Neuroprotective Effect of Docosahexaenoic Acid–Enriched Phospholipids in Experimental Diabetic Neuropathy

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A deficiency in essential fatty acid metabolism has been widely reported in both human and animal diabetes. Fish oil supplementations (n-3 fatty acids), containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were less effective on diabetic neuropathy than (n-6) fatty acids. This partial effect of (n-3) fatty acids might be attributed to the presence of EPA, a competitor of arachidonic acid, which enhanced the diabetes-induced decrease of this fatty acid in serum and tissues. For determining whether a supplementation with DHA alone could prevent neuropathy in streptozotocin-induced diabetes, diabetic rats were given daily, by gavage, liposomes containing DHA phospholipids, at a dose of 60 mg/kg. Eight weeks of diabetes induced significant decreases in nerve conduction velocity (NCV), nerve blood flow (NBF), and sciatic nerve and erythrocyte (red blood cells [RBCs]) Na,K-ATPase activities. DHA phospholipids totally prevented the decrease in NCV and NBF observed during diabetes when compared with the nonsupplemented diabetic group. DHA phospholipids also prevented the Na,K-ATPase activity decrease in RBC but not in sciatic nerve. Moreover, DHA level in sciatic nerve membranes was correlated with NCV. These results demonstrate a protective effect of daily doses of DHA on experimental diabetic neuropathy. Thus, treatment with DHA phospholipids could be suitable for evaluation in clinical trials.
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In type 1 diabetes, hyperglycemia and hypoinsulinemia lead to a spectrum of metabolic and vascular abnormalities, including an increase of the polyol pathway, abnormalities in lipid metabolism, advanced glycosylated end product formation, increased oxidative damage, defects in growth factors, and endoneurial hypoxia (1,2). Although the cause of diabetic neuropathy remains unknown, its correlation with these aforementioned changes seems to occur in a similar temporal sequence (3). Diabetes impairs essential fatty acid metabolism by decreasing activities of Δ6 and Δ5 desaturases, enzymes that convert dietary linoleic acid (LA) and α-linolenic acid to long-chain polyunsaturated fatty acids (PUFA), including γ-linolenic acid (GLA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (4). As a result, AA and DHA levels are reduced in membrane phospholipids of several tissues, including erythrocyte and sciatic nerve, in patients with type 1 diabetes and in diabetic animals (5–7).

It was demonstrated recently that dietary supplementation with GLA (8,9) and with fish oil, containing EPA and DHA (9,10), prevented completely for GLA and partially for fish oil the diabetes-induced decrease in nerve conduction velocity (NCV), a physiological marker of diabetic neuropathy. These results could be explained in part by a normalization of eicosanoid synthesis, which is depressed in diabetic nerve, and/or by a direct effect on incorporation of these fatty acids into the plasma membranes (1,8,11). By changing membrane properties, PUFA can modify the activity of transmembrane enzymes, such as the Na,K-ATPase, which is implicated in the propagation of nerve impulses. We previously reported that diets deficient in α-linolenic acid or, in contrast, rich in EPA and DHA modulate the functional properties of Na,K-ATPase isoenzymes (12,13). In diabetic neuropathy, Na,K-ATPase activity is dramatically decreased in rat and human sciatic nerve and erythrocytes (14–16).

Nevertheless, we previously found that the preventive effect of fish oil on NCV and Na,K-ATPase activity is only partial in diabetic rats (10,17). Moreover, fish oil treatment has been demonstrated to have some deleterious effects in retinopathy (18). This could be due to a counteracting effect of EPA, which enhances the decrease in AA levels in plasma and tissue phospholipids, by competition for desaturase enzyme, as a result of a structural homology.

In the present study, we investigated the effects of DHA supplementation using liposomes containing DHA phospholipids on neurophysiological, i.e., NCV and nerve blood flow (NBF), and biological parameters, i.e., Na,K-ATPase activity and erythrocyte/sciatic nerve membrane fatty acid compositions. Our results present evidence for a marked neuroprotective effect of DHA on diabetic neuropathy.

RESEARCH DESIGN AND METHODS

Animals. The 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 A 13823. The principles of laboratory

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AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; NBF, nerve blood flow; NCV, nerve conduction velocity; P_i, inorganic phosphate; PPAR, peroxisome proliferator–activated receptor; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SFA, saturated fatty acid; STZ, streptozotocin.

TABLE 1
Fatty acid composition in diet supplementations

Fatty acid	DHA 60	Standard diet
C16:0	135.6	156
C18:0	81.4	30
C18:1	174.6	480
C18:2 n-6 (LA)	90.2	870
C18:3 n-3 (ALA)	ND	Traces
C20:4 n-6 (AA)	21.2	ND
C20:5 n-3 (EPA)	9.6	ND
C22:6 n-3 (DHA)	60	ND
Σ PUFA	181	870
Σ n-6	111.4	870
Σ n-3	69.6	Traces
n-6/n-3	1.6	

Data are expressed in $mg \cdot kg^{-1} \cdot day^{-1}$ for each supplementation. ALA, α-linolenic acid; ND, not detectable.

animal care (National Institutes of Health) were followed. Male Sprague-Dawley rats ($n = 40$; Iffa Credo, Saint Germain de l'Arbresle, France) were entered in the study after acclimatization for 1 week. Their body weight at the beginning of the study averaged 232 ± 12 g, and they were randomly assigned to four weight-matched groups ($n = 10$). For the two diabetic groups, diabetes was induced by a single intravenous injection of streptozotocin (STZ; 65 mg/kg; Sigma, St. Louis, MO), freshly dissolved in citrate sodium buffer (0.01 mol/l, pH 5.5). Control rats received an injection of buffer only. All diabetic rats were maintained without insulin. Diabetes was checked 3 days 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). Animals were given a standard nonpurified rodent diet (A04; UAR, Epinay sur Orge, France) and water ad libitum. Gavage was started on the day of STZ or buffer administration. Two groups were given no supplementation, i.e., the control (C) and diabetic (D) groups. The control (CDHA) and diabetic (DDHA) groups were given DHA phospholipids at a daily dose of $0.8\ g \cdot kg^{-1} \cdot day^{-1}$ corresponding to 60 mg/kg DHA per day. DHA supplementation was administered daily at 0900. No difference in food intake was observed between the supplemented groups. After 8 weeks of supplementation, rats were killed by intraperitoneal anesthesia using pentobarbital (50–100 mg/kg).

