

## Polyunsaturated (n-3) Fatty Acids Susceptible to Peroxidation Are Increased in Plasma and Tissue Lipids of Rats Fed Docosahexaenoic Acid-Containing Oils

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**ABSTRACT** Docosahexaenoic acid [DHA, 22:6(n-3)], a major component of membrane phospholipids in brain and retina, is profoundly susceptible to oxidative stress *in vitro*. The extent of this peroxidation in organs when DHA is ingested in mammals, however, is not well elucidated. We investigated the effect of dietary DHA-containing oils (DHA 7.0–7.1 mol/100 mol total fatty acids), in the form of triacylglycerols (TG), ethyl esters (EE) and phospholipids (PL), on tissue lipid metabolism and lipid peroxidation in rats. Groups of Sprague-Dawley rats were fed semipurified diets containing 15 g/100 g test oils and were compared with those fed 80% palm oil and 20% soybean oil as the control (unsupplemented group) for 3 wk. The DHA oil diets markedly increased ( $P < 0.05$ ) the levels of DHA in the plasma, liver and kidney, 1.5–1.9, 2.5–3.8 and 2.2–2.5 times the control values, respectively, whereas there was a concomitant reduction ( $P < 0.05$ ) in arachidonic acid. All forms of DHA oil caused lower TG concentrations in plasma ( $P < 0.05$ ) and liver ( $P < 0.05$ ), but had no effect in kidney. The DHA oil-fed rats had greater phospholipid hydroperoxide accumulations in plasma (191–192% of control rats), liver (170–230%) and kidney (250–340%), whereas the  $\alpha$ -tocopherol level was reduced concomitantly (21–73% of control rats). Consistent with these results, rats fed DHA-containing oils had more thiobarbituric reactive substances in these organs than the controls. Thus, high incorporation of (n-3) fatty acids (mainly DHA) into plasma and tissue lipids due to DHA-containing oil ingestion may undesirably affect tissues by enhancing susceptibility of membranes to lipid peroxidation and by disrupting the antioxidant system. *J. Nutr.* 130: 3028–3033, 2000.

**KEY WORDS:** • chemiluminescence • fish oil • lipid peroxidation • phospholipid hydroperoxide •  $\alpha$ -tocopherol • rats

In pathophysiologic studies, it has been suggested that (n-3) polyunsaturated fatty acids (PUFA),<sup>2</sup> such as docosahexaenoic acid [DHA, 22:6(n-3)], may prevent atherosclerosis, heart disease and tumor growth (Boreson et al. 1989, Harris 1996, Kinsella et al. 1990, Reddy et al. 1991, Sakaguchi et al. 1990). Increased intake of DHA may ameliorate lipid abnormalities such as hyperlipidemia and imbalances of (n-6) and (n-3) fatty acids (Lands 1990, Lees and Karel 1986). However, because DHA has six double bonds and five methylene carbons, it is highly susceptible to peroxidation. DHA is more readily oxidized and results in more toxic oxidation products than linoleic, linolenic and arachidonic acids (Cho et al. 1987, Witting and Horwitt 1964). Furthermore, polyunsaturated oil intake decreases the concentration of vitamin E in animal tissues (Leibowitz 1990, Miyazawa 1993). There are several different opinions

about whether DHA ingestion in mammals affects the oxidative state of organs.

In this study, we tested the effect of DHA-enriched oils as triacylglycerols (TG), ethyl esters (EE) and phospholipids (PL) with identical constituent fatty acids, on lipid profiles and peroxidative status in rat plasma, liver and kidney. These are critical organs in the regulation and synthesis of lipids, and their constituent fatty acids are influenced by the oils ingested.

The phospholipid on the surface of membranes of tissues should be an ideal target for peroxidation. To evaluate peroxidation *in vivo*, a chemiluminescence-HPLC (CL-HPLC) system was developed previously as a method for the quantitative analysis of hydroperoxide in biomembranes (Miyazawa 1989). This method was shown to be highly sensitive and specific for phospholipid hydroperoxide (PLOOH) (Miyazawa et al. 1992). In this study, we used the CL-HPLC assay to determine the concentrations of PLOOH as key products for oxidative injury in the plasma, liver and kidney of rats. Given the important structural and functional roles of fatty acids in tissue membranes, alterations in fatty acid composition may contribute to the peroxidative effect of DHA.

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<sup>2</sup> Abbreviations used:  $\alpha$ -TOC,  $\alpha$ -tocopherol; CL, chemiluminescence; DHA, docosahexaenoic acid; EE, ethyl ester; FAME, fatty acid methyl esters; MDA, malondialdehyde; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; PI, peroxidizability index; PL, phospholipid; PLOOH, phospholipid hydroperoxide; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid reactive substance; TG, triacylglycerol.

