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Differential Effect of ω3 PUFA Supplementations on Na,K-ATPase and Mg-ATPase Activities: Possible Role of the Membrane ω6/ω3 Ratio

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Abstract. Several functional properties of Na,K-ATPase are strongly dependent on membrane fatty acid composition, but the underlying mechanism is still not well defined. We have studied the effects of two types of supplementations enriched in the ω3 polyunsaturated fatty acids on the Na,K-ATPase and Mg-ATPase activities in sciatic nerve (SN) and red blood cells (RBC). Eight groups of rats, controls and diabetics, received a standard diet, supplemented or not with 30 or 60 mg/kg/day of docosahexaenoic acid (DHA) or with soybean for eight weeks. Diabetes induced significant decrease of Na,K-ATPase activity in SN (−23%) and RBC (−25%), without affecting Mg-ATPase activity. In RBC, soybean and DHA supplementations caused significant increases in Na,K-ATPase activity (in various range, +13% to +145%) in all groups, and in Mg-ATPase activity in control soybean (+65%), control and diabetic DHA high dose (+39%, +53%) and diabetic DHA low dose (+131%) groups. In SN, the soybean caused a significant decrease in Na,K-ATPase activity (−26%) and still more in the diabetic group (−53%). The DHA diet induced a slight decrease in activity in control groups, whilst during diabetes, at high dose, we noted an aggravation of this decrease (−36%). Mg-ATPase activity was not modified by supplementations except for the low dose of DHA where the activity was slightly decreased in the control group (−16%). The supplementations induced multiple tissue-specific modifications in the membrane fatty acid composition of RBC and of SN homogenates. Several specific correlations have been found between variations in fatty acids amounts and Na,K-ATPase activity in these tissues but only in RBC for Mg-ATPase activity. Indeed, we observed that the variations in Na,K-ATPase activity are positively and significantly correlated with changes in the ω6/ω3 ratio in SN as well as in RBC. These data clearly show, for the first time, that the diet could modulate the Na,K-ATPase activity via the ω6/ω3 ratio in the membranes. A similar correlation was observed with Mg-ATPase activity in RBC, suggesting also a dietary regulation of the enzyme; but for the SN, this activity might be regulated by a different ω6/ω3 ratio or by another pathway.

Key words: Na,K-ATPase — Mg-ATPase — ω3 PUFA — Diabetes — Diet — ω6/ω3 ratio

Introduction

Na,K-ATPase (EC 3.6.1.37) and Mg-ATPase (a subgroup of EC 3.6.1.3.), are transmembranous enzymes in contact with the lipids present in biological membranes. In fact, the enzyme structures are surrounded by membrane phospholipids. Several functional properties of the Na,K-ATPase are strongly dependent on membrane structure, such as the transport (Jorgensen et al., 1998; Pedersen et al., 1998) and the sodium or ouabain affinity (Gerbi et al., 1993, 1997, 1998, 1999a,c).

Nevertheless, the mechanism involved in the regulation of this enzyme by the lipid environment is still not well defined. To our knowledge, the implication of membrane properties in Mg-ATPase regulation has not been described in the literature. Moreover, with the increasing number of implications of lipids in neurological disorders such as schizophrenia (Opler & Opler 2001, Peet & Horrobin 2002), bipolar disorder (Berk et al., 2001) and dia-

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betic neuropathy (Gerbi et al., 1998; Coste et al., 1999; Sima & Sugimoto, 1999), it will be very useful to better understand the relationship between the lipid membrane composition and the stucture/function of ATPases, membrane-bound enzymes implicated in numerous physiological process in the nervous system.

Extraphysiological procedures, consisting of direct lipid incubations of cells (Dwight & Hendry, 1995; Nguyen et al., 1998; Mayol et al., 1999) or vesicles (Swarts, Schuurmans-Stekhoven & De Pont, 1990) or specific phospholipase digestions (Harris, 1985), induced several modifications in Na,K-ATPase properties, but did not give any reliable results for the comprehension of this regulation. The fatty acids (FA) may act on Na,K-ATPase activity by modifying its potassium sensitivity (Swarts et al., 1990) and its affinity towards ouabain (Swarts et al., 1990; Mayol et al., 1999).

In physiological studies concerning the variations in lipid membrane composition resulting from lipid-enriched diets, it is more difficult to characterize the specific roles in Na,K-ATPase activity of the different FA present in biological membranes (Gerbi et al., 1998; Coste et al., 1999) because these variations are multiple and occur simultaneously. It is commonly accepted that the length (Abeywardena et al., 1984) as well as the number of double bonds of the carbon chains (Swann, 1984) participate in the Na,K-ATPase regulation by changing various parameters such as sodium and ouabain affinity (Gerbi et al., 1993, 1998, 1999c).

In physiopathological states such as diabetes, Na,K-ATPase activity is altered in sciatic nerve and red blood cells (RBC) (Raccah et al., 1994; Gerbi et al., 1998; Coste et al., 1999; Djemli-Shipkolye et al., 2001). This low activity is associated with a modification of membrane FA composition (Djemli-Shipkolye et al., 2001), which results from a well-known decrease in Δ5 and Δ6 desaturase activities (Eck et al., 1979). However, we have also observed that the simultaneous decrease in activity in sciatic nerve and RBC membranes is associated with tissue-specific membrane fatty acid composition modifications (Djemli-Shipkolye et al., 2001).

Dietary supplementations enriched in ω6 and ω3 polyunsaturated fatty acids (PUFA) were conducted to better understand the effects of a direct supply of these PUFA on Na,K-ATPase activity and to palliate the deficiency in these PUFA induced by diabetes. The use of fish oil in diabetic rats was aimed at compensating for the limiting steps in the metabolism of ω3 PUFA by providing the end products of the ω3 metabolic pathway, i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This diet results in a limited decrease in Na,K-ATPase activity in sciatic nerve, likely by enhancing the expression of α1 and α3 isoforms (Gerbi et al., 1998). It has been hypothesized that this partial effect may be induced by an inhibitory effect of EPA, mainly present in fish oil, on Na,K-ATPase activity as described by Mayol et al. (1999), or by an adverse effect of EPA on arachidonic acid (AA) metabolism (James, Gibson & Cleland, 2000), while a full preservation of Na,K-ATPase activity might be expected in the absence of EPA.

