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Dietary Docosahexaenoic Acid Enhances Ferric Nitrilotriacetate-Induced Oxidative Damage in Mice but not when Additional α-Tocopherol is Supplemented

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Weaning mice were fed a diet supplemented with beef tallow (BT) or BT plus docosahexaenoic acid (DHA) containing 100 mg α-tocopherol/kg (α-Toc100) or 500 mg α-tocopherol/kg (α-Toc500) for 4 wk to modify membrane fatty acid unsaturation, and then were administered ferric nitrilotriacetate (Fe-NTA). The mortality caused by Fe-NTA was higher in the group fed the DHA (α-Toc100) diet than in the BT diet groups but the DHA (α-Toc500) diet suppressed this increase. Serum and kidney α-tocopherol contents were slightly influenced by the dietary fatty acids but not significantly. These results indicate that the increased unsaturation of tissue lipids enhances oxidative damage induced by Fe-NTA in mice fed DHA (α-Toc100) but not when additional α-tocopherol is supplemented. The apparent discrepancy between the observed enhancement by dietary DHA of oxidative damage and the beneficial effects of dietary DHA on the so-called free radical diseases is discussed in terms of strong bolus oxidative stress and moderate chronic oxidative stress.

Keywords: α-tocopherol, docosahexaenoic acid, ferric nitrilotriacetate, lipid peroxidation

Abbreviations: α-Toc100 and α-Toc500, 100 or 500 mg α-tocopherol/kgBT, beef tallowDHA, docosahexaenoic acidEPA, eicosapentaenoic acidFe-NTA, ferric nitrilotriacetatePI, peroxidizability indexPUFA, polyunsaturated fatty acids

INTRODUCTION

n-3 Polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (20 : 5n-3, EPA) and docosahexaenoic acid (22 : 6n-3, DHA) are more easily autoxidized in air than other less unsaturated fatty acids.[1] Feeding EPA and DHA-rich fish oil to animals leads to accumulation of n-3 PUFA in

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cellular membranes. Several studies have suggested that dietary fish oil or DHA increases lipid peroxide levels *in vivo*.[2–4] These observations led to a suggestion that feeding n-3 PUFA increases tissue vulnerability to oxidative stress causing lipid peroxidation. However, long-term feeding of n-3 PUFA, as compared with n-6 PUFA, has been shown to have beneficial effects against the so-called free radical diseases such as aging, carcinogenesis and atherosclerosis.[5] Obviously, we need more direct evidence to define the *in vivo* conditions under which membrane unsaturation exerts favorable and unfavorable effects on animal physiology.

Ferric nitrilotriacetate (Fe-NTA) has been used to induce renal tubular injury in rodents.[6] Thiobarbituric acid-reactive substances (TBARS) and 4-hydroxy-2-nonenal, both products of lipid peroxidation, contents increased in kidneys of rats following Fe-NTA injection.[7,8] Moreover, several antioxidants such as vitamin E[9,10] and probucol[11] have been reported to decrease lipid peroxide levels and inhibit renal injury in rodents treated with Fe-NTA. Free radicals and reactive oxygen species generated by Fe-NTA have been proposed to be responsible for the renal injury.[6] On the other hand, we have observed that DHA-rich fish oil and carbon tetrachloride (CCl_4) treatments do not synergistically enhance liver injury and accumulation of oxidation products in mice.[12,13] In this study, we have examined the effects of dietary DHA and α-tocopherol on mice treated with Fe-NTA.

MATERIALS AND METHODS

Animals and Diets

Female ICR mice at 3 wk of age (Shizuoka Laboratory Animals Co., Ltd., Shizuoka, Japan) were fed test diets for 4 wk. Test diets were prepared by mixing a conventional diet containing 3.0% (w/w) fatty acids and 109 mg α-tocopherol/kg (CE-2, Clea Japan, Inc., Tokyo) with 10% (w/w) beef tallow (BT) (Wako Pure Chemical Co., Osaka, Japan), or 6.0% (w/w) BT plus 4.0% (w/w) ethyl docosahexaenoate (Harima Chemicals Inc., Ibaraki, Japan). The final fatty acid content was 12.7% (w/w), corresponding to 29.7% of energy content of the test diet. The fatty acid composition of the diets is shown in Table I. A peroxidizability index was calculated as follows: % of monoenoate $\times$ 0.025 + % of dienoate $\times$ 1 + % of