## MATERIALS AND METHODS

**Animals and diets.** Male Sprague-Dawley rats (4 wk old; Funabashi Farm, Chiba, Japan), weighing 79–82 g, were divided into four groups ( $n = 6$  rats/group). They were kept in an air-conditioned room (22–23°C) with a 12-h light:dark cycle. The experimental protocols were approved by Tohoku University Animal Care Committee. Rats in the experimental groups were fed a diet containing 15 g/100 g of different types of lipids (~8.31 g total food mass/100 g rat mass containing 1.25 g fat including 90 mg DHA) for 3 wk according to a paired-feeding protocol to ensure similar energy intakes in the groups (30.6% of the total energy). Briefly, another group of rats ( $n = 6$ ) was allowed to consume the control oil diet ad libitum; food consumption was monitored daily. Experimental rats were fed corresponding amounts the next day. The diet composition was based on the AIN-76A formula for rats (AIN 1997). The composition of the diet (g/100 g diet) was as follows: vitamin-free casein, 20;  $\alpha$ -cornstarch, 45; sucrose, 10; cellulose, 5; oil, 15; mineral mixture (AIN-76), 3.5; vitamin mixture (AIN-76, free of  $\alpha$ -tocopherol), 1.2; and DL-methionine, 0.3. All dietary components were purchased from Oriental Yeast (Tokyo, Japan) except for the DHA oils, which were a kind gift from the Bizen Chemical (Okayama, Japan). The DHA oils in the form of TG, EE and PL, which contained similar fatty acid compositions and equal amounts of DHA content in their constituent fatty acids, were used. The fatty acid compositions of DHA oils as TG and EE were quite similar to each other; the DHA concentration was 7.0 mol/100 mol total fatty acids as given in Table 1. The peroxidizability index (PI) of DHA oil in these three forms (PI, 95.1–112.2) was ~5 times that of the control oil (PI, 20.4). The test oils contained  $\alpha$ -,  $\gamma$ - and  $\delta$ -tocopherols, among which  $\gamma$ -tocopherol was predominant in

the TG and EE oils. The total tocopherols in DHA oils and control oils were equivalent. The control group received palm oil supplemented with 20% soybean oil (i.e., 80 g palm oil mixed with 20 g soybean oil), which contained no DHA. The diets were freshly prepared every 5 d and stored at -25°C. During the feeding period, the rats received fresh food daily. The peroxide value of test oil in the diet was kept below 6.0 mequiv/kg throughout the animal trial (Song and Miyazawa 1997).

At the end of the 3-wk feeding period, rats were food deprived overnight and anesthetized with ether. Heparinized blood was withdrawn by heart puncture and was centrifuged at low speed (800  $\times$  g) at 4°C for 10 min to separate plasma. Liver and kidney were perfused in situ with ice-cold 0.15 mol/L saline, rinsed, blotted and weighed. Tissue samples were immediately frozen with dry ice and then stored at -80°C until analysis.

**Lipid analysis.** Plasma total lipid was extracted with a mixture of chloroform/methanol (2:1, v/v) as previously described (Kates 1986; Miyazawa 1993). Briefly, 5 mL of chloroform/methanol (2:1, v/v) containing 10 mg BHT was added to 1 mL of plasma, which was obtained by the centrifugation of heparinized rat blood. The mixture was centrifuged at 1000  $\times$  g for 10 min. The lower chloroform layer was concentrated in a rotary evaporator and dried under a nitrogen stream. Liver and kidney total lipids were extracted as described (Miyazawa 1993). Then, 2 mL of 0.15 mol/L saline containing 20 mg BHT was added to 200 mg tissue, and the mixture was homogenized in a teflon-glass homogenizer with ice cooling. The homogenate obtained was added to 5 mL of chloroform/methanol (2:1, v/v) and was centrifuged at 1000  $\times$  g for 10 min. The lower chloroform layer was concentrated in a rotary evaporator and dried under a nitrogen stream.

Fatty acid composition of the total lipids was analyzed by gas-liquid chromatography (Song and Miyazawa 1997). Fatty acid methyl esters (FAME) derived from total lipid in the presence of 1 mmol/L pentadecanoic acid (15:0) as internal standard were prepared by incubation with 10% methanolic-HCl in sealed vials at 85°C for 1 h. Methyl esters were extracted with hexane and were analyzed using a GC 380 gas chromatograph (GL Sciences, Tokyo, Japan) equipped with a flame ionization detector and a CP-SIL 88 capillary column (0.25 mm  $\times$  50 m, Chrompack, Bergen op Zoom, The Netherlands). The column temperature was increased from 170 to 225°C at 2°C/min. FAME were identified by comparison of their retention times with those of standards.

Total cholesterol, free cholesterol, TG and HDL cholesterol were analyzed enzymatically using cholesterol E-test, free cholesterol E-test, triacylglycerol E-test and HDL-cholesterol E-test (Wako Pure Chemical, Osaka, Japan), respectively. Phospholipid phosphorus was determined by the method of Bartlett (1959).

Tissue  $\alpha$ -tocopherol ( $\alpha$ -TOC) was determined by the fluorescence detection-HPLC method (Ave 1975), using 2,2,5,7,8-pentamethyl-6-hydroxychroman (Wako Pure Chemical) as an internal standard.