Moreover, after pooling data (FA composition, ouabain affinity, and activity of Na,K-ATPase isoforms) from several tissues during dietary fish oil supplementation, a positive correlation between the levels of membrane ω6 PUFA and the increase in α1 isoform affinity for ouabain has been found (Gerbi & Maixent, 1999).

To better understand the regulation of the enzyme by ω3 PUFA, a study of the effects of two types of supplementations was undertaken. The first diet enriched in DHA, the end-product of ω3 PUFA metabolism, and the second one enriched in its precursor α-linolenic acid (ALA), were evaluated on the activities of Na,K-ATPase and Mg-ATPase, and on the membrane fatty acid composition in sciatic nerve homogenates and RBC membranes. Diabetic and control rats, their diet supplemented with DHA and ALA, were used to assess the specific modifications induced by each supplementation in the absence and presence of diabetes. Contrary to previous studies (Gerbi et al., 1998; Coste et al., 1999), these modifications were compared to those of diabetic and control rats, fed on a standard diet without supplementation.

Thanks to the number of supplementations used in this study, we were able to show that the FA modifications in membranes, induced by these supplementations, could correlate significantly with the variations observed in Na,K-ATPase and Mg-ATPase activities.

Materials and Methods

Animals

This study was done according to the guidelines of the French department of Agriculture, Fishing and Diet on the experimental use of laboratory rats with agreement number A13823. The principles of laboratory animal care (NIH) were followed. Male Sprague-Dawley rats ($n = 80$; Iffa Credo, Saint Germain de l'Arbresle, France) weighing ~190 g were entered in the study after acclimatization for one week. Their body weight at the beginning of the study averaged 220 ± 12 g, and they were randomly assigned to eight age-matched groups ($n = 10$). In the four diabetic groups, diabetes was induced by a single intravenous injection of streptozotocin (STZ, 65 mg/kg; Sigma, St. Louis, MO), freshly dissolved in sodium citrate buffer (0.01 mol/l, pH 5.5). Control rats were injected with buffer only. All diabetic rats were maintained without insulin. Diabetes was checked 3d after the STZ-induction and on the last day of the study by the presence of hyperglycemia (> 25 mmol/l) in blood samples collected from the tip of the tail (Reflolux, Boehringer Mannheim, Mannheim, Germany). Rats con-

sumed food and water ad libitum. The food was standard nonpurified rodent diet (AO4, UAR, Epinay sur Orge, France). Gavage was started on the day of STZ or buffer administration. Two groups, a control (C) and a diabetic group (D), were given no supplementation. A control (CS) and a diabetic group (DS) were given soybean phospholipids at a daily dose of 0.8 g/kg/day. A control (CD60) and a diabetic group (DD60) were given DHA phospholipids at a daily dose of 0.8 g/kg/day. And the two last groups, control (CD30) and diabetic (DD30), were given DHA phospholipids at a daily dose of 0.4 g/kg/day. These two doses correspond, respectively, to 60 mg/kg and 30 mg/kg of DHA per day. Soybean or DHA phospholipids have similar caloric value. These supplementations were administered daily at 9:00 am. No differences were observed in food intake between groups supplemented with these preparations. After 8 weeks, rats were anesthetized with pentobarbital (50–100 mg/kg) by intraperitoneal injection to obtain blood by cardiac puncture and sciatic nerves.

Preparation and Composition of Supplementations

Two different phospholipid supplementations were used. The first one consisted of an egg-phospholipid preparation enriched in DHA and obtained by feeding chicken with a special diet. The egg-phospholipids were extracted with alcohol. The alcohol was then eliminated with a rotavapor and the phospholipids were hydrated at 60% with distilled water.

The second supplementation consisted of soybean phospholipids hydrated at 60% with distilled water.

It should be noted that the phospholipids, and in particular DHA phospholipids, are more stable than triglycerides to peroxydation (Song, Inoue & Miyazawa, 1997).

The phospholipid preparations were prepared every week, maintained under nitrogen, in the dark and at 4°C, and analyzed regularly to monitor lipid peroxidation. The FA composition of these preparations is given in Table 1. Following this analysis, we were able to determine, in mg, the daily intake of the principal FA concerned with the study.

Table 1. Fatty acid composition of diet supplementations

Fatty acid	(mg/kg/day)			
	DHA 30	DHA 60	Soybean	Standard diet
C16:0	67.8	135.6	89.3	156
C16:1	–	–	–	Traces
C18:0	40.7	81.4	28.5	30
C18:1 ω9	87.3	174.6	64.5	480
C18:2 ω6 (LA)	45.1	90.2	346.8	870
C18:3 ω3 (ALA)	–	–	43.2	Traces
C20:4 ω6 (AA)	10.6	21.2	–	–
C20:5 ω3 (EPA)	4.8	9.6	–	–
C22:6 ω3 (DHA)	30	60	–	–
Σ PUFA	90.5	181	390	870
Σ ω6	55.7	111.4	346.8	870
Σ ω3	34.8	69.6	43.2	Traces
ω6/ω3	1.6	1.6	8.02	

Tissue Preparations

Blood was collected by cardiac puncture into tubes with 0.11 mol/l sodium citrate. Sciatic nerves from the spine to the peroneal bifurcation were dissected and, after removal of adherent tissue, frozen in liquid nitrogen and conserved at −80°C until use.

Red Blood Cells and Plasma

Plasma was separated by centrifugation at $1500 \times g$ for 15 min. Leukocytes and platelets were removed from the blood samples by filtration through a microcrystalline cellulose column (Beutler, West & Blume, 1976). Red blood cells (RBC) were hemolyzed by Tris buffer 11 mmol/l and centrifuged ($30{,}000 \times g$ for 30 min at 4°C) and the membrane pellet was resuspended in 30 ml of buffer. This centrifugation step was repeated 3 times, as previously described by Raccah et al. (1992). The RBC membranes were then stored for Na,K-ATPase activity measurement and FA composition determination.

Sciatic Nerve

On the day of the homogenate preparation, sciatic nerve segments were measured, weighed and rinsed in ice-cold saline solution. The sciatic nerve was chopped into small pieces and then homogenized in 2 ml of ice-cold saline containing 11 mmol/l Tris buffer, pH 7.4, at 4°C with a motorized Potter homogenizer (model 94348, Heidolph, Germany) using three 15-sec bursts. The resulting homogenate was passed through a cellulose filter (600F4252, Fioroni, La Chapelle St Mesmin, France) to remove impurities. The homogenate was then aliquoted for Na,K-ATPase activity measurement and FA composition determination.

