the activity inhibitable by high ouabain concentrations (Table 2). We could reasonably speculate that some α1-isoenzyme was not assembled with β1 into functional enzyme units since β1 levels were decreased by diabetes, and this could be a limiting factor for full activity [46, 47]. Indeed, the stoichiometry could be affected by diabetes [48]. Furthermore, the lower activity of the Na,K-ATPase induced by diabetes could result from different enzymatic rates [49] or phosphorylation status [50].

The diabetes-induced alterations in total fatty acid content observed in this study may result from both Δ6- and Δ5-desaturase inhibitions (Table 3) because substrates of these enzymes were increased, C18:1 (n-9) (oleic acid), C18:2(n-6) (linoleic acid), and C20:3(n-6) and the final product of the reaction was decreased, C20:4(n-6) (arachidonic acid). Such an inhibition of Δ6 and Δ5-desaturases [40, 51] has been attributed either to the glucose-rich medium and/or insulin deficiency [52]. These alterations in the lipid environment are comparable to those previously described in membranes from STZ-treated rats and erythrocytes of diabetic patients [18, 38, 40]. Other factors not investigated in this study such as membrane fluidity or the phospholipid profile could also participate in the enzyme-linked membrane changes [40].

One may speculate that the reduced activity of both isoenzymes impairs myocyte contractility and plays a role in the development of diabetic cardiomyopathy [7, 53]. Indeed it seems reasonable to assume that intracellular Na^+ and K^+ homeostasis is altered, given the recent evidence for differences in the affinities of isoforms for Na^+ [30].

The second purpose of this study was to assess the ability of fish oil supplementation (n-3 fatty acids) 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. Supplementation had no effect on plasma characteristics or on heart and body weight (Table 1).

In the enzymological study, Na,K-ATPase activities (native membranes) measured in the fish oil supplemented diabetic group were the same as the olive oil supplemented diabetic group (Table 2). However, in SDS-treated membranes, Na,K-ATPase was markedly lower in the fish oil supplemented diabetic group relative to either the olive oil supplemented diabetic group or the control group. This suggests that fish-oil-supplementation changes the orientation of vesicles during the membrane preparation. The proportion of inside-out vesicles was lower in the fish oil supplemented groups.

The modification of the relative proportion of α1- and α2-isoenzymes could explain the opposite effects of fish oil supplementation, i. e. a large decrease in α1-isoenzyme activity and a prevention and/or restoration of α2-isoenzyme activity (Table 2). These effects were achieved without a change in protein and RNA abundances as compared to the olive oil supplemented diabetic group. In the fish oil supplemented diabetic group, the ouabain affinity for the α2-isoenzyme was the same as in the olive oil supplemented diabetic group whereas that of the α1-isoenzyme was higher. Such changes in ouabain affinity have already been described as a result of fatty acid membrane changes [54].

The increase in β1 polypeptide levels is not related to changes in its mRNA expression, since its mRNA abundance was not modified after fish oil diet. This result is in favour of a post-transcriptional regulation of the β1 subunit by fish oil. For α2-isoenzymes, the mechanism underlying a restored activity without a change in expression could be: 1) an increased turnover of α2-isoenzymes via covalent modification (e.g. phosphorylation processes) [49] or by an increase in membrane fluidity with incorporation of EPA; or 2) an increase in active $\alpha 2\beta 1$ complexes produced by the elevated abundance of β1.

The large increase in the membrane incorporation of EPA (+ 50 %) and its consequences (as a structural component of tissue lipids or precursors of prostaglandins) could modify the membrane lipid environment of Na,K-ATPase isoenzyme, and explain vesicle orientation, restoration of α2-isoenzyme activity, and a1-isoenzyme ouabain affinity, as well as increased β1 levels. These studies suggest that fish oil therapy may be effective in preventing or treating some of the adverse consequences of diabetes.

Acknowledgements. This work was supported in part by Pierre Fabre Santé, the institution of "Fondation de France" (Paris, France) and the UF 1638 (Ministère de la Santé, Paris, France). We thank Drs. K. Sweadner, P. Martin-Vasallo, R. Mercer for providing antibodies and cDNA. We would like to thank Pr. F. Couraud (INSERM U374, Marseille, France), Dr. F. Paganelli (Laboratoire de Recherches Cardiologiques) and Dr J. Palmari (INSERM CJF 93–11, Marseille, France) for their support and advice. We would also like to thank Dr. I. Bertrand (Smith Kline Beecham, Rennes, France), Dr. R.M. Kawamoto (Procter and Gamble Pharmaceuticals, Norwich, N.Y., USA) and Dr. T. Pressley (Texas Technical University, Lubbock, Tx., USA) for their critical reading of this manuscript.