**PLOOH determination.** Phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) in total lipid extracts from plasma and tissue homogenates were determined by the CL-HPLC method using a chemiluminescence cocktail consisting of cytochrome c and luminol (Miyazawa et al. 1992 and 1994). Briefly, the column was a JASCO Finepak SIL NH<sub>2</sub>-5 (250  $\times$  4.6 mm, 5  $\mu$ m; JASCO, Tokyo, Japan). The column mobile phase was hexane/2-propanol/methanol/water (5:7:2:1, v/v/v/v), and the flow rate was maintained at 1 mL/min by a JASCO 880PU pump. The luminescent reagent was prepared by dissolving cytochrome c (from horse heart, type IV; Sigma, Tokyo, Japan) and luminol (3-aminophthaloyl hydrazine; Wako Pure Chemical) in an alkaline borate buffer (pH 10) and was added at a flow rate of 1.2 mL/min. The column eluate was mixed with the luminescent reagent at a postcolumn mixing joint (Y-type; Kyowa Seimitsu, Japan) with the temperature controlled at 40°C in a JASCO 860 column oven. The chemiluminescence generated by the reaction of the hydroperoxide with the luminescent reagent at the postcolumn was measured with a CLD-110 chemiluminescence detector (Tohoku Electronic Industries, Sendai, Japan). Because antioxidants and neutral lipids elute earlier, these components did not interfere with the assay. Calibrations of PCOOH and PEOOH were done with an authentic

TABLE 1

Fatty acid composition and tocopherol concentration of dietary oils<sup>1</sup>

	TG	EE	PL	Control <sup>2</sup>
	mol/100 mol			
Fatty acid				
16:0	42.3	43.7	39.1	40.4
16:1	0.8	1.3	0.6	0.2
18:0	5.1	5.2	9.2	4.3
18:1(n-9)	23.0	23.1	26.5	35.9
18:2(n-6)	14.0	12.7	11.4	17.3
18:3(n-3)	0.9	0.8	0.2	1.1
20:4(n-6)	1.4	1.3	1.8	—
20:5(n-3)	3.8	3.1	1.9	—
22:5(n-6)	1.2	1.1	0.7	—
22:5(n-3)	0.7	0.7	0.5	—
22:6(n-3)	7.0	7.0	7.1	—
P:M:S <sup>3</sup>	0.6:0.5:1	0.5:0.5:1	0.5:0.6:1	0.5:1:1
(n-6)/(n-3)	1.3	1.3	1.0	16.8
PI <sup>4</sup>	112.2	105.5	95.1	20.4
	mg/100 g			
Tocopherol				
$\alpha$ -	3.0	2.0	2.7	2.9
$\delta$ -	10.1	7.8	2.5	2.2
$\gamma$ -	4.2	4.4	0.2	1.2
Total	17.3	14.2	5.4	6.3

<sup>1</sup> The docosahexaenoic (DHA) oils in the diet are supplemented in the forms of TG (triacylglycerols), EE (ethyl esters) or PL (phospholipids).

<sup>2</sup> Palm oil supplemented with 20 g/100 g soybean oil.

<sup>3</sup> P:M:S denotes polyunsaturated/monounsaturated/saturated acids.

<sup>4</sup> Peroxidizability index (PI) was calculated from following equation: PI = (% monoenoic  $\times$  0.025) + (% dienoic  $\times$  1) + (% trienoic  $\times$  2) + (% tetraenoic  $\times$  4) + (% pentaenoic  $\times$  6) + (% hexaenoic  $\times$  8).

TABLE 2

Plasma, liver and kidney lipids in rats fed different docosahexaenoic acid (DHA) oils in the form of triacylglycerols (TG), ethyl esters (EE) or phospholipids (PL) for 3 wk<sup>1</sup>

	Diet group				Pooled SEM
	TG	EE	PL	Control	
	mmol/L				
Plasma					
Total cholesterol	2.00	1.95	2.30	2.16	0.13
Free cholesterol	0.66 <sup>a</sup>	0.46 <sup>a</sup>	0.51 <sup>a</sup>	0.91 <sup>b</sup>	0.07
Cholesterol ester	1.35 <sup>a</sup>	1.49 <sup>a</sup>	1.79 <sup>b</sup>	1.25 <sup>a</sup>	0.10
HDL cholesterol	1.15	0.97	0.81	1.19	0.06
Triacylglycerol	0.96 <sup>b</sup>	0.67 <sup>a</sup>	1.29 <sup>c</sup>	1.54 <sup>d</sup>	0.05
Phospholipid	1.20	1.09	1.38	1.26	0.13
	μmol/g				
Liver					
Total cholesterol	7.06	6.98	8.14	7.21	0.33
Free cholesterol	2.47	2.75	3.05	3.49	0.27
Triacylglycerol	7.47 <sup>b</sup>	4.11 <sup>a</sup>	8.65 <sup>b</sup>	12.02 <sup>c</sup>	1.10
Phospholipid	24.35	28.68	25.54	31.12	2.08
Kidney					
Total cholesterol	7.21	6.77	6.93	7.69	0.33
Free cholesterol	1.87	2.02	2.05	1.93	0.07
Triacylglycerol	2.79	2.43	2.24	3.53	0.43
Phospholipid	13.57	13.23	12.47	11.51	0.46