Preparation and composition of the supplementation. The supplementation consisted of an egg-phospholipid preparation enriched in DHA. Egg phospholipids were extracted with alcohol. After evaporation of the alcoholic phase using a rotative evaporator, the phospholipids were hydrated at 60% with distilled water. Note that phospholipids, in particular DHA phospholipids, are reported to be more stable than triglycerides toward peroxidation (19). The phospholipid preparation was prepared freshly every week, maintained under nitrogen in the dark and at 4°C, and regularly analyzed to monitor lipid peroxidation. The fatty acid composition of the standard diet and supplementation are given in Table 1. After these analyses, we were able to determine the daily intake of the principal fatty acids concerned with this study.

NCV measurement. After anesthesia, rat backs were shaved and motor NCV was recorded as previously described (8) in a temperature-controlled environment from the left sciatic tibial nerve by a modified noninvasive method adapted from Stevens et al. (20). Briefly, the rectal temperature was maintained at 37°C, and the left sciatic nerve was stimulated proximally at the sciatic notch and distally at the knee via bipolar electrodes by a Neuromatic 2000C (Disa, Skovlunde, Denmark). The muscle action potential was recorded from the ankle by unipolar pin electrodes. NCV was calculated as the ratio of the distance in millimeters between both sites of stimulation divided by the difference between proximal and distal latencies measured in milliseconds, giving a value for NCV in meters per second.

NBF measurement. After NCV was recorded, NBF was assessed according to Yasuda et al. (21) using a laser Doppler flowmeter (Periflux, model 4001 master; Perimed, Stockholm, Sweden) as previously described (11). Briefly, the left sciatic nerve was exposed without bleeding, and the probe was lowered at a right angle to the surface of the perineurium at 1 cm below the sciatic notch. NBF was then recorded continuously for at least 10 min, and the values were averaged to one value.

Tissue preparations. After physiological measurements, blood was collected by cardiac puncture into 0.11 mol/l sodium citrate tubes. Sciatic nerves from the spine to the peroneal bifurcation were dissected and frozen in liquid nitrogen after removal of adherent tissue. Samples were preserved at −80°C until use.

Erythrocyte and plasma. Plasma was separated by centrifugation at 1,500*g* for 15 min. Leukocytes and platelets were removed from the blood samples by filtration through a microcrystalline cellulose column (22). Erythrocytes were hemolyzed in 11 mmol/l Tris buffer and centrifuged (30,000*g* for 30 min at 4°C), and the membrane pellet obtained was resuspended in 30 ml of buffer. The centrifugation step was repeated three times, as previously described (23). The washed erythrocyte membranes were then stored for Na,K-ATPase activity measurement and fatty acid composition determination.

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

Na,K-ATPase activity measurement. Na,K-ATPase activity was measured on purified plasma membranes from erythrocytes or sciatic nerves as previously described (23). Briefly, the release of inorganic phosphate (P_i) from ATP was measured using spectrophotometry with or without 1 mmol/l ouabain (Sigma), a specific Na,K-ATPase inhibitor. After incubation with 4 mmol/l 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 (5,500*g*, 10 min) at 4°C, the amount of P_i in the supernatant was determined according to the method of Hurst (24). Na,K-ATPase activity was calculated as the difference between P_i released per milligram of protein per hour in the presence and absence of ouabain. Membrane protein concentration was determined using a protein assay (Bio-Rad Laboratories, Munich, Germany). All assays were performed in triplicate, and blanks were included to determine the endogenous phosphate and non-enzyme–related breakdown of ATP.

Membrane fatty acid phospholipid composition. Total plasma, erythrocyte membranes, and sciatic nerve homogenate lipids were extracted with methanol and chloroform according to the method of Bligh and Dyer (25), modified by the use of a sonicator. Fatty acid composition was determined after methylation with BF3-methanol (Sigma) according to Ohta et al. (26). 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 BPX 70 (60 m × 0.22 mm inner diameter; SGE, Villeneuve St. Georges, France) equipped with a flame ionization detector and using hydrogen as the carrier gas. The temperature program ranged from 160 to 205°C with a rise of 1°C/min. Peak areas from the resulting chromatogram were measured with a Perkin Elmer 1022S integrator. Fatty acids were identified by their retention times on the column with respect to appropriate standards.

Statistical analysis. Results are expressed as means ± SEM. A Kolmogorov-Smirnov test for normality and a Bartlett test for homogeneous variance were performed for each group. All of the data were analyzed using a nonparametric Kruskal-Wallis test, and differences between groups were tested using the Mann-Whitney U test. $P < 0.05$ was considered significant. All analyses were done using the STATVIEW software (Abacus Concepts, Berkeley, CA) on a Macintosh iMac (Apple Computer, Les Ulis, France).