Measurement of Na,K-ATPase and Mg-ATPase Activities

Na,K-ATPase activity was measured in a final volume of 1 ml as previously described (Raccah et al., 1992). Membranes (25 and 300 μg for SN and RBC, respectively) were preincubated for 10 min at 37°C in a mixture containing (in mM) 92 Tris HCl (pH 7.4), 100 NaCl, 20 KCl, 5 $MgSO_4 \times 7H_2O$ and 1 EDTA. Assays were performed with or without ouabain, a specific Na,K-ATPase inhibitor. After incubation with 4 mM vanadate-free ATP (Sigma) at 37°C for 10 min, the reaction was stopped by addition of ice-cold trichloroacetic acid at a final concentration of 5%. After centrifugation at 4°C and $5500 \times g$ for 10 min, the amount of inorganic phosphate in the supernatant was determined according to the method of Hurst (1964).

Na,K-ATPase activity was calculated as the difference between Pi released per milligram protein per hour in the presence and absence of ouabain. Membrane protein concentration was determined using Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany). The Mg-ATPase activity was calculated as the difference between the ouabain-insensitive ATPase activity and the ATP hydrolysis without membranes.

All assays were performed in triplicate and blanks were included in each experiment to determine the endogenous phosphate and the nonenzyme-related breakdown of ATP.

Membrane Fatty Acid Composition

Total lipids of plasma, RBC membranes and sciatic nerve homogenates were extracted with methanol and chloroform according to the method of Bligh & Dyer (1959), modified by the use of a sonicator. FA composition was determined after methylation with BF3-methanol (Sigma) according to Ohta et al. (1990). The fatty acid methyl esters were analyzed by gas chromatography on a Perkin Elmer Autosystem XL (Perkin Elmer, Courtaboeuf, France), using a fused silica capillary column (60 m × 0.22 mm

Table 2. Effects of ω3 PUFA supplementations on plasma glucose, lipid levels and body weight

	C	D	CD30	DD30	CD60	DD60	CS	DS
Weight (g)	433 ± 5	248 ± 12*	448 ± 11	270 ± 15*	464 ± 9	255 ± 15*	455 ± 16	251 ± 10*
Blood glucose (mmol/l)	9 ± 0.3	33 ± 3*	8.3 ± 0.3	36.8 ± 2*	7.7 ± 0.4	36.9 ± 2*	7.9 ± 0.3	38.6 ± 2.8*
Cholesterol (mmol/l)	1.4 ± 0.1	1.8 ± 0.2*	1.5 ± 0.1	1.9 ± 0.2*	1.4 ± 0.1	2 ± 0.1*	1.5 ± 0.1	1.9 ± 0.1*
Triglycerides (mmol/l)	0.7 ± 0.1	1.6 ± 0.5*	0.8 ± 0.2	2.1 ± 0.5*	1 ± 0.15	2.7 ± 1*	1.1 ± 0.2	3.5 ± 0.6*
Phospholipids (mmol/l)	1.5 ± 0.06	2 ± 0.2*	1.6 ± 0.06	2.1 ± 0.2*	1.5 ± 0.15	2.4 ± 0.2*	1.4 ± 0.1	2.18 ± 0.1*
C18:2 ω6 LA	19.9 ± 0.3	32.3 ± 0.7*	21.8 ± 0.6*	35.3 ± 1.3*	21.8 ± 0.6*	30.5 ± 1.1*	27 ± 1.1*	39.4 ± 1.2*
C20:4 ω6 AA	13.1 ± 0.7	11.3 ± 0.6	20.1 ± 2.4*	11.3 ± 1.2	16.6 ± 0.9*	14.6 ± 1.5	15.1 ± 1.1	10.2 ± 1.2
C20:5 ω3 EPA	1.7 ± 0.1	2.4 ± 0.2*	1.3 ± 0.1*	1.9 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	1.5 ± 0.1
C22:6 ω6 DHA	5.4 ± 0.2	6.1 ± 0.2	5.5 ± 0.2	5.8 ± 0.3	6.3 ± 0.3	6.5 ± 0.5	4.2 ± 0.1*	4.5 ± 0.2*
ω6/ω3	3.7 ± 0.1	4.3 ± 0.2*	5.2 ± 0.3*	5.6 ± 0.2*	4.2 ± 0.2*	5.1 ± 0.4*	6.2 ± 0.4*	7.4 ± 0.4*

Values are means ± SEM for each group ($n = 10$).
*Significant difference ($p < 0.05$) compared to control group (C).

inner diameter), BPX 70 (SGE, Villeneuve Saint Georges, France) equipped with a flame ionization detector and using hydrogen as the carrier gas. The temperature program ranged from 160°C to 205°C at 1°C/min. Peak areas from the resulting chromatogram were measured with a Perkin Elmer 1022 S integrator. FA were identified by their retention times on the column with respect to appropriate standards.

Statistical Analysis

Results are expressed as mean ± SEM. Before assessing the different variables, we did a Kolmogornov-Smirnov test for normality and a Bartlett test for homogeneous variance for each group. The differences between two groups were tested by the nonparametric Mann–Whitney U test. *p*-values of less than 0.05 were considered significant.

Correlations were evaluated by a single linear regression analysis; values of $p < 0.05$ were considered significant. All analyses were done by STATVIEW software (Abacus Concepts, Berkeley, CA) on Macintosh Performa 6400 (Apple Computer, Les Ulis, France).

Abbreviations

AA: Arachidonic acid; ALA: α-Linolenic acid; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; FA: Fatty acid; LA: Linolenic acid; PUFA: polyunsaturated fatty acid; RBC: Red blood cell; SN: Sciatic nerve; STZ: Streptozotocin.

Results

Effect of ω3-enriched Supplementations on Weight, Plasma Glucose, and Lipid Levels

Eight weeks of diabetes leads to hyperglycemia, hypercholesterolemia and an increase in the levels of triglycerides and phospholipids, as shown in Table 2. As compared to controls, weight gain is less important in diabetic groups. DHA-enriched supplementations, at high dose, as well as the soybean supplementation, lead to an increase in the level of plasmatic triglycerides in the diabetic (25 to 100%) and control (~50%) groups. The level of DHA in the plasma follows the dietary intake. Comparison between supplemented and non-supplemented groups (groups C and D) shows that there are no changes in the other parameters considered.