<sup>1</sup> Values are means and pooled SEM,  $n = 6$ . Means in a row without common superscripts are significantly different,  $P < 0.05$ .

standard PCOOH according to the method of Miyazawa et al. (1992). Thiobarbituric acid reactive substances (TBARS) were measured as a marker of lipid peroxidation in plasma by fluorometric determination (Yagi 1976) and tissues by colorimetric determination (Buege and Aust 1978). Two different methods were applied for determining TBARS in plasma and in tissues to avoid interference by iron ions

and to use a sufficiently sensitive assay to obtain a quantitative measure of the lower levels of lipid peroxide in plasma.

**Statistical analysis.** Differences among the four groups were identified using a one-way ANOVA (StatView, version 4.5, Abacus Concepts, Berkeley, CA) and the significance of differences was evaluated by Duncan's multiple range test. For comparisons between two groups, Student's  $t$  test was used. Differences of  $P < 0.05$  were considered to be significant.

## RESULTS

All rats showed steady growth during the 3-wk feeding period, and final body weight gains among the groups did not differ (285–314 g,  $P > 0.05$ ). Groups did not differ in the relative weight gains of liver (4.6–5.3 g/100 g body,  $P > 0.05$ ) or kidney (0.73–0.82 g/100 g body,  $P > 0.05$ ). Plasma total cholesterol and PL levels did not differ among the groups, but free cholesterol was significantly lower in all DHA oil-fed rats than in control oil-fed rats (60–70% of control) (Table 2). Although plasma TG concentration was reduced in all DHA-fed groups, it was most dramatically affected in the rats fed the EE diet (Table 2). The major constituent fatty acids in total plasma lipids of all groups were as follows: 16:0 (26.9–32.9 mol/100 mol); 20:4(n-6) (14.4–26.0 mol/100 mol); 18:2(n-6) (11.8–15.1 mol/100 mol); 18:1(n-9) (10.0–15.1 mol/100 mol); and 18:0 (12.2–13.0 mol/100 mol) (Table 3). Plasma DHA concentrations of rats fed DHA-containing oil as TG and EE were 1.5–1.9 times that of control oil-fed rats. The PL values of TG and EE DHA-fed rats were not different from controls (Table 3). The DHA oil-fed rats had more than three times the control concentration of 20:5(n-3) in plasma total lipids.

The groups did not differ in hepatic total cholesterol, free cholesterol or PL, although the TG concentration of the DHA-fed rats was significantly reduced, especially in those fed DHA in the form of EE (Table 2). The DHA concentration in the liver total lipids was higher in the DHA oil-fed groups, and the PI values of fatty acid were 1.9–2.7 times the control group (Table 3). Regardless of the dietary oil ingested, the arachidonic acid [20:4(n-6)] concentration in the liver was lower than that in plasma.

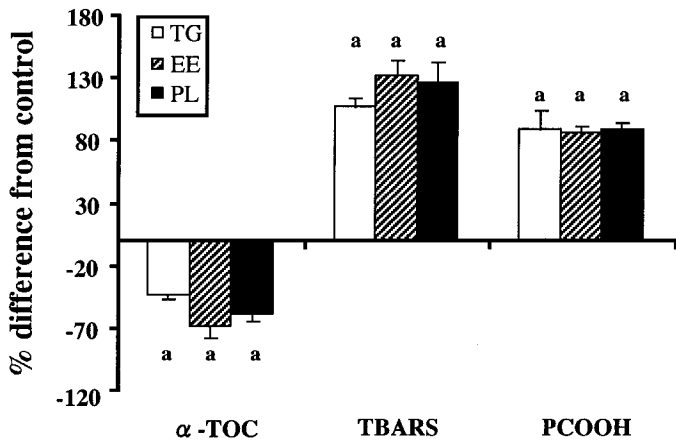
TABLE 3

Fatty acid composition of plasma, liver and kidney total lipids in rats fed different docosahexaenoic acid (DHA) oils in the form of triacylglycerols (TG), ethyl esters (EE) or phospholipids (PL) for 3 wk<sup>1</sup>