RESULTS

DHA does not correct abnormal diabetes-induced metabolic characteristics. Plasma glucose levels in both diabetic groups were increased ~500% relative to all nondiabetic control groups (36 ± 1.8 vs. 8.5 ± 0.3 mmol/l; $P < 0.0001$). Body weight gain was decreased in diabetic groups as compared with control groups (252 ± 10 vs. 444 ± 5 g; $P < 0.0001$). Lipid supplementation had no effect on these parameters with the exception of DHA, which increased body weight by 7% in DHA-supplemented control animals compared with nonsupplemented ones (464 ± 9 vs. 434 ± 5; $P = 0.01$).

The observed decrease in sciatic NCV in diabetic rats is totally prevented by DHA supplementation. Eight weeks of diabetes induced a 30% decrease in sciatic NCV

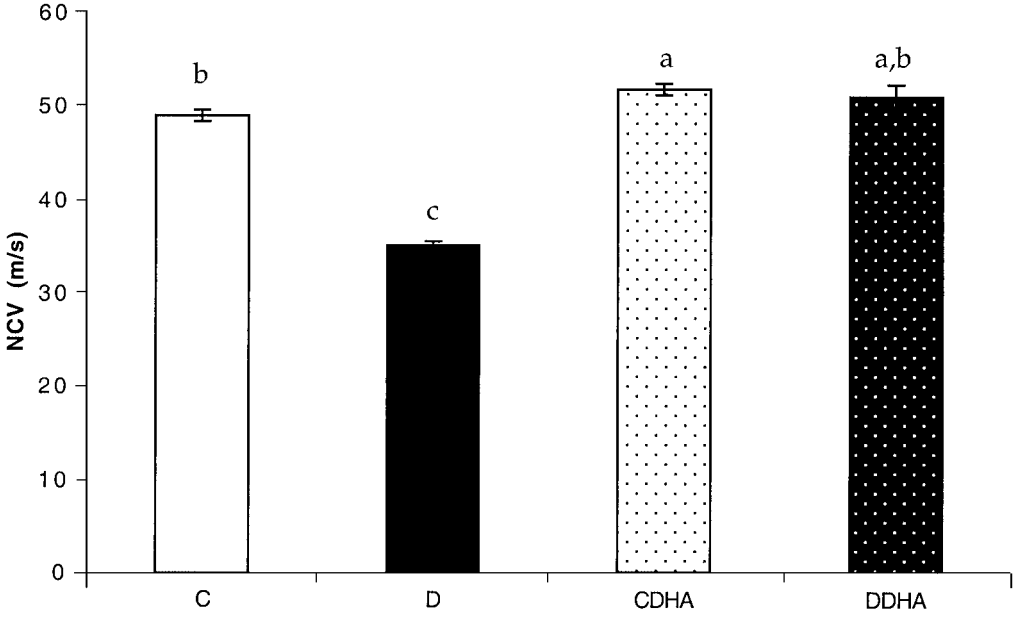

FIG. 1. NCV was recorded from control (C) and diabetic (D) rats supplemented or not with DHA liposomes (60 mg · kg^{-1} · d^{-1}). Results, expressed in m/s, are means ± SEM (n = 10). Bars not bearing at least one same superscript letter are significantly different.

($P < 0.0001$; Fig. 1). This deficit was completely prevented by supplementation with 60 mg/kg DHA ($P < 0.0002$). Moreover, DHA supplementation slightly increased the NCV in control rats (6%; $P < 0.006$).

DHA supplementation corrects the sciatic NBF. Similar to the NCV, a decrease in NBF was observed in diabetic rats (−50%; $P < 0.0001$; Fig. 2). DHA supplementation completely prevented the decrease in NBF ($P < 0.01$). Contrary to NCV, DHA supplementation had no effect on control rat NBF.

Sciatic NCV and NBF are correlated. When the results from all rats were plotted, a significant positive correlation between NCV and NBF was found ($r = 0.54$, $P = 0.0005$; data not shown).

The alteration in sciatic nerve Na,K-ATPase activity in diabetic rats is not prevented with DHA supplementation. Na,K-ATPase activity was decreased in sciatic nerve homogenates from diabetic rats (−25%; $P < 0.0001$; Fig. 3). DHA supplementation was unable to prevent the diabetes-induced decrease in Na,K-ATPase activity in the diabetic group ($P < 0.01$). Moreover, DHA supplementation decreased the basal level of Na,K-ATPase activity in control and diabetic groups when compared with the control and diabetic group without supplementation. Nevertheless, these decreases did not affect NCV.

DHA supplementation prevents the diabetes-induced decrease in red blood cell Na,K-ATPase activity. Similar to the sciatic nerve, 8 weeks of diabetes resulted in a decrease in red blood cell (RBC) Na,K-ATPase activity (−30%; $P < 0.006$; Fig. 4). DHA supplementation induced a slight but not significant increase in the control group and a threefold increase in the diabetic group ($P < 0.001$) when compared with their respective controls without supplementation.