FA Modifications Induced by ω3-Enriched Supplementations

Diabetes induced modifications in membrane FA that are tissue-specific. In sciatic nerve, we note an increase in the levels of C22:4 ω6 and C18:4 ω3 (Table 3). In RBC membranes, we have an accumulation of LA (C18:2 ω6) (Table 4). All the supplementations induced profound differences in the lipid composition of membranes as compared to non-supplemented groups, as shown in Tables 3 and 4.

In sciatic nerve, DHA-enriched supplementations cause a significant decrease in the amounts of PUFA, which is essentially due to a decrease in the level of ω6 PUFA in the membranes (Table 3). The incorporation of DHA is significantly increased in the membranes. The soybean supplementation favors the incorporation of ω3 PUFA in the membranes with significant increases in EPA (C20:5 ω3), docosapentaenoic acid (DPA, C22:5 ω3) and DHA (C22:6 ω3). Moreover, as for the DHA-enriched supplementations, we also observe a decrease in the level of LA.

In RBC membranes, ω3 PUFA supplementations cause a significant increase in the incorporation of PUFA (Table 4). The amounts of AA (C20:4 ω6) are significantly increased in all the groups and also more specifically the level of LA is increased in the supplemented diabetic rats (DD30, DD60, and DS). Paradoxically, the incorporation of ω3 PUFA is diminished in the membranes. In fact, the use of DHA-enriched supplementations leads to a significant decrease in the incorporation of EPA. The soybean supplementation in the CS group causes a similar decrease in EPA level, but this time the decrease in DHA and DPA is more pronounced.

Table 3. Fatty acid modifications induced by ω3 PUFA supplementations in sciatic nerve homogenates

Fatty acids	C	D	CD30	DD30	CD60	DD60	CS	DS
C16:0	15 ± 0.4^{a}	14 ± 0.5^{a,b,c}	14.5 ± 0.4^{a,b,c}	13.3 ± 0.1^{b,d}	14.4 ± 0.7^{a}	13 ± 0.1^{c}	13.3 ± 0.2^{b}	12.3 ± 0.1^{c}
C18:0	9.0 ± 0.4^{a}	10.3 ± 1^{b}	10 ± 0.6^{a,b,c}	11.6 ± 0.3^{b,c}	9.9 ± 0.6^{a,c,d}	11.7 ± 0.4^{b,c,d}	10.2 ± 0.3^{d}	11.8 ± 0.4^{b}
C18:2 ω6	6.27 ± 0.8^{a}	6.96 ± 1^{a,c}	4.6 ± 0.6^{a,b,c}	2.74 ± 0.28^{b,d}	5.44 ± 1.05^{a,c}	2.74 ± 0.46^{b,c,d}	3.95 ± 0.37^{c}	2.86 ± 0.24^{d}
C18:3 ω3	0.27 ± 0.03^{a,d}	0.22 ± 0.01^{a,c,d}	0.18 ± 0.06^{a,b}	0.20 ± 0.06^{b}	0.15 ± 0.03^{b,c}	0.31 ± 0.08^{a,b,d}	0.28 ± 0.04^{a,d}	0.41 ± 0.06^{d}
C18:4 ω3	1.79 ± 0.1^{a}	2.12 ± 0.1^{b,d}	1.95 ± 0.1^{a,b}	2.22 ± 0.06^{b,d}	1.74 ± 0.1^{a,c}	2.24 ± 0.13^{b,d}	2.12 ± 0.07^{b,c}	2.36 ± 0.04^{d}
C20:4 ω6	3.57 ± 0.18^{a}	3.86 ± 0.17^{a}	3.76 ± 0.1^{a}	4.0 ± 0.07^{a}	3.71 ± 0.16^{a}	4.35 ± 0.1^{b}	3.95 ± 0.1^{a}	3.98 ± 0.06^{a}
C20:5 ω3	3.46 ± 0.16^{a,b}	3.81 ± 0.2^{a,b}	3.44 ± 0.19^{a,b}	3.66 ± 0.09^{a}	3.18 ± 0.16^{b}	4.52 ± 0.1^{c}	4.59 ± 0.20^{c}	4.60 ± 0.1^{c}
C22:5 ω3	1.82 ± 0.08^{a}	1.77 ± 0.11^{a}	1.1 ± 0.07^{b}	1.16 ± 0.03^{b}	1.08 ± 0.06^{b}	1.77 ± 0.16^{a}	2.62 ± 0.2^{c}	2.51 ± 0.11^{c}
C22:4 ω6	0.91 ± 0.06^{a}	1.10 ± 0.08^{b}	0.73 ± 0.04^{c}	0.88 ± 0.02^{a}	0.70 ± 0.05^{c}	0.89 ± 0.05^{a}	0.91 ± 0.04^{a}	0.91 ± 0.04^{a}
C24:1 ω9	2.2 ± 0.2^{a}	2.4 ± 0.2^{a,d}	1.7 ± 0.08^{b,f}	1.8 ± 0.04^{c}	1.6 ± 0.2^{b,c}	2.6 ± 0.1^{d,e,f}	3 ± 0.25^{e}	2.9 ± 0.1^{e,f}
C22:6 ω3	1.23 ± 0.05^{a,b}	1.16 ± 0.05^{a}	1.36 ± 0.04^{b,c}	1.35 ± 0.03^{b,c}	1.42 ± 0.05^{c,d}	1.52 ± 0.03^{d}	1.37 ± 0.05^{b,c}	1.31 ± 0.03^{b}
Σ SFA	24.8 ± 0.1^{a,b}	24.3 ± 1.04^{a}	24.4 ± 1.4^{a,b,d}	24.8 ± 0.3^{a,b,d}	24.3 ± 0.3^{b,c,d}	24.4 ± 0.3^{a,b,d}	23.5 ± 0.2^{c}	23.9 ± 0.2^{c,d}
Σ MUFA	46.5 ± 0.8^{a,b}	45.8 ± 1.1^{a,b}	47.6 ± 0.6^{a}	46.8 ± 1.0^{b}	47.5 ± 1.1^{a,b}	46.9 ± 0.8^{a,b}	46.7 ± 0.4^{a,b}	46.9 ± 0.4^{a,b}
Σ PUFA	19.36 ± 0.4^{a,d}	21.02 ± 0.1^{a}	17.10 ± 0.6^{b,c,e}	16.25 ± 0.34^{b}	16.9 ± 0.2^{b,c}	18.6 ± 0.7^{a,c,d}	19.81 ± 0.5^{a,d}	18.95 ± 0.4^{d,e}
Σ ω6	10.76 ± 0.6^{a}	11.9 ± 1.2^{a}	9.06 ± 0.6^{a,b,c,d}	7.65 ± 0.29^{b,d}	8.99 ± 0.23^{c}	7.99 ± 0.5^{b,c,d}	8.81 ± 0.33^{c}	7.75 ± 0.27^{b,d}
Σ ω3	8.59 ± 0.3^{a,b,c}	9.08 ± 0.46^{a}	8.06 ± 0.5^{a,b}	8.60 ± 0.20^{a}	7.52 ± 0.38^{b}	10.41 ± 0.41^{c,d}	10.99 ± 0.52^{d}	11.19 ± 0.28^{d}
ω6/ω3	1.31 ± 0.12^{a}	1.38 ± 0.2^{a}	1.17 ± 0.1^{a,b}	0.89 ± 0.04^{b,c}	1.17 ± 0.06^{a}	0.79 ± 0.06^{b,c,d}	0.82 ± 0.6^{c,d}	0.69 ± 0.03^{d}