Fatty acid	Plasma					Pooled SEM	Liver					Pooled SEM	Kidney					Pooled SEM
	TG	EE	PL	Control	TG		EE	PL	Control	TG	EE		PL	Control				
	mol/100 mol																	
16:0	31.3 <sup>ab</sup>	32.9 <sup>b</sup>	29.2 <sup>ab</sup>	26.9 <sup>a</sup>	1.3	29.0 <sup>b</sup>	26.2 <sup>a</sup>	26.1 <sup>a</sup>	26.5 <sup>a</sup>	1.0	28.2	28.9	27.0	27.5	0.4			
16:1	1.4	1.0	1.3	1.4	0.3	2.8 <sup>a</sup>	1.6 <sup>a</sup>	5.0 <sup>b</sup>	1.6 <sup>a</sup>	0.4	2.2 <sup>ab</sup>	2.1 <sup>ab</sup>	3.4 <sup>b</sup>	1.3 <sup>a</sup>	0.3			
18:0	13.0	12.5	12.8	12.2	0.8	8.2 <sup>a</sup>	8.4 <sup>a</sup>	7.9 <sup>a</sup>	11.3 <sup>b</sup>	0.4	13.9	13.9	14.7	13.0	0.3			
18:1(n-9)	10.0 <sup>a</sup>	10.7 <sup>a</sup>	17.3 <sup>c</sup>	15.1 <sup>b</sup>	0.7	18.0 <sup>a</sup>	17.8 <sup>a</sup>	26.1 <sup>b</sup>	27.8 <sup>b</sup>	0.6	13.6 <sup>a</sup>	14.1 <sup>a</sup>	16.2 <sup>ab</sup>	19.0 <sup>b</sup>	0.7			
18:2(n-6)	13.8 <sup>ab</sup>	12.3 <sup>ab</sup>	15.1 <sup>b</sup>	11.8 <sup>a</sup>	0.6	10.3 <sup>b</sup>	10.2 <sup>b</sup>	8.7 <sup>a</sup>	11.8 <sup>c</sup>	0.3	10.8	10.8	10.0	11.2	0.3			
18:3(n-3)	0.6	0.3	0.7	0.5	0.2	0.7	0.5	0.6	0.5	0.1	0.3	0.4	0.3	0.5	0.0			
20:4(n-6)	18.1	17.4	14.4	26.0	0.8	6.6 <sup>a</sup>	7.1 <sup>a</sup>	7.9 <sup>a</sup>	14.1 <sup>b</sup>	0.4	16.7 <sup>a</sup>	17.3 <sup>ab</sup>	18.4 <sup>ab</sup>	21.6 <sup>b</sup>	0.6			
20:5(n-3)	2.9 <sup>c</sup>	3.6 <sup>c</sup>	2.4 <sup>b</sup>	0.8 <sup>a</sup>	0.5	4.4 <sup>c</sup>	4.5 <sup>c</sup>	3.1 <sup>b</sup>	0.4 <sup>a</sup>	0.2	8.2 <sup>c</sup>	6.2 <sup>bc</sup>	4.1 <sup>b</sup>	1.9 <sup>a</sup>	0.5			
22:5(n-6)	0.3	0.5	0.4	0.6	0.1	0.6	0.7	0.4	0.4	0.1	0.2	0.2	0.4	0.5	0.1			
22:5(n-3)	1.0 <sup>b</sup>	1.0 <sup>b</sup>	0.3 <sup>a</sup>	0.6 <sup>ab</sup>	0.1	3.9 <sup>c</sup>	4.4 <sup>c</sup>	2.2 <sup>b</sup>	0.7 <sup>a</sup>	0.3	0.9	0.8	0.7	1.2	0.1			
22:6(n-3)	7.6 <sup>c</sup>	7.3 <sup>c</sup>	6.1 <sup>b</sup>	4.1 <sup>a</sup>	0.4	15.5 <sup>c</sup>	18.6 <sup>c</sup>	12.1 <sup>b</sup>	4.9 <sup>a</sup>	0.8	5.0 <sup>b</sup>	5.4 <sup>b</sup>	4.8 <sup>b</sup>	2.2 <sup>a</sup>	0.3			
(n-6)/(n-3)	2.8 <sup>a</sup>	2.5 <sup>a</sup>	3.2 <sup>a</sup>	6.9 <sup>b</sup>	0.5	0.7 <sup>a</sup>	0.6 <sup>a</sup>	1.0 <sup>a</sup>	4.1 <sup>b</sup>	0.1	2.0 <sup>a</sup>	2.3 <sup>a</sup>	3.0 <sup>a</sup>	5.8 <sup>b</sup>	0.2			
PI <sup>2</sup>	173.7 <sup>b</sup>	173.0 <sup>b</sup>	141.9 <sup>a</sup>	162.0 <sup>b</sup>	5.1	215.9 <sup>c</sup>	246.4 <sup>c</sup>	172.7 <sup>b</sup>	92.3 <sup>a</sup>	8.8	174.6 <sup>b</sup>	167.0 <sup>b</sup>	154.0 <sup>ab</sup>	138.3 <sup>b</sup>	5.1			

<sup>1</sup> Values are means and pooled SEM,  $n = 6$ . Means in a row without common superscripts are significantly different,  $P < 0.05$ .

<sup>2</sup> PI, peroxidizability index.



**FIGURE 1** Plasma lipid peroxidation in rats fed different docosahexaenoic acid (DHA) oils in the form of triacylglycerols (TG), ethyl esters (EE) or phospholipids (PL) for 3 wk. Data are expressed as the percentage difference from the values of control oil-fed rats [ $\alpha$ -tocopherol ( $\alpha$ -TOC), 21.3  $\pm$  2.8  $\mu$ mol/L; thiobarbituric acid reactive substances (TBARS), 4.0  $\pm$  0.2  $\mu$ mol malondialdehyde/L; phosphatidylcholine hydroperoxide (PCOOH), 46.7  $\pm$  8.4 nmol/L]. Values are means  $\pm$  SEM, *n* = 6. <sup>a</sup>Significantly different (*P* < 0.05) from the values of control oil-fed group.