Diabetes and DHA supplementation induce large changes in the plasma fatty acid composition. Diabetes induced an accumulation in LA ($P = 0.001$) and di-homo GLA (C20:3 [n-6]; $P = 0.02$), likely as a result of

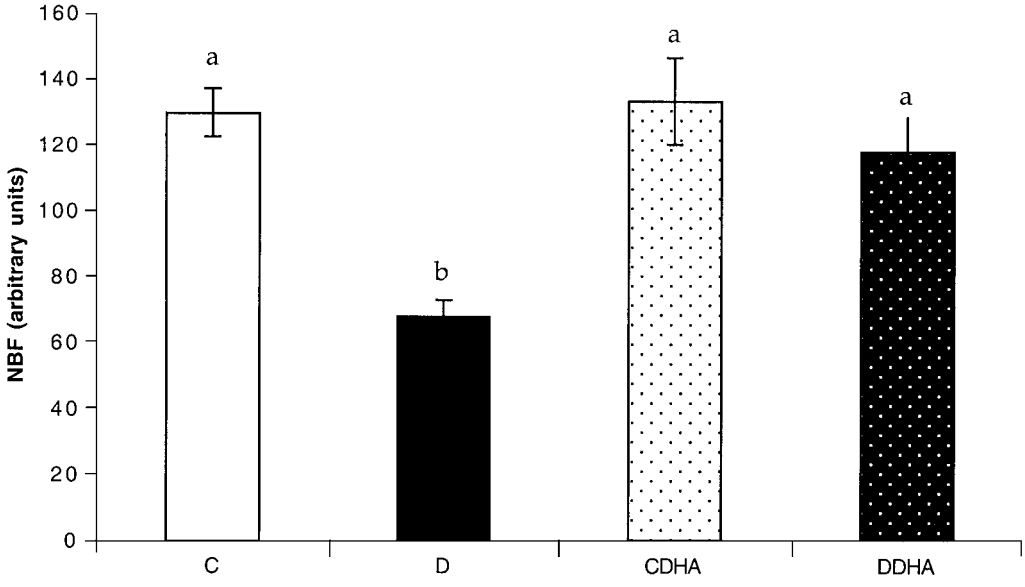

FIG. 2. NBF was recorded from control (C) and diabetic (D) rats supplemented or not with DHA liposomes (60 mg · kg^{-1} · day^{-1}). Results, expressed in arbitrary units, are means ± SEM (n = 10). Bars not bearing at least one same superscript letter are significantly different.

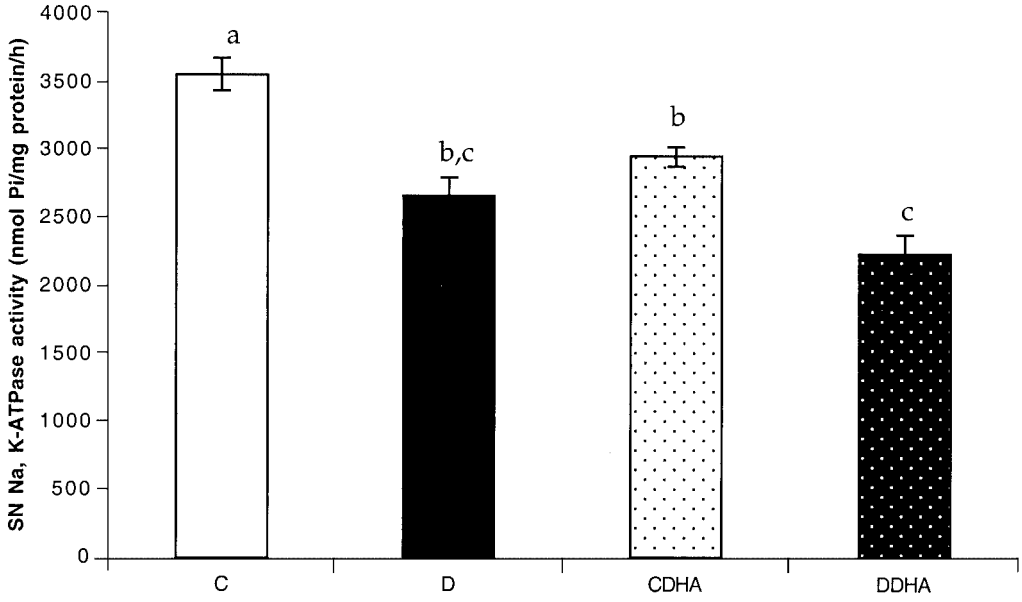

FIG. 3. Sciatic Nerve (SN) Na,K-ATPase activity was recorded from control (C) and diabetic (D) rats supplemented or not with DHA liposomes (60 mg · kg^{-1} · day^{-1}). Results, expressed in nmol Pi · mg protein^{-1} · h^{-1}, are mean ± SEM (n = 10). Bars not bearing at least one same superscript letter are significantly different.

decreased desaturase activities (4). A slight but nonsignificant decrease in AA was also observed. Overall, diabetic animals exhibited substantial decreases in monounsaturated fatty acids (MUFAs; P = 0.001), whereas PUFA levels increased (P = 0.001). Saturated fatty acid (SFA) levels remained unchanged (Table 2). DHA supplementation increased the plasma LA level and, hence, the level of PUFA in the control group, whereas the diabetes-induced decrease in MUFA was attenuated. DHA supplementation prevented the diabetes-induced decrease in AA levels. Plasma DHA levels remained unchanged (Table 2).

Diabetes and DHA supplementation modify RBC membrane fatty acid composition. As described for plasma fatty acids, diabetes induced an increase in LA level (P = 0.006) and a decrease in MUFA level (P = 0.002) in erythrocyte membranes. Although AA levels tended to decrease after induction of diabetes, it did not reach significance (Table 2). DHA supplementation decreased the RBC membrane SFA level in both diabetic and control groups. Moreover, a significant increase in PUFA level, resulting from increases in LA and AA levels, was observed (Table 2).

Changes in sciatic nerve membrane fatty acid composition are only minor. Eight weeks of diabetes was not long enough to observe substantial changes in the fatty acid composition of sciatic nerve membranes. DHA supplementation slightly influenced the sciatic nerve membrane fatty acid composition with a decrease in PUFA level and an increase in DHA level in both control and diabetic groups (Table 3).