TABLE I Fatty acid composition of experimental diets (% of total fatty acids)[a]

Fatty acid	Beef tallow (BT) diet	DHA diet
14:0	2.0	1.2
16:0	23.5	13.6
16:1	2.2	1.4
18:0	13.6	6.4
18:1n-9[b]	39.3	22.6
18:2n-6	15.8	17.1
18:3n-3	1.2	1.4
20:1	0.6	0.6
20:5n-3	0.6	2.5
22:6n-3	0.6	32.4
n-6/n-3[c]	6.43	0.48
PI[d]	30.0	294.6

[a] The fatty acid composition of the diets was analyzed by gas–liquid chromatography.
[b] The position of the double bond numbered from the methyl terminus is designated as n-9, n-6, or n-3.
[c] n-6/n-3 indicates the total n-6 fatty acids/total n-3 fatty acids ratio.
[d] Peroxidizability index = (% monoenoate $\times$ 0.025) + (% dienoate $\times$ 1) + (% trienoate $\times$ 2) + (% tetraenoate $\times$ 4) + (% pentaenoate $\times$ 6) + (% hexaenoate $\times$ 8).

trienoate × 2 + % of tetraenoate × 4 + % of pentaenoate × 6 + % of hexaenoate × 8.[14] α-Tocopherol contents in the test diets were adjusted to 100 or 500 mg/kg by adding α-tocopherol acetate (Sigma, St. Louis, MO). The peroxide values of the BT (α-Toc100), BT (α-Toc500), DHA (α-Toc100) and DHA (α-Toc500) diets were 3.4, 3.6, 5.8 and 5.9 meq/kg, respectively. The diets were kept frozen at −20°C for less than 10 days before serving, and replaced every day to keep the peroxide values of ingested diets minimal. Mice were kept at 23 ± 2°C and given free access to the test diets.

After being fed the test diets for 4 wk, mice were sacrificed to obtain blood and kidneys. The kidneys were frozen immediately in liquid nitrogen. Sera and kidneys were stored at −20°C and −80°C, respectively, until analysis.

Preparation and Administration of Ferric Nitrilotriacetate (Fe-NTA)

A Fe-NTA solution was prepared using the method reported by Li *et al.*[15] Briefly, 75 mM iron nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9H_2O$) (Wako) and 150 mM nitrilotriacetic acid disodium salt (Aldrich, Milwaukee, WI), dissolved in deionized water, were mixed at a ratio of 1 : 2 (molar ratio 1 : 4), and the pH was adjusted to 7.4 with sodium bicarbonate. After being fed the test diets for 4 wk, mice were injected intraperitoneally with the Fe-NTA solution at a dose of 5 or 10 mg of Fe/kg body weight, and the mortality was followed at 1 h intervals throughout the day.

Analysis of Phospholipid Fatty Acid in Kidney

Total lipid was extracted from kidney according to the method of Bligh and Dyer.[16] Phospholipid (PL) was separated by thin-layer chromatography on a Silica Gel G plate (E. Merck, Darmstadt, Germany) using petroleum ether/diethyl ether/acetic acid (80 : 30 : 1, by vol) as a developing solvent. The PL was visualized under an ultraviolet light after spraying with primuline (Nacalai Tesque Co., Kyoto, Japan) and was extracted from the adsorbent according to the method of Bligh and Dyer.[16] Fatty acids in the PL fraction were converted to methyl esters by treatment with 5% (w/v) HCl in methanol for 1 h in boiling water and then quantitated by gas–liquid chromatography with a capillary column (DB-225, J&W Scientific, Folsom, CA), using 1,2-diheptadecanoyl-L-α-phosphatidylcholine (Sigma) as an internal standard.

Determination of α-Tocopherol

Tocopherols were extracted from serum with *n*-hexane and those from diet and kidney were extracted with chloroform/methanol.[16] The diet extract was saponified with one mole ethanolic KOH in the presence of 5.5% (w/v) pyrogallol, and tocopherols were extracted with *n*-hexane. α-Tocopherol content was measured by high performance liquid chromatography (HPLC)[17,18] on a LiChrosorb $5NH_2$ column (4.6 × 150 mm) (GL Sciences Inc., Tokyo) equilibrated with *n*-hexane/isopropanol (98 : 2, v/v) at a flow rate of 0.8 ml/min and at 30°C. The elution was monitored fluorometrically (excitation at 298 nm and emission at 325 nm). 2,2,5,7,8-Pentamethyl-6-hydroxychroman (Wako) was used as an internal standard.