References

1. Fein FS, Sonnenblick EH (1985) Diabetic cardiomyopathy. Prog Cardiovasc Dis 27: 255–270
2. Giacomelli F, Weiner J (1979) Primary myocardial disease in the diabetic mouse. Lab Invest 40: 460–468
3. Imanaga I, Kamegawa Y, Kamei R, Kuroiwa M (1991) Cardiac sarcolemmal Na,K-adenosine triphosphatase (ATPase) activity in diabetic dog. In: Nagano M, Dhalla NS (eds) The diabetic heart. Raven Press, Ltd., New York, pp 237–247
4. Ku DD (1980) Decreased sodium pump activity in heart and thoracic aorta of streptozotocin (STZ)-induced diabetic rats. Pharmacologist 22: 288 (Abstract)
5. Ku DD, Sellers BM (1982) Effects of streptozotocin diabetes and insulin treatment on myocardial sodium pump and contractility of the rat heart. J Pharmacol Exp Ther 222: 395–400
6. Onji T, Liu MS (1980) Effects of alloxan-diabetes on the sodium-potassium adenosine triphosphatase enzyme system in dog hearts. Biochem Biophys Res Commun 96: 799–804
7. Pierce GN, Dhalla NS (1983) Sarcolemmal Na,K-ATPase activity in diabetic rat heart. Am J Physiol 245:C241–C247
8. Pierce GN, Dhalla NS (1984) The association of membrane alterations with heart dysfunction during experimental diabetes mellitus. In: Dhalla NS, Signal PK, Beamish RE (eds) Pathophysiology of heart disease. Boston, Nijhoff pp 177–184
9. Ng YC, Tolerico PH, Book CB (1993) Alterations in levels of Na,K-ATPase isoforms in heart, skeletal muscle, and kidney of diabetic rats. Am J Physiol 265:E243–E251
10. Murphy MG (1990) Dietary fatty acids and membrane protein function. J Nutr Biochem 1: 68–79
11. Sweadner KJ (1989) Isozymes of the Na,K-ATPase. Biochim Biophys Acta 988: 185–220
12. Gerbi A, Zérouga M, Debray M et al. (1993) Effect of dietary α-linolenic acid on functional characteristics of Na,K-ATPase isoenzymes in whole brain membranes of weaned rats. Biochim Biophys Acta 1165: 291–298
13. Gerbi A, Zérouga M, Debray M et al. (1994) Effect of fish oil diet on fatty acid composition of phospholipids of brain membranes and on kinetic properties of Na,K-ATPase isoenzymes of weaned and adult rats. J Neurochem 62: 1560–1569
14. Stubbs CD, Smith AD (1985) The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 779: 89–137
15. Corrocher R, Ferrari S, Bassi A et al. (1987) Membrane polyunsaturated fatty acids and lithium-sodium countertransport in human erythrocytes. Life Sci 41: 1172–1178
16. Corrocher R, Guadagnin L, De Gironcoli M et al. (1989) Membrane fatty acids, glutathione-peroxidase activity and cation transport system of erythrocytes and malondialdehyde production by platelets in Laurence Moon Barter Biedl syndrome. J Endocrinol Invest 12: 475–481
17. Corrocher R, Bassi A, Gandini A et al. (1990) Transmembrane cation fluxes and fatty acid composition of erythrocytes in psoriatic patients. Clin Chim Acta 186: 335–344
18. Ruiz-Gutierrez V, Stiefel P, Villar J et al. (1993) Cell membrane fatty acid composition in type 1 (insulin-dependent) diabetic patients: relationship with sodium transport abnormalities and metabolic control. Diabetologia 36: 850–856
19. Borkman M, Storlien LH, Pan DA et al. (1993) The relation between insulin sensitivity and the fatty acid composition of skeletal muscle phospholipids. New Engl J Med 328: 238–244
20. Storlien LH, Jenkins AB, Chisholm DJ et al. (1991) Influence of dietary fat composition on development of insulin resistance in rats. Diabetes 40: 280–289
21. Pan DA, Hulbert AJ, Storlien LH (1994) Dietary fats, membrane phospholipids and obesity. J Nutr 124: 1555–1565
22. Simopoulos AP (1991) ω3 fatty acids in health and disease and in growth and development. Am J Clin Nutr 54: 438–463
23. Leaf A, Weber PC (1988) Cardiovascular effects of n-3 fatty acids. New Engl J Med 31: 549–557
24. Weaver BJ, Holub BJ (1988) Health effects of dietary eicosapentaenoic acid. Prog Food Nutr Sci 12: 111–150
25. Hallaq H, Sellmayer A, Smith TW, Leaf A (1990) Protective effect of eicosapentaenoic acid on ouabain toxicity in neonatal rat cardiac myocytes. Proc Natl Acad Sci USA 87: 7834–7838
26. Black SC, Katz S, McNeill JH (1989) Cardiac performance and plasma lipids of omega-3 fatty acid-treated streptozotocin-induced diabetic rats. Diabetes 38: 969–974
27. McLennan PL, Abeywardena MY, Charnock JS (1985) Influence of dietary lipids on arrhythmias and infarction after coronary ligation in rats. Can J Physiol Pharmacol 63: 1411–1417