Physiological and biochemical parameters are correlated. When the results from all rats were plotted, some correlations were observed. RBC Na,K-ATPase was correlated with NCV (r = 0.47, P = 0.02) but not with NBF (r = 0.30, P = 0.16). There was a positive correlation between sciatic nerve and RBC Na,K-ATPase activities (r = 0.46, P = 0.02). Moreover, NCV was positively correlated with DHA level in sciatic nerve membranes (r = 0.44, P = 0.004). Some strong correlations between RBC Na,K-ATPase activity and levels of SFA (r = −0.55, P = 0.002),

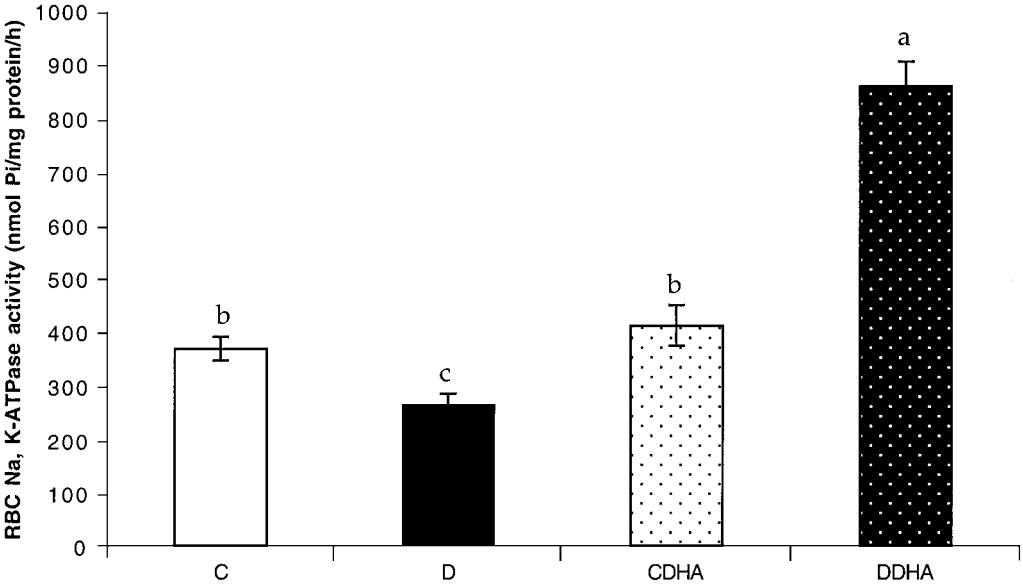

FIG. 4. RBC Na,K-ATPase activity was recorded from control (C) and diabetic (D) rats supplemented or not with DHA liposomes (60 mg · kg^{-1} · day^{-1}). Results, expressed in nmol Pi · mg protein^{-1} · h^{-1}, are means ± SEM (n = 10). Bars not bearing at least one same superscript letter are significantly different.

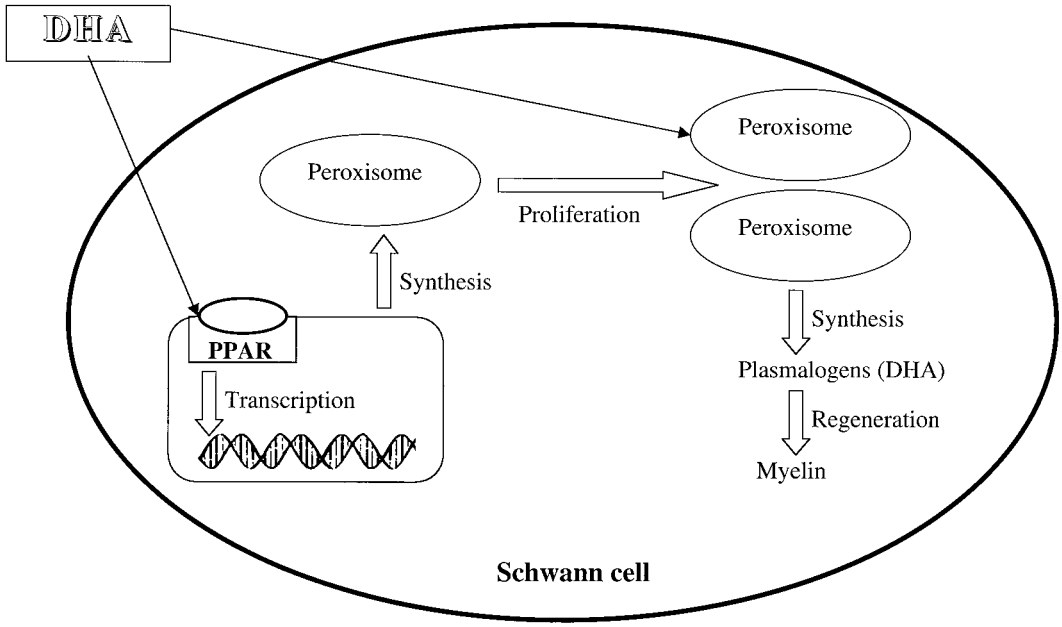

FIG. 5. DHA may prevent the development of diabetic neuropathy.

PUFA ($r = 0.72$, $P < 0.0001$), and n-6 fatty acids ($r = 0.71$, $P < 0.0001$) in erythrocyte membranes were found.

DISCUSSION

The present study demonstrates a neuroprotective effect of egg phospholipids enriched with DHA in rat diabetic neuropathy. We showed a beneficial effect of DHA on two neurophysiological indexes of diabetic neuropathy, NCV and NBF.