Protein Determination

Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Statistical Analysis

Data are presented as means ± SD. Statistical analysis of the mortality data was performed by Wilcoxon signed rank method using the computer program JMP3.0, Statistic Made Visual (SAS Institute Inc., Cray, NC). Statistical analysis of other data was performed by one-way ANOVA

using Bonferroni's multiple comparison (StatView-J 4.11, Abacus Concepts Inc., Berkeley, CA), and probability values below 0.05 were considered significant.

RESULTS

Mortality of Mice Treated with Fe-NTA

The body weights of the mice were not significantly different among the diet groups (data not shown). When mice were treated with Fe-NTA at a dose of 5 mg Fe/kg body weight, the mortality rate was significantly higher in the DHA (α-Toc100) diet group than in other diet groups (Figure 1A). No significant difference in the mortality rate was observed among the DHA (α-Toc500), BT (α-Toc100) and BT (α-Toc500) diet groups. At the higher dose of 10 mg Fe/kg body weight, the mortality rate increased, and that of the DHA (α-Toc100) diet group was significantly higher than those of the other groups (Figure 1B), indicating that dietary DHA stimulated the mortality rate induced by Fe-NTA at a marginal level of α-tocopherol (0.8 mg α-tocopherol/g oil) but an additional α-tocopherol supplement suppressed the stimulating effect of DHA.

Fatty Acid Composition of Phospholipids in Kidney

Since kidney has been reported to be the major target organ of Fe-NTA,[6] we analyzed the fatty acid composition of kidney phospholipids (Table II). The proportion of arachidonic acid was significantly lower but those of EPA and DHA were higher in the DHA diet groups compared with the BT diet groups; the difference in dietary α-tocopherol levels did not affect the fatty acid composition. Peroxidizability index was calculated to be about 25% higher in the DHA diet groups than in the BT diet groups.

α-Tocopherol Contents in Serum and Kidney

Reflecting the dietary α-tocopherol contents, tissue α-tocopherol contents were significantly higher in the α-Toc500 groups than in the α-Toc100 groups (Figure 2). Serum α-tocopherol

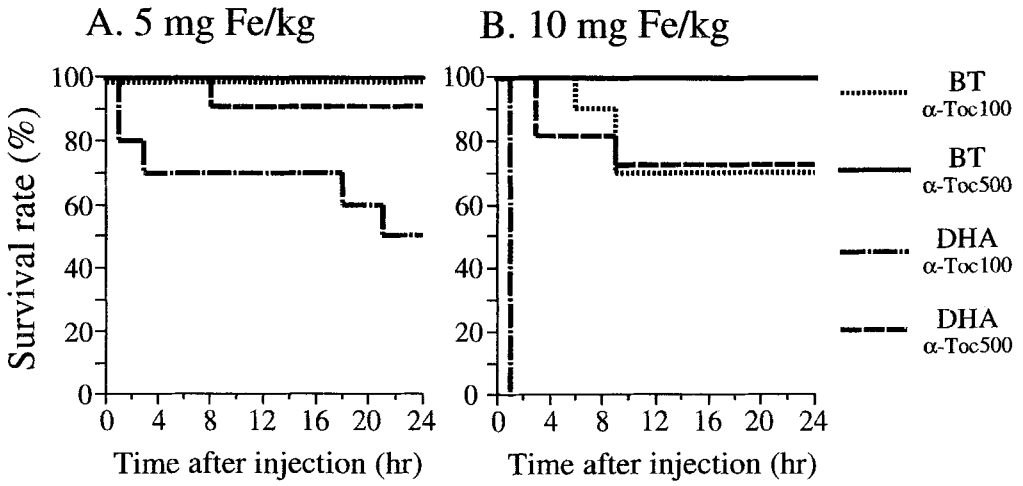

FIGURE 1 Mortality rate of mice fed the test diets for 4 wk and treated with Fe-NTA at a dose of 5 mg (A) or 10 mg (B) Fe/kg body weight. Statistical significance of the differences among the dietary groups was $p < 0.05$ for DHA (α-Toc100) vs. BT (α-Toc100), BT (α-Toc500) and DHA (α-Toc500) in the Wilcoxon test (A). Statistical significance of the differences among the dietary groups was $p < 0.01$ for DHA (α-Toc100) vs. BT (α-Toc100), BT (α-Toc500) and DHA (α-Toc500) in the Wilcoxon test (B). There were 10 to 11 mice per group.