Values represent relative amounts, expressed as percentage of the total identified fatty acids by weight, and are means ± SEM from each group (n = 10), C (control), D (diabetic), CD30 (control + DHA 30 mg/kg/day), DD30 (diabetic + DHA 30 mg/kg/day), CD60 (control + DHA 60 mg/kg/day), DD60 (diabetic + DHA 60 mg/kg/day), CS (control + soybean) and DS (diabetic + soybean). SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, ND: not detectable. Values in the same row not sharing a same superscript letter were significantly different from each other.

Table 4. Fatty acid modifications induced by ω3 PUFA supplementations on red blood cells membranes

Fatty acids	C	D	CD30	DD30	CD60	DD60	CS	DS
C16:0	25.7 ± 0.3^{a}	24.1 ± 1^{a}	20.4 ± 0.2^{b}	18.6 ± 0.5^{c}	21.4 ± 0.4^{b,e}	19.2 ± 0.2^{d}	22.5 ± 0.5^{e}	17.3 ± 0.5^{c}
C18:0	14.2 ± 0.2^{a}	13.5 ± 0.6 ^{a,c}	14.9 ± 0.3^{a,c}	16.1 ± 0.1^{b}	15.3 ± 0.5^{a,c,d}	15 ± 0.26^{c,e}	16.3 ± 0.3^{b,d}	16 ± 0.3^{b,d,e}
C18:2 ω6	9.15 ± 0.2^{a}	12.42 ± 0.7^{b}	9.96 ± 0.18^{c}	15.36 ± 0.6^{d}	10.36 ± 0.3^{c,e}	14.56 ± 0.4^{d}	11.3 ± 0.4^{b,e}	15.17 ± 0.66^{d}
C20:4 ω6	18.42 ± 0.8^{a}	16.92 ± 0.9^{a}	29.3 ± 0.1^{b}	22.7 ± 0.8^{c,e}	26.4 ± 0.67^{d}	25.3 ± 0.4^{c,d}	23.0 ± 0.67^{e}	25.4 ± 0.96^{d,e}
C20:5 ω3	1.66 ± 0.1^{a}	1.68 ± 0.35^{a}	0.73 ± 0.04^{b}	0.89 ± 0.06^{b}	0.9 ± 0.08^{b}	0.82 ± 0.05^{b}	0.89 ± 0.08^{b}	0.87 ± 0.05^{b}
C22:5 ω3	3.07 ± 0.2^{a,b}	3.31 ± 0.28^{a}	2.75 ± 0.05^{b,f}	2.23 ± 0.06^{c}	2.51 ± 0.07^{d}	2.79 ± 0.11^{a,b,d}	1.94 ± 0.08^{e}	2.51 ± 0.16^{c,d,f}
C22:6 ω3	5.25 ± 0.26^{a,b,d}	5.55 ± 0.57^{a,b,d}	5.58 ± 0.15^{a}	4.75 ± 0.47^{b,d}	5.20 ± 0.19^{a,b,d}	5.77 ± 0.24^{a,b}	3.14 ± 0.24^{c}	5.03 ± 0.17^{d}
Σ SFA	39.8 ± 0.3^{a}	37.6 ± 1.5^{a,c,e}	35.2 ± 0.4^{b,d}	34.6 ± 0.5^{b,c,f}	36.7 ± 0.6^{d,e}	34.3 ± 0.2^{b,c,f}	38.8 ± 0.63^{a}	32.9 ± 0.8^{f}
Σ MUFA	10.9 ± 0.13^{a}	7.9 ± 0.27^{b}	10.6 ± 0.3^{a}	8.4 ± 0.2^{b,d}	12.43 ± 0.4^{c}	8.7 ± 0.18^{d}	11 ± 0.45^{a,c}	7.7 ± 0.25^{b}
Σ PUFA	37.5 ± 1.0^{a}	40.2 ± 0.95^{a}	46.3 ± 0.25^{b,e}	45.9 ± 0.7^{b,c}	44.3 ± 0.9^{c}	49.2 ± 0.34^{d}	40.28 ± 0.79^{e}	49.0 ± 0.5^{d}
Σ ω6	27.6 ± 0.98^{a}	29.3 ± 1.04^{a}	37.2 ± 0.19^{b,c}	38.1 ± 0.49^{b}	35.7 ± 0.7^{c,e}	39.8 ± 0.34^{d}	34.3 ± 0.6^{e}	40.61 ± 0.39^{d}
Σ ω3	9.97 ± 0.27^{a}	9.98 ± 0.97^{a,b,c}	9.05 ± 0.15^{b}	7.87 ± 0.47^{c}	8.61 ± 0.21^{b,e}	9.37 ± 0.3^{a,b}	5.97 ± 0.31^{d}	8.41 ± 0.19^{c,e}
ω6/ω3	2.77 ± 0.12^{a}	3.16 ± 0.35^{a}	4.12 ± 0.03^{b}	4.94 ± 0.28^{c,e}	4.15 ± 0.08^{b}	4.27 ± 0.15^{b,c}	5.83 ± 0.25^{d}	4.83 ± 0.19^{e}

Values represent relative amounts, expressed as percentage of the total identified fatty acids by weight, and are means ± SEM from each group (n = 10), C (control), D (diabetic), CD30 (control + DHA 30 mg/kg/day), DD30 (diabetic + DHA 30 mg/kg/day), CD60 (control + DHA 60 mg/kg/day), DD60 (diabetic + DHA 60 mg/kg/day), CS (control + soybean) and DS (diabetic + soybean). SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, ND: not detectable. Values in the same row not sharing a same superscript letter were significantly different from each other.