In experimental diabetic neuropathy, dramatic decreases in NCV and NBF have been widely reported (3,8,27,28). For explaining these defects in nerve physiology, numerous hypotheses have been proposed as reviewed by Cameron and Cotter (1). Among these, the importance of a decrease in Na,K-ATPase activity has been actively debated (1,3,27). Indeed, it has been shown that the sciatic nerve and RBC Na,K-ATPase activities are decreased in diabetes, in both human and animal studies (8,14–16). Das et al. (29) first described this decrease in sciatic nerve from diabetic rat, and this hypothesis was then incorporated by Greene et al. (30) in their physiopathological scheme of the polyol pathway. A decrease in sciatic nerve Na,K-ATPase activity could alter the normal membrane axon repolarization after the depolarization induced by an action potential, and one therefore could expect a decrease in NCV. Moreover, a similar decrease in RBC Na,K-ATPase activity could alter the NBF by increasing erythrocyte volume, which in turn reduces their deformability (31).

Wright and Nukada (28) found significant reductions in NCV after 16 weeks of diabetes, with trends apparent after 4 weeks. Because diabetes was induced in mature rats in this study, there was a delay before the NCV decrease became significant. Indeed, when diabetes is induced in

TABLE 2
Fatty acid composition of plasma and red blood cell membranes

	Plasma				Red blood cell membranes			
Fatty acid	C	D	CDHA	DDHA	C	D	CDHA	DDHA
C16:0	19.6 ± 0.5^{a}	17.6 ± 0.3^{b}	14.9 ± 0.3^{c}	14.6 ± 0.4^{c}	25.7 ± 0.3^{a}	24.1 ± 0.9a	21.4 ± 0.4^{b}	19.2 ± 0.3^{c}
C16:1	3.5 ± 0.3^{a}	0.8 ± 0.1^{c}	2.5 ± 0.2^{b}	0.6 ± 0.1^{c}	1.0 ± 0.1a	0.4 ± 0.2^{b}	0.4 ± 0.1^{b}	ND
C18:0	6.9 ± 0.2^{c}	7.7 ± 0.2^{b}	8.0 ± 0.3^{b}	10.2 ± 0.4^{a}	14.1 ± 0.2^{b}	13.5 ± 0.7^{a,b}	15.3 ± 0.5^{a,b}	15.1 ± 0.3^{a}
C18:1	15.1 ± 0.7^{b}	9.7 ± 0.4^{c}	19.3 ± 0.9^{a}	13.2 ± 1.0^{b}	9.9 ± 0.1^{b}	7.5 ± 0.3^{d}	11.2 ± 0.3^{a}	8.7 ± 0.2^{c}
C18:2(n-6)	19.9 ± 0.3^{c}	31.5 ± 1.0^{a}	21.8 ± 0.6^{b}	30.5 ± 1.1^{a}	9.1 ± 0.2^{d}	12.4 ± 0.7^{b}	10.4 ± 0.3^{c}	14.6 ± 0.4^{a}
C20:3(n-6)	0.8 ± 0.1^{b}	1.2 ± 0.1^{a}	1.3 ± 0.1^{a}	1.4 ± 0.1^{a}	ND	ND	ND	ND
C20:4(n-6)	13.1 ± 0.7^{b}	11.8 ± 0.8^{b}	16.6 ± 0.9^{a}	14.6 ± 1.5^{a,b}	18.4 ± 0.8^{b}	16.9 ± 1.0^{b}	25.4 ± 0.7^{a}	25.3 ± 0.4^{a}
C20:5(n-3)	1.7 ± 0.1^{b}	2.4 ± 0.2^{a}	1.5 ± 0.1^{b}	1.6 ± 0.1^{b}	1.7 ± 0.1^{a}	1.7 ± 0.3^{a}	0.9 ± 0.1^{b}	0.8 ± 0.1^{b}
C22:5(n-3)	1.9 ± 0.1^{a}	2.0 ± 0.1^{a}	1.6 ± 0.1^{b}	1.4 ± 0.1^{b}	3.1 ± 0.2^{a}	3.3 ± 0.3^{a}	2.5 ± 0.1^{b}	2.8 ± 0.1^{a,b}
C22:6(n-3)	5.4 ± 0.2^{a}	6.0 ± 0.2^{a}	6.3 ± 0.3^{a}	6.5 ± 0.5^{a}	5.3 ± 0.3^{a}	5.6 ± 0.6^{a}	5.2 ± 0.2^{a}	5.8 ± 0.2^{a}
SFA	26.5 ± 0.5^{a}	25.3 ± 0.3^{a,b}	23.0 ± 0.4^{c}	24.8 ± 0.4^{b}	39.8 ± 0.4^{a}	37.6 ± 1.6^{a,b}	36.7 ± 0.7^{b}	34.3 ± 0.2^{b}
MUFA	18.7 ± 1.0^{b}	10.5 ± 0.4^{c}	21.8 ± 1.0^{a}	14.5 ± 2.2^{b}	10.9 ± 0.1^{a}	7.9 ± 0.3^{b}	11.3 ± 0.4^{a}	8.7 ± 0.2^{b}
PUFA	42.8 ± 1.2^{c}	55.0 ± 0.6^{a}	49.1 ± 0.9^{b}	55.9 ± 1.2^{a}	37.5 ± 1.0^{c}	40.2 ± 1.0^{c}	44.3 ± 0.9^{b}	49.2 ± 0.3^{a}
n-6	33.8 ± 0.9^{c}	44.5 ± 0.7^{a}	39.7 ± 1.0^{b}	46.4 ± 1.3^{a}	27.6 ± 1.0^{a}	29.3 ± 1.0^{a}	35.7 ± 0.8^{b}	39.8 ± 0.3^{c}
n-3	9.0 ± 0.4^{b}	10.5 ± 0.3^{a}	9.4 ± 0.2^{b}	9.5 ± 0.6^{a,b}	10.0 ± 0.3^{a}	10.0 ± 1.0^{a,b}	8.6 ± 0.2^{b}	9.4 ± 0.3^{a,b}
n-6/n-3	3.8 ± 0.1^{b}	4.3 ± 0.2^{a}	4.2 ± 0.2^{a}	5.1 ± 0.4^{a}	2.8 ± 0.1^{b}	3.2 ± 0.4^{b}	4.2 ± 0.1^{a}	4.3 ± 0.2^{a}