TABLE II Fatty acid composition of kidney phospholipids from mice fed the beef tallow (BT) diet or DHA diet containing 100 or 500 mg α-tocopherol/kg (% of total fatty acid)[a]

Fatty acid	α-Tocopherol (mg/kg diet)							
	BT diet				DHA diet			
	100		500		100		500	
14:0	0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	
16:0DMA	0.9 ± 0.2		0.9 ± 0.1		0.7 ± 0.1		0.7 ± 0.2	
16:0	17.8 ± 1.0		18.4 ± 0.3		17.4 ± 1.0		17.6 ± 0.9	
16:1	0.3 ± 0.1		0.3 ± 0.1		0.2 ± 0.0		0.2 ± 0.0	
18:0DMA	1.3 ± 0.2	c	1.3 ± 0.1	bc	0.9 ± 0.2	a	0.9 ± 0.2	ab
18:1DMA	0.7 ± 0.2		0.7 ± 0.2		0.4 ± 0.1		0.5 ± 0.1	
18:0	17.0 ± 0.7		16.6 ± 0.5		17.7 ± 0.9		17.7 ± 0.9	
18:1	10.6 ± 0.5	b	10.4 ± 0.3	ab	8.8 ± 0.4	a	9.6 ± 1.6	ab
18:2n-6	11.0 ± 0.6	a	11.4 ± 0.4	ab	12.3 ± 0.6	b	11.4 ± 0.6	ab
18:3n-3	0.2 ± 0.1		0.2 ± 0.1		0.2 ± 0.0		0.2 ± 0.0	
20:0	0.3 ± 0.0		0.3 ± 0.0		0.3 ± 0.0		0.3 ± 0.0	
20:1	0.2 ± 0.0	b	0.2 ± 0.0	b	0.1 ± 0.0	a	0.1 ± 0.0	a
20:3n-6	1.2 ± 0.2	b	1.2 ± 0.1	b	0.7 ± 0.1	a	0.7 ± 0.0	a
20:4n-6	18.6 ± 0.6	b	18.0 ± 0.3	b	4.2 ± 0.4	a	4.3 ± 0.5	a
20:5n-3	0.9 ± 0.5	a	1.2 ± 0.2	a	8.5 ± 1.1	b	8.3 ± 0.7	b
22:0	1.1 ± 0.0		1.0 ± 0.0		1.0 ± 0.0		1.0 ± 0.2	
22:4n-6	0.2 ± 0.0	b	0.2 ± 0.0	b	ND	a	ND	a
22:5n-6	0.4 ± 0.0	b	0.4 ± 0.0	b	0.3 ± 0.0	a	0.3 ± 0.1	ab
22:5n-3	0.5 ± 0.1	b	0.5 ± 0.0	b	0.3 ± 0.0	a	0.4 ± 0.0	a
22:6n-3	13.7 ± 0.7	a	13.5 ± 0.6	a	22.8 ± 1.4	b	22.4 ± 1.0	b
24:0	2.0 ± 0.1		2.0 ± 0.2		2.3 ± 0.1		2.3 ± 0.3	
24:1	1.0 ± 0.1		1.0 ± 0.0		0.8 ± 0.1		0.9 ± 0.2	
n-6	31.3 ± 1.0	b	31.2 ± 0.6	b	17.5 ± 0.8	a	16.7 ± 0.8	a
n-3	15.4 ± 0.5	a	15.5 ± 0.6	a	31.8 ± 1.3	b	31.2 ± 1.0	b
n-6/n-3	2.04 ± 0.10	b	2.02 ± 0.11	b	0.55 ± 0.03	a	0.54 ± 0.03	a
PI	210 ± 4	a	208 ± 3	a	268 ± 11	b	264 ± 8	b
Fatty acid (μg/mg protein)	191.2 ± 4.4		204.5 ± 21.0		199.3 ± 35.1		185.2 ± 18.1	

[a] Values are means ± SD from 5 mice. Values with different letters are significantly different from each other among different dietary groups at $p < 0.05$ by one-way ANOVA using Bonferroni's multiple comparison.ND, not detected; PI, peroxidizability index.

content in the BT (α-Toc500) group tended to be higher than that in the DHA (α-Toc500) diet group ($p = 0.07$), but the differences in the dietary and tissue fatty acid composition brought about no significant effects on kidney α-tocopherol contents.