28. McLennan PL, Abeywardena MY, Charnock JS (1988) Dietary fish oil prevents ventricular fibrillation following coronary artery occlusion and reperfusion. Am Heart J 116: 709–717
29. Maixent J-M, Fenard S, Kawamoto RM (1991) Tissue localisation of active Na,K-ATPase isoenzymes by determination of their profile of inhibition with ouabain, digoxin, digitoxigenin and cordil, a new aminosteroid cardiotonic. J Rec Res 11: 687–698
30. Gerbi A, Debray M, Maixent J-M et al. (1993) Heterogeneous Na sensitivity of Na,K-ATPase isoenzymes in whole brain membranes. J Neurochem 60: 246–252
31. Berrebi-Bertrand I, Maixent J-M, Christe G, Lelievre LG (1990) Two active Na,K-ATPase of high affinity for ouabain in adult rat brain membranes. Biochim Biophys Acta 1021: 148–156
32. Schwarz G (1978) Estimating the dimension of a model. Ann Stat 6: 461–464
33. Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497–509
34. Hagenfeldt L (1966) A gas chromatographic method for the determination of fatty acids in plasma. Clin Chim Acta 13: 266–268
35. Chomczynski P, Sacchi V (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
36. Ouafik L'H, May V, Keutman HT, Eipper BA (1989) Developmental regulation of peptidylglycine a-amidating monooxgenase (PAM) in rat heart atrium and ventricle. J Biol Chem 264: 5839–5845
37. Vincent S, Marty L, Fort P (1993) S26 ribosomal protein RNA: an invariant control for gene regulation experiments in eucaryotic cells and tissues. Nucleic Acids Res 21: 1498
38. Holman RT, Johnson SB, Gerrard J-M et al. (1983) Arachidonic acid deficiency in streptozotocin-induced diabetes. Proc Natl Acad Sci USA 80: 2375–2379
39. Horrobin DF (1988) The roles of essential fatty acids in the development of diabetic neuropathy and other complications of diabetes mellitus. Prostaglandins Leukotr Essent Fatty Acids 31: 181–197
40. Brenner RR (1981) Nutritional and hormonal factors influencing desaturation of essential fatty acids. Prog Lip Res 20: 41–47
41. Fein FS, Strobeck JE, Malhotra A et al. (1981) Reversibility of diabetic cardiomyopathy with insulin in rats. Circ Res 49: 1251–1261
42. Penpargkul ST, Schaible T, Yipintsoi T, Scheuer J (1980) The effect of diabetes on performance and metabolism of rat hearts. Circ Res 47: 911–921
43. Pierce GN, Dhalla NS (1981) Cardiac myofibrillar ATPase activity in diabetic rats. J Mol Cell Cardiol 13: 1063–1069
44. Malasanos TH, Stacpoole PW (1991) Biological effects of ω3 fatty acids in diabetes mellitus. Diabetes Care 14: 1160–1179
45. Foussard-Gilbert F, Ermias A, Laget P et al. (1982) Detergent effects of kinetic properties of Na,K-ATPase from kidney membranes. Biochim Biophys Acta 692: 296–304
46. Geering K (1991) The functional role of the beta-subunit in the maturation and intracellular transport of Na,K-ATPase. FEBS Lett 285: 189–193
47. Mircheff AK, Bowen JW, Yiu SC, McDonough AA (1992) Synthesis and translocation of Na,K-ATPase α- and β-subunits to plasma membrane in MDCK cells. Am J Physiol 262:C470–C483
48. Sun Y, Ball WJ (1992) Determination of Na,K-ATPase α and β isoforms and kinetic properties in mammalian liver. Am J Physiol 262:C1491–C1499
49. Maixent J-M, Berrebi-Bertrand I (1993) Turnover rates of the canine cardiac Na,K-ATPases. FEBS Lett 330: 297–301
50. Borghini I, Geering K, Gjinovci A et al. (1994) In vivo phosphorylation of the Na,K-ATPase α subunit in sciatic nerves of control and diabetic rats: effects of protein kinase modulators. Proc Natl Acad Sci USA 91: 6211–6215
51. Black SC, Katz S, McNeill JH (1993) Influence of ω3 fatty acid treatment on cardiac phospholipid composition and coronary flow of streptozotocin-diabetic rats. Metabolism 42: 320–326
52. Eck MG, Wynn JO, Carter WJ, Faas FH (1979) Fatty acid desaturation in experimental diabetes mellitus. Diabetes 28: 479–485
53. Shaffer SW (1991) Cardiomyopathy associated with noninsulin-dependent diabetes. Mol Cell Biochem 107: 1–20
54. Abeywardena MY, Charnock JS (1983) Modulation of cardiac glycoside inhibition of Na,K-ATPase by membrane lipids. Difference between species. Biochim Biophys Acta 729: 75–84