Data are means ± SEM ($n = 10$). Values in the same row not bearing at least one same superscript letter are significantly different. ND, not detectable.

TABLE 3
Fatty acid composition of sciatic nerve

Fatty acid	C	D	CDHA	DDHA
C16:0	15.0 ± 0.4[a]	14.0 ± 0.5[a,b]	14.4 ± 0.3[a]	12.9 ± 0.2[b]
C16:1	2.5 ± 0.2[a,b]	1.4 ± 0.2[c]	3.2 ± 0.4[a]	1.8 ± 0.3[b,c]
C18:0	9.9 ± 0.4[b]	10.3 ± 1.1[a,b]	9.9 ± 0.6[b]	11.5 ± 0.4[a]
C18:1	41.9 ± 0.9[a]	41.8 ± 1.1[a]	42.7 ± 1.4[a]	41.9 ± 1.0[a]
C18:2(n-6)	6.3 ± 0.8[a]	6.6 ± 1.4[a]	5.4 ± 1.1[a]	3.9 ± 0.8[a]
C18:4(n-3)	1.8 ± 0.1[b]	2.1 ± 0.1[a]	1.7 ± 0.1[b]	2.2 ± 0.1[a]
C20:4(n-6)	3.6 ± 0.2[b]	3.9 ± 0.2[a,b]	3.7 ± 0.2[b]	4.2 ± 0.1[a]
C20:5(n-3)	3.5 ± 0.2[b,c]	3.8 ± 0.2[b]	3.2 ± 0.2[c]	4.3 ± 0.2[a]
C22:5(n-3)	1.8 ± 0.1[a]	1.8 ± 0.1[a]	1.1 ± 0.1[b]	1.7 ± 0.2[a]
C22:6(n-3)	1.2 ± 0.1[b]	1.2 ± 0.1[b]	1.4 ± 0.1[a]	1.5 ± 0.1[a]
SFA	24.9 ± 0.1[a]	24.3 ± 1.0[a]	24.3 ± 0.3[a]	24.4 ± 0.3[a]
MUFA	46.6 ± 0.8[a]	45.8 ± 1.1[a]	47.5 ± 1.2[a]	46.2 ± 0.8[a]
PUFA	19.4 ± 0.4[a]	21.0 ± 1.0[a]	17.6 ± 0.7[b]	18.7 ± 0.6[a,b]
n-6	11.0 ± 0.6[a]	12.2 ± 1.3[a,b]	10.2 ± 1.0[a,b]	9.1 ± 0.7[b]
n-3	8.3 ± 0.4[b,c]	9.0 ± 0.4[a,b]	7.4 ± 0.4[c]	9.6 ± 0.4[a]
n-6/n-3	1.4 ± 0.1[a]	1.5 ± 0.2[a]	1.5 ± 0.3[a]	1.0 ± 0.1[b]

Data are means ± SEM (n = 10). Values in the same row not bearing at least one same superscript letter are significantly different.

growing rats, a significant decrease in NCV is observed as early as 2 weeks (32). Here, we found a similar impairment in the NCV in the diabetic group after 8 weeks of disease, in agreement with our previous studies (8,10,11). Although only partial, fish oil supplementation has shown a beneficial effect on NCV (9,10). In contrast to the fish oil supplementation, DHA supplementation was able to totally prevent the diabetes-induced decrease in NCV. Lastly, DHA (60 mg · kg^{-1} · day^{-1}) significantly induced a slight increase in NCV in nondiabetic control rats. These findings could be explained in part by the positive correlation between DHA level in sciatic nerve membranes and NCV. This correlation remained when the results from additional groups, i.e., soybean and DHA 30 mg · kg^{-1} · day^{-1} groups, were included (data not shown).

Disturbances in Na,K-ATPase activity in the sciatic nerve have been proposed to be partially but not fully responsible for the slowing of NCV in the diabetic rat (1,3) because, in some studies, the slowing of motor NCV seems to precede the decrease in enzyme activity (28,32). In our studies, we always observe a partial restoration of Na,K-ATPase activity associated with either partial (10) or total (8) restoration of conduction velocity in sciatic nerve of diabetic rats. In addition, Coppey et al. (32) showed that the temporal decrease between these two parameters was similar, although the decrease in motor NCV became significant more rapidly (14 days) than the decrease in Na,K-ATPase activity (28 days). All of these observations argue for a partial rather than a primordial implication of a decrease in Na,K-ATPase as the cause of the diabetes-induced decrease in NCV. Some authors hypothesized that the early defect in NBF could be responsible for the decrease in NCV (1,32) and that a treatment reversion of nerve ischemia, i.e., an increase in NBF, could gradually improve NCV by an action on nerve microvessels (33). However, we have observed that the decrease in NCV can be partially prevented by a half-dose of DHA phospholipids (30 mg · kg^{-1} · day^{-1}) or soybean phospholipid supplementations without correcting the NBF in diabetic rats (data not shown). So, like Na,K-ATPase activity, NBF seems to be only partially implicated in diabetes-induced changes in NCV. It therefore seems likely that several parameters contribute to the diabetes-induced changes in NCV.