DISCUSSION

Isolated tissues from animals fed fish oil are vulnerable to lipid peroxidation *in vitro*.[19,20] However, whether or not oxidative stresses induced by iron has adverse effects in animals fed n-3 PUFA needs to be examined *in vivo*. In the present study, we estimated the mortality of mice induced by Fe-NTA treatment as an *in vivo* end point. When the dietary level of α-tocopherol was 100 mg/kg (α-Toc100, approximately 0.8 mg α-tocopherol/g oil), the Fe-NTA treatment induced no statistically significant change in the mortality of the BT diet group, but dietary DHA increased the mortality as well as tissue PI values. Additional supplementation of α-tocopherol (α-Toc500, approximately 3.9 mg α-tocopherol/g oil) significantly suppressed the Fe-NTA induced increase in mortality observed only in the DHA diet group. These results support the interpretation that elevated unsaturation of tissue phospholipid acyl chains by dietary manipulation results

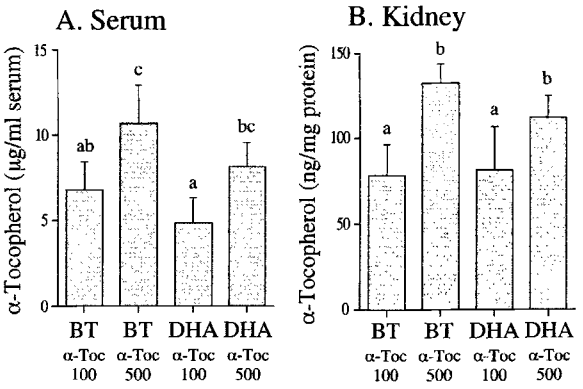

FIGURE 2 Serum (A) and kidney (B) α-tocopherol contents in mice fed the test diets for 4 wk. Each column with a bar represents the mean ± SD for 6 to 7 mice. Values with different letters are significantly different ($p < 0.05$) by one-way ANOVA using Bonferroni's multiple comparison.

in increased tissue lipid peroxides by bolus loading of oxidative stress and increased damage *in vivo* when endogenous antioxidative vitamins are limited. Common vegetable oils are known to be stabilized at ~0.5 mg α-tocopherol/g oil but several-fold higher levels of α-tocopherol are added to stabilize fish oils. On the other hand, the very low arachidonate content of kidney phospholipids in the DHA diet group (Table II) might have exerted additional unfavorable effects because eicosanoids derived from arachidonate have essential roles in kidney function.[21] When signal decay of carbamoyl-PROXYL, a nitroxide radical, was estimated as an indicator of the degree of free radical reaction by *in vivo* ESR measurements, the signal decay rates did not reflect the mortality rates of the mice treated with Fe-NTA (data not shown).

Our previous studies demonstrated that CCl_4 treatment and dietary DHA did not synergistically enhance liver injury and lipid peroxidation.[12,13] These distinct effects of CCl_4 and Fe-NTA administrations may be ascribed to the difference in their tissue distribution, the species of free radicals generated, and/or their efficiency to cause tissue damages. CCl_4 is metabolized by liver microsomal cytochrome P-450 to the trichloromethyl radical (CCl_3•), which immediately forms the trichloromethylperoxyl radical (CCl_3OO•) following reaction with O_2, and then CCl_3OO• initiates lipid peroxidation.[22]

A large amount of evidence has been accumulated supporting that long-term feeding of n-3 PUFA (α-linolenic acid, EPA and DHA) is beneficial for the suppression of so-called free radical diseases (i.e., aging, carcinogenesis and atherosclerosis) as summarized elsewhere.[5] These diseases are interpreted to be due mainly to relatively weak but persistent oxidative stresses associated with ischemia and inflammation, which are suppressed by dietary n-3 PUFA in the presence of appropriate amounts of endogenous antioxidants and a coupled antioxidative enzyme system. When the endogenous antioxidative systems are impaired or bolus oxidative stresses over the endogenous antioxidative capacity are loaded, as in the Fe-NTA treatment examined in the present study, increased unsaturation of tissue fatty acids accelerates lipid peroxidation and peroxidative damage. We should be careful in applying the results of animals loaded with extremely strong oxidative stresses to natural processes of free radical diseases in which moderate and chronic oxidative stresses are possibly involved, and eicosanoid tone related to n-6 and n-3 PUFA balance is mainly involved.

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