Kidney lipids did not differ among groups (Table 2). DHA in kidney lipids of the DHA oil-fed rats was 4.8–5.4 mol/100 mol of the total fatty acids, higher than that in the control oil-fed rats (Table 3). PI values of kidney lipids of DHA oil-fed rats were 1.1–1.3 times those of the control oil-fed rats.

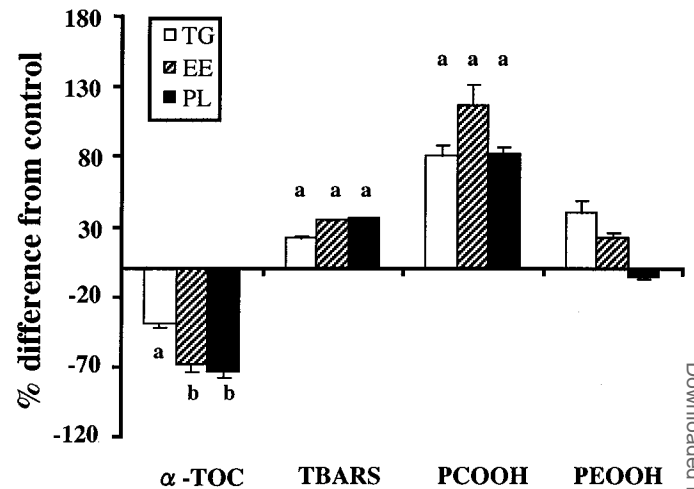
Plasma  $\alpha$ -TOC concentrations were significantly lower in all three DHA oil-fed groups, decreasing 44–70% (21.3  $\pm$  2.8  $\mu$ mol/L plasma, Fig. 1). The plasma TBARS levels of the DHA groups were 107–131% greater than those of the control group [4.0  $\pm$  0.2  $\mu$ mol malondialdehyde (MDA)/L plasma]. Similarly, the plasma PCOOH concentration was 90% greater in DHA oil-fed rats than that of control oil-fed rats (46.7  $\pm$  8.4 nmol/L plasma). No significant differences were observed among the rats fed the DHA oil-containing diets (Fig. 1).

Liver  $\alpha$ -TOC concentrations were 40–74% lower among the DHA oil-fed groups relative to the control group (48.8  $\pm$  3.3 nmol/g liver, Fig. 2). The concentrations of liver TBARS were 22–35% greater in DHA oil-fed rats relative to control rats (397  $\pm$  12 nmol MDA/g liver). Liver PCOOH levels were also higher among the DHA-fed groups (70–110%) relative to the control, although no significant difference was found among liver PEOOH levels (Fig. 2).

Kidney  $\alpha$ -tocopherol concentrations were 21–28% lower in DHA-fed rats than in control rats (27.1  $\pm$  1.5 nmol/g kidney, Fig. 3). Kidney TBARS levels were slightly higher in DHA oil-fed rats, whereas PCOOH levels were higher by 63–156% in DHA oil-fed rats relative to control rats (TBARS, 529  $\pm$  16 nmol MDA/g kidney; PCOOH, 265  $\pm$  40 pmol/g kidney). PEOOH levels in kidney were higher (93–223%) in the DHA-fed rats than in controls (PEOOH, 203  $\pm$  20 pmol/g kidney).

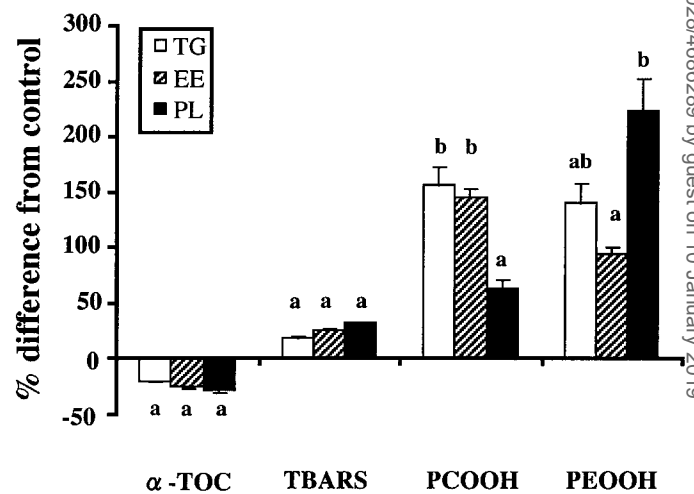
**DISCUSSION**

We reported previously that the in vitro oxidative stabilities of DHA-enriched oils in the form of PL were comparable to that of control palm oil (Song et al. 1997, Song and Miyazawa 1997). From these results, we expected beneficial resistance of tissue membrane lipids toward oxidative stress