The role of NBF and the presence of peripheral nerve ischemia in triggering early diabetic neuropathy are controversial (1,34). Some groups have postulated that early reductions in NBF account for pathogenesis of diabetic neuropathy (35,36), and a 50% reduction in NBF as early as 3 days after the induction of diabetes has been reported (32). Others, in contrast, have not identified early ischemia in animal (37) or human (38) studies. These discrepancies may be explained, in part, by the different methods used as well as by the duration and severity of diabetes in animals and humans (1). Cameron and Cotter (1) proposed that during the course of diabetes, there is first a hyperperfusion, which results in an increase in blood flow, followed by a hypoperfusion as vessel disease progresses; the hyperperfusion period could be dramatically shortened by the severity of hyperglycemia. With the laser Doppler flowmetry method used in the present study, we showed a decrease in NBF in rats after 8 weeks of experimental diabetes. Importantly, we showed that DHA supplementation (60 mg · kg^{-1} · day^{-1}) fully prevented the decrease in NBF in diabetic animals. Alterations in membrane fluidity have been implicated in the regulation of Na,K-ATPase activity, and a positive correlation between DHA incorporation into RBC membranes and membrane deformability has been reported (39,40). Nevertheless, our results demonstrated that an increase in Na,K-ATPase activity did not correlate with increased NBF. Furthermore, we found no relationship between the RBC level of DHA and NBF. It is possible that the DHA effect reflects a direct role of this fatty acid rather than one mediated via alteration in membrane properties and/or Na,K-ATPase activity. Supplementation with DHA could be expected to lead to step retroconversion of DHA to EPA. This retroconversion could reverse the diabetes-induced vasoconstriction by enhancing the production of vasoactive eicosanoids from series 1 and 3 and inducing a diminution of eicosanoids from series 2 (9). These altered eicosanoid profiles may therefore affect the vascular endothelium-dependent relaxation and thus improve NBF (41).

Previous studies demonstrated dramatic changes in

PUFA composition of diabetic tissues and also in phosphatidylinositol turnover in sciatic nerve (42,43). In this study, only minor changes in fatty acid levels occurred in the sciatic nerve of diabetic rats. We did not find the slight but significant decrease in AA level as previously described by Gerbi et al. (44). However, we have examined the fatty acid composition of sciatic nerve homogenates as compared with purified sciatic nerve membranes, and this particularity could explain the observed differences. Moreover, DHA supplementation induced an increase of its incorporation into the sciatic nerve membranes and could explain in part the observed decrease in basal level of sciatic nerve Na,K-ATPase activity. In contrast, DHA supplementation induced a twofold increase in RBC Na,K-ATPase activity in the diabetic group and totally prevented the diabetes-induced decrease. Further studies will be required to link this finding to increased Na,K-ATPase subunit membrane expression or in Na,K-ATPase phosphorylation mediated by protein kinase C α isoform. The effect of DHA supplementation on RBC Na,K-ATPase activity and NCV and the positive correlation between these two parameters are interesting for human studies. Indeed, RBCs are readily accessible in humans and could represent a reliable surrogate for the assessment of DHA supplementation–induced effects on nerve function in clinical studies (15).

But how can we explain the effectiveness of DHA in the prevention of experimental diabetic neuropathy? Although, at present, the mechanism is not known, the following scenario may apply: Diabetes is known to induce histological damage to the sciatic nerve fibers, including endoneural edema and axonal degeneration, with occasional secondary segmental demyelination (45). Plasmalogens are lipids that are important components of myelin (46). It is interesting that peroxisomes are involved in the breakdown of long-chain PUFAs, by the process called β-oxidation, and particularly in the last step of DHA synthesis, i.e., from C24:6 (n-3) to C22:6 (n-3) (47), and that they are also required for the synthesis of plasmalogens. Indeed, it has been suggested that peroxisomal synthesis of plasmalogens plays an important role in the prevention of demyelination (48), and DHA is preferentially incorporated in sn-2 position in ethanolamine plasmalogens, relative to other PUFAs. Moreover, peroxisome proliferation is regulated by the peroxisome proliferator–activated receptors (PPARs), which in turn are regulated by polyunsaturated C20 and C22 fatty acids, including DHA (49). Thus, one could expect that nutritional supplementation with DHA leads to an upregulation of PPARs, causing proliferation of the peroxisomes and therefore a rise in synthesis of ethanolamine plasmalogens containing DHA. This would therefore result in the prevention of segmental demyelination in sciatic nerve induced by diabetes (Fig. 5). This hypothesis could also explain the prevention of segmental demyelination observed in diabetic rats supplemented with fish oil (10).

DHA and EPA supplementations have already shown beneficial effects on cardiovascular diseases such as atherosclerosis and coronaropathy in patients with diabetes (50). Diabetic neuropathy is among the most common complication of diabetes but is also the least treatable. Advanced distal sensory, motor, and autonomic deficits underlie most foot ulcers and amputations in patients with diabetes. These patients are long awaiting a treatment independent of glycemic control. Recently, Goss et al. (51) indicated a protective effect of nerve growth factor gene transfer on experimental diabetic neuropathy, and DHA supplementation could enhance this nerve growth factor effect. We have demonstrated in this study that nutritional DHA supplementation is effective in preventing experimental diabetic neuropathy and could be suitable for clinical study with two advantages, harmlessness and low cost.

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