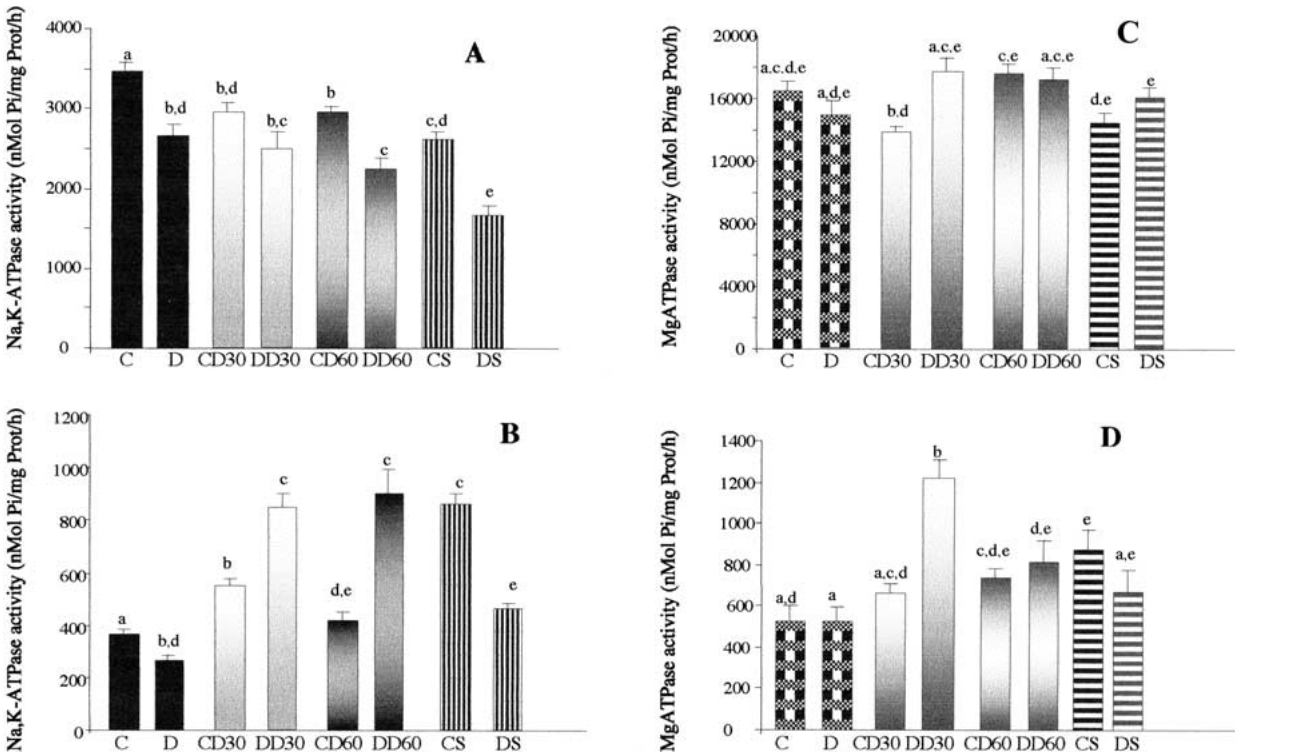

Fig. 1. Effects of ω3-enriched supplementation on Na,K-ATPase and Mg-ATPase activities. Na,K-ATPase activity was measured as the difference between inorganic phosphate released in the presence and absence of ouabain in sciatic nerve (*A*) and RBC (*B*). MgATPase activity was measured as the difference between inorganic phosphate released in the presence of ouabain and in blanks (ATP and buffer) in sciatic nerve (*C*) and RBC (*D*). Data are represented as mean ± SEM of activities performed in triplicate by spectrophotometric method, ($n = 10$ for each group). Values not sharing a same superscript differ significantly, with $p < 0.05$.

Effects of DHA-enriched Supplementation on Na,K-ATPase Activity

Diabetes induces a significant decrease in Na,K-ATPase activity in sciatic nerve and RBC membranes in group D (Fig. 1*A*, *B*).

In the sciatic nerve, the DHA-enriched supplementations induce a slight decrease in Na,K-ATPase activity in the control groups, CD30 and CD60, in a dose-independent manner (Fig. 1A). In the diabetic groups, DHA, at low dose, does not have any consequence on the decrease in activity observed during diabetes, whilst at high dose, we note an aggravation of this decrease.

In RBC membranes, in contrast, the DHA-enriched supplementations induce a significant stimulation of Na,K-ATPase activity in all the groups (Fig. 1*B*). However, the increases observed in the DD30 and DD60 groups are clearly more important as compared to the control groups, CD30 and CD60.

Effects of Soybean Supplementation on Na,K-ATPase Activity

In sciatic nerve, the soybean supplementation causes a significant decrease (−36%) in the Na,K-ATPase activity in the CS group, which is equivalent to the decrease observed during diabetes (D), as shown in Fig. 1*A*. This supplementation has a negative effect on the enzyme activity in the DS group, thus aggravating the decrease due to diabetes.

In RBC membranes, the soybean supplementation causes a significant stimulation of Na,K-ATPase activity that is more marked (+130%) in the CS group (Fig. 1*B*).

Effects of ω3-enriched Supplementation on Mg-ATPase Activity

Mg-ATPase activity is not significantly altered during diabetes (Fig. 1*C*, *D*). In the sciatic nerve, only the DHA-enriched supplementation at lowdose induces a significant diminution in activity, in control group (CD30) (Fig. 1*C*). In RBC membranes, the supplementations increased Mg-ATPase significantly in all groups except in CD30 and DS (Fig. 1*D*).