**FIGURE 2** Liver lipid peroxidation in rats fed different docosahexaenoic acid (DHA) oils in the form of triacylglycerols (TG), ethyl esters (EE) or phospholipids (PL) for 3 wk. Data are expressed as the percentage difference from the values of control oil fed rats [ $\alpha$ -tocopherol ( $\alpha$ -TOC), 48.8  $\pm$  3.3 nmol/g; thiobarbituric acid reactive substances (TBARS), 397  $\pm$  12 nmol malondialdehyde/g; phosphatidylcholine hydroperoxide (PCOOH), 398  $\pm$  43 pmol/g; phosphatidylethanolamine hydroperoxide (PEOOH), 327  $\pm$  97 pmol/g]. Values are means  $\pm$  SEM, *n* = 6. <sup>a,b</sup>Significantly different (*P* < 0.05) from the values of the control oil-fed group and among the different DHA oil-fed groups. Values with different letters within each measure are significantly different and a letter indicates different from control (*P* < 0.05).

when DHA oil was ingested in the form of PL. Therefore, we examined whether dietary DHA oil in the form of TG, EE and PL affected lipid metabolism and tissue lipid peroxidation differently in rats.



**FIGURE 3** Kidney lipid peroxidation in rats fed different docosahexaenoic acid (DHA) oils in the form of triacylglycerols (TG), ethyl esters (EE) or phospholipids (PL) for 3 wk. Data are expressed as the percentage difference from the values of control oil-fed rats [ $\alpha$ -tocopherol ( $\alpha$ -TOC), 27.1  $\pm$  1.5 nmol/g; thiobarbituric acid reactive substances (TBARS), 529  $\pm$  16 nmol malondialdehyde/g; phosphatidylcholine hydroperoxide (PCOOH), 265  $\pm$  40 pmol/g; phosphatidylethanolamine hydroperoxide (PEOOH), 203  $\pm$  20 pmol/g]. Values are means  $\pm$  SEM, *n* = 6. <sup>a,b</sup>Significantly different (*P* < 0.05) from the values of the control oil-fed group. Values with different letters within each measure are significantly different and a letter indicates different from control (*P* < 0.05).

Treatment with DHA oils changed the PUFA pattern of the tissue membranes by increasing DHA [(n-3) fatty acid] content and by decreasing concomitantly arachidonic acid [(n-6) fatty acid]. We confirmed the findings of others that increases in (n-3) fatty acids such as DHA and EPA in total plasma lipids occur in rats supplemented with DHA oil in the form of TG, EE and PL (Table 3) (Arbuckle et al. 1991, Innis et al. 1995). DHA [22:6(n-3)] incorporation into plasma and liver was lower in DHA-PL-fed relative to DHA-TG- and DHA-EE-fed rats. It is possible that DHA oil in the form of PL was absorbed in the intestine less efficiently than DHA oil in the form of TG and EE as a result of differences in the enzyme activities of pancreatic lipases for TG and EE and of phospholipase for PL. DHA-PL administration increased the oxidative stress of cell membranes to the same extent as did DHA-TG or DHA-EE, even though it did not increase (n-3) fatty acids relative to other DHA oils. Perhaps there is a tissue concentration of DHA that is sufficient for increased oxidative stress vulnerability.

Plasma TG are generally accepted as an independent risk factor for heart disease (Davignon and Cohn 1996). The previously described hypotriacylglycerolemic effect of DHA-containing oil is thought to be mediated by its inhibition of VLDL synthesis and secretion in the liver (Herold and Kinsella 1986, Simopoulos 1991, Thiery and Seidel 1987, Tremoli et al. 1995, Vidgren et al. 1997) and by its enhancement of  $\beta$ -oxidation of fatty acids in the liver (Sanders et al. 1985, Surette et al. 1992). We found that dietary DHA oils exerted a significant hypotriacylglycerolemic effect in the rats whose diets were supplemented with DHA (2.14% of total energy intake), although kidney lipid profiles were not affected (Table 2). When DHA was ingested in the form of EE-type oil, plasma TG concentration was lowered more than it was when it was ingested as TG- or PL-type oil. This may be due to differences in rates of hydrolysis of DHA-containing oil by lipases in the intestinal digest. Imaizumi et al. (1982) suggested that dietary PL absorption may be inhibited by an increase in the particle size of chylomicrons in the intestine. Ikeda et al. (1995) reported that digestion and lymphatic transport of DHA were influenced by lipid types in rats. We found that plasma free cholesterol levels were decreased in rats fed the DHA oil diets relative to those fed the control oil diet. This is not surprising because the (n-3) fatty acid content of the DHA oil was higher than that of the control oil. Dietary (n-3) fatty acids have previously been demonstrated to reduce the amount of free cholesterol through its conversion to cholesterol ester (Sugano et al. 1997). These results indicate that reduction of plasma TG and free cholesterol by DHA oil is an important intervention in reducing the risk of atherosclerosis, in view of the relationship to cardiovascular disease. Although the ingestion of highly unsaturated oils such as fish and DHA-containing oils may be part of interesting therapeutic approaches for the prevention of this disease, there is the potential to cause tissue membrane PL peroxidation and exhaust  $\alpha$ -TOC in mammals. In our examination of oxidative stress, results in plasma, liver and kidney showed no significant differences in lipid peroxidation among the three different DHA oil forms, even though the PL-type DHA oil had a lower PI value than TG- or EE-type DHA oils. The PI values may not necessarily correlate with the lipid peroxidation values. These discrepancies may be due to different concentrations of the (n-3) and (n-6) fatty acids in the DHA oil-fed groups and the control oil-fed group. The PI value is obtained from both (n-3) and (n-6) fatty acids values; however, the (n-3) fatty acids contribute a greater portion of those oxidized. There was a close correlation between DHA oil-fed groups and the control