Relations between Membrane FA Composition and Na,K-ATPase and Mg-ATPase Activities

In view of the FA variations observed in our study, we expected to find an evident link between these

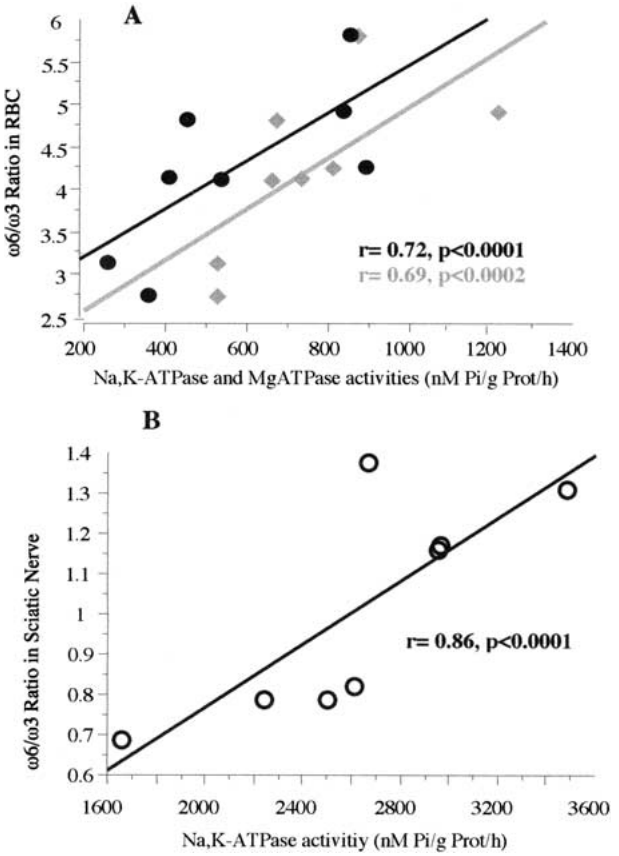

Fig. 2. Correlations between total ω6/ω3 ratio in RBC membranes and in sciatic nerve homogenates and ATPase activities. Correlations were evaluated by a single linear regression analysis. Correlation analysis has been tested with average values of ω6/ω3 ratio and ATPase activities from each group: Na,K-ATPase activity in RBC (*A*) (●), in sciatic nerve (*B*) (○) and Mg-ATPase activity in RBC (*A*) (♦).

variations and the modifications in enzymatic activities. We have used simple regression analysis to detect a correlation between Na,K-ATPase activity and the FA present in these tissues (Table 5). Several specific correlations have thus been observed in each tissue, with also similar variations in these two tissues, associating, essentially, PUFA of the ω3 and ω6 family and the variations in Na,K-ATPase activity. Indeed, we observe that the variations in the enzyme activity are positively and significantly correlated with changes in the total ω6/ω3 ratio in sciatic nerve ($p < 0.0001$) as well as in RBC membranes ($p < 0.0001$) (Fig. 2). These data clearly show that the Na,K-ATPase activity may be essentially modulated by the ω6/ω3 ratio in the membranes.

Using the same approach, we find a positive correlation between the variations of Mg-ATPase activity in RBC membranes and the total ω6/ω3 ratio present in the membranes ($p < 0.0002$) (Fig. 2). In sciatic nerve, we did not find any correlation, because the Mg-ATPase activity was not altered significantly

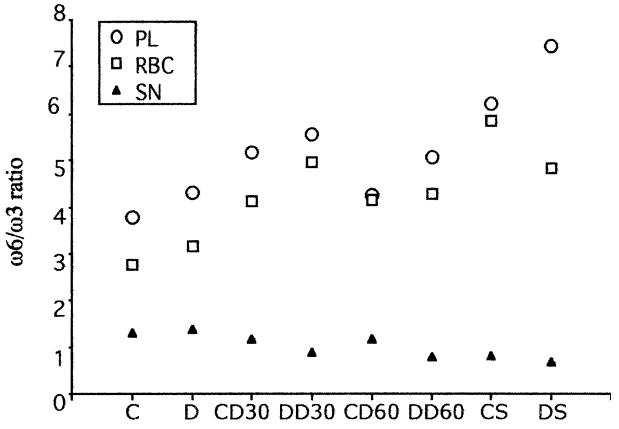

Fig. 3. Variations of ω6/ω3 ratio by ω3-enriched supplementations. Mean values of ω6/ω3 ratio of each group in plasma (PL), red blood cell (RBC) and sciatic nerve (SN) are represented.

by diabetes or supplementations, suggesting that the FA modifications in this tissue did not affect this activity.

Discussion

The different ω3-enriched supplementations used either as precursors or as very long-chain FA have different repercussions on Na,K-ATPase and Mg-ATPase activities in sciatic nerves and in erythrocyte membranes. The effects observed are also tissue-specific. We have also observed that each supplementation has a distinct effect on the lipid composition of the membranes, which is again tissue-specific. However, these modifications do not palliate the specific alterations due to diabetes although a DHA supplementation prevents the diabetes-induced defect in nerve conduction velocity (Gerbi et al., 2002).

It is of interest that DHA and soybean supplementations modify the incorporation of ω3 and ω6 PUFA in the membranes in a tissue-specific manner; they both cause a decrease in the level of ω6 PUFA in the sciatic nerve but an increase in RBC membranes. The effect of DHA supplementation on RBC membrane AA level is greatly different from that with fish-oil supplementation where EPA decreases the AA level (Gerbi et al., 1997). The regulation of the level of ω3 PUFA in the membranes is dependent on the type of supplementation used. Thus, the use of the ω3 PUFA precursor, in the soybean supplementation, leads to an increase in the incorporation of the metabolic end products of ω3 PUFA, namely, EPA, DPA and DHA in sciatic nerve membranes, whilst the levels of these PUFA are diminished in RBC membranes. On the other hand, the use of the metabolic end-product, DHA, essentially leads to its incorporation in sciatic nerve membranes and a decrease in the incorporation of DPA and EPA in RBC membranes.

Table 5. Relationship between membrane fatty acid and Na,K-ATPase and Mg-ATPase activities

Fatty acid	Na,K-ATPase				Mg-ATPase	
	Sciatic nerve		Red blood cell		Red blood cell	
	r	*p*	*r*	*p*	*r*	*P*
C16:0	+0.51	< 0.0001	−0.34	0.008	−0.28	0.03
C18:0			+0.45	0.003	+ 0.43	0.0006
C18:2 ω6	+0.36	0.001	+ 0.34	0.009	+ 0.32	0.01
C18:3 ω3	−0.30	0.01				
C18:4 ω3	−0.41	0.0002				
C20:4 ω6			+ 0.3	0.02		
C20:5 ω3	−0.46	< 0.0001	−0.37	0.004		
C22:5 ω3	−0.31	0.006	−0.47	0.002	−0.36	0.004
C24:1 ω9	−0.32	0.004				
C22:6 ω3			−0.34	0.008	−0.4	0.0017

Correlations were evaluated by single regression analysis. The correlations have been tested between membranous FA of each tissue and the corresponding Na,K-ATPase or Mg-ATPase activities.

Rather than a molecular or a structural modification in the enzyme itself, the variations in the activities observed can be due to a change in the composition of the FA constituting the surrounding membrane environment. So the membrane could regulate the enzyme through its structural composition or by modifications on its biophysical properties.

It has been shown that a deficiency in ω3 PUFA is associated with a variation in Na,K-ATPase properties (Gerbi et al., 1993, 1994, 1999a,b). Furthermore, several supplementation studies have shown specific effects of ω3 PUFA on Na,K-ATPase. Indeed, the number of enzyme units present in the membranes (Gerbi et al., 1997, 1998; Sennoune et al., 2000), the ouabain-affinity of the enzyme isoforms (Gerbi et al., 1997, 1998; Sennoune et al., 2000) as well as its sodium sensitivity (Gerbi et al., 1993, 1994, 1999c) and activity (Abeywardena et al., 1984; Gerbi et al., 1998; Sennoune et al., 2000) are modulated by the diet. All these observations suggest the existence of a physiological ω3 PUFA regulation for Na,K-ATPase.

We have used egg and soybean phospholipids, both rich in ω3 PUFA, to determine the effects of an ω3-enriched supplementation on two ATPase activities, Na,K-ATPase and Mg-ATPase, which are membrane-bound proteins whose activity is in part dependent on the FA membrane composition.

Several significant associations have been found between the variations in the FA-specific levels and Na,K-ATPase and Mg-ATPase activities. Up to date, only heterogeneous results have been obtained concerning the direct effects of FA on Na,K-ATPase activity. However, some effects obtained by in vitro studies are confirmed by dietary manipulations, which modify the enzyme membrane environment. Here we observe that the enzymatic activity of Na,K-ATPase is negatively correlated with the amounts of EPA and DHA in the membranes, as already observed in vitro (Mayol et al., 1999), while, on the contrary, a positive correlation exists with the LA level in the membranes. However, it must be kept in mind that contradictory results have been described in vitro (Swarts et al., 1990; Nguyen et al., 1998).

Subsequently, a positive correlation between the ratio of ω6/ω3 PUFA in the tissue membranes and Na,K-ATPase activity was found for both tissues. Gerbi & Maixent (1999) have already shown that, although dietary fish oil had a variable effect on Na,K-ATPase activity in the heart, kidney, nerve and brain, the level of membrane ω6 PUFA in all these tissues was positively correlated to an increase in the affinity of α1 isoform for ouabain. No other relationship has been deduced from this study.

The modification of the ω6/ω3 ratio by deficiency in ω3 PUFA has also been shown by Gerbi et al. (1999c) in brain. The role of this ratio in the regulation of Na,K-ATPase activity was only suspected then. Here we show, for the first time, the implication of this ratio in the regulation of enzyme activity of two ATPases. Indeed, such an implication of this ratio in modulation of other enzymatic activities has already been shown in heart for succinate-cytochrome c reductase (McMurchie et al., 1983) and calcium-magnesium ATPase activity (Swanson, Lokesh & Kinsella, 1989).

Given that ω6 and ω3 PUFA have different spatial conformations, we can suggest that the ratio between these two PUFA families participates in variations of membrane properties, which in turn could modulate Na,K- and Mg-ATPase activities in RBC membranes. These structural properties would be necessary for movements of Na,K-ATPase during its enzymatic cycle.

ω3 PUFA-enriched supplementations not only cause modifications in the membrane environment of

the enzyme but seem also to modify the pool of enzyme present in the membrane. Fish oil restores the proportion of α1 and α3 isoforms in diabetic rats as compared to controls supplemented with olive oil (Gerbi et al., 1998) and α2 and β2 isoforms have been found to be upregulated in the nerve fibers of diabetic rats supplemented with fish oil, as detected by confocal microscopy (Gerbi et al., 1999b). The expression of the α1 isoform, representative of 50% of the enzyme activity in purified membranes of nerve (Gerbi et al., 1998), was evaluated. Compared to the effect of fish oil, we note an increase in the amounts of the α1 isoform in the diabetic groups supplemented with DHA. Furthermore, this increase is also observed in the control groups, suggesting a regulatory effect of DHA on the amount of α1 isoform in membranes (*data not shown*).

However, we must keep in mind that in diabetic rats, and in the absence of any supplementation, the early decrease in activity is essentially induced by the insulinopenic state (Sima & Sugimoto, 1999). We must pay attention to the fact that the regulation of Na,K-ATPase activity implies several factors (Therien & Blostein, 2000), but in the light of our results we can suggest that, following the supplementations with DHA and soybean phospholipids, the ω6/ω3 ratio participates in the regulation of the enzyme activity in diabetic as well as in control rats. In RBC membranes, Mg-ATPase activity seems to be regulated the same way as Na,K-ATPase activity. Nevertheless, in the SN homogenates, there is no relationship between this activity and the ω6/ω3 ratio. So, these ATPase activities are not similarly sensitive to regulation by lipids in spite of their extensive structure homology.

During experimental insulin-dependent diabetes, it has been shown that the decreases in Na,K-ATPase activities in sciatic nerve and RBC membranes are correlated (Scarpini et al., 1993; Raccah et al., 1994; Djemli-Shipkolye et al., 2001). In type 1 diabetic patients, since RBC membrane Na,K-ATPase activity was positively correlated with tibial and peroneal nerve conduction, Raccah et al. (1996) suggested that the enzyme activity in RBC membranes could be a reliable surrogate of the activity in sciatic nerve. However, we clearly show that ω3 PUFA supplementations induce completely different modifications in Na,K-ATPase activity in RBC membranes and sciatic nerve in the rat. The use of the Na,K-ATPase activity in RBC membranes as a marker of the activity in sciatic nerve, during treatment with ω3 PUFA, must be done with caution. Na,K-ATPase regulation by PUFA being tissue-specific, it is important to study this regulation in each tissue and very likely in each animal species. In spite of these tissue-specific differences, we show that the ω6/ω3 ratio correlation with Na,K-ATPase activity is the same in the two tissues studied, i.e., sciatic nerve and RBC membranes. We thus suggest that the dietary modulation of this ratio may be important in Na,K-ATPase activity in both tissues and in Mg-ATPase activity in RBC membranes. Further studies are underway to determine if this modulation is tissue-specific or is a common pathway for these ATPases.

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