oil-fed group associated with corresponding amounts of DHA (plasma,  $r = 0.980$ ,  $P < 0.05$ ; liver,  $r = 0.969$ ,  $P < 0.05$ ; kidney,  $r = 0.965$ ,  $P < 0.05$ ) or values of PLOOH (plasma,  $r = 0.985$ ,  $P < 0.05$ ; liver,  $r = 0.971$ ,  $P < 0.05$ ; kidney,  $r = 0.984$ ,  $P < 0.05$ ) or TBARS (plasma,  $r = 0.966$ ,  $P < 0.05$ ; liver,  $r = 0.965$ ,  $P < 0.05$ ; kidney,  $r = 0.986$ ,  $P < 0.05$ ). These results are consistent with our previous observation that a fish oil diet increased the PLOOH concentration of liver and plasma more than safflower oil, perilla oil and olive oils, supporting the idea that a diet rich in DHA oils significantly affects lipid peroxidation in vivo (Miyazawa et al. 1993).

The incorporation of PUFA into tissue membranes after DHA oil ingestion increases their susceptibility to free radical attack,  $\alpha$ -TOC exhaustion and subsequent peroxidation in the digestive tract, blood and tissue organelles (Alexander-North et al. 1994, Buckingham 1985, Mouri et al. 1984). These results suggest that PUFA and tocopherol levels influence tissue membrane susceptibility to lipid peroxidation. However, the different forms of DHA oil did not cause different degrees of lipid peroxidation. Thus, tissue peroxidative susceptibilities in oil-fed rats were related to increases in corresponding tissue membrane unsaturation as a result of dietary oil ingestion. Our present results showed that the  $\alpha$ -TOC concentration in plasma, liver and kidney of DHA oil-fed groups and the control oil-fed group was inversely associated with corresponding amounts of DHA (plasma,  $r = 0.746$ ,  $P < 0.05$ ; liver,  $r = 0.700$ ,  $P < 0.05$ ; kidney,  $r = 0.605$ ,  $P < 0.05$ ) or values of PCOOH (plasma,  $r = 0.700$ ,  $P < 0.05$ ; liver,  $r = 0.650$ ,  $P < 0.05$ ; kidney,  $r = 0.678$ ,  $P < 0.05$ ) or TBARS (plasma,  $r = 0.771$ ,  $P < 0.05$ ; liver,  $r = 0.857$ ,  $P < 0.05$ ; kidney,  $r = 0.524$ ,  $P < 0.05$ ).

The similarity of  $\alpha$ -TOC reductions and the degree of lipid peroxidation resulting from the ingestion of different forms of DHA oil suggest that the decrease in  $\alpha$ -TOC or other changes in antioxidant activity may be central to the increased lipid peroxidation in DHA oil-fed rats. These observations are consistent with a study in which a fish oil-supplemented diet increased the activities of catalase, glutathione peroxidase and glutathione-S-transferase and decreased glutathione levels in rat tissues, whereas vitamin E cosupplementation with fish oil decreased antioxidant enzyme activities (Atalay et al. 2000). These data suggest that dietary oils significantly affect the antioxidant defense in biomembranes, which in turn was caused by a decrease of  $\alpha$ -TOC. Ando et al. (2000) also observed that intake of fish oil (50 g/kg diet) when supplemented with vitamin E (4.3 g/kg fish oil) did not increase lipid peroxidation in rat organs, compared with rats fed safflower oil, which was rich in (n-6) fatty acids. These results support the hypothesis that feeding DHA-containing oil is sufficient to change tissue membrane lipid content to high amounts of (n-3) fatty acids, which can enhance the production of hydroperoxides and decrease the antioxidant  $\alpha$ -TOC in target membranes. In addition, (n-3) fatty acids, mainly DHA, are more likely targets for peroxidation than (n-6) fatty acids. Therefore, the degree of DHA incorporation affects the extent of lipid peroxidation, which is consistent with an increased susceptibility to an oxidative stress found when certain cells were cultured with DHA in the absence of  $\alpha$ -TOC (Igarashi and Miyazawa 2000). Moreover, other clinical studies have demonstrated that high doses of DHA-rich fish oil in the human diet, as well as long-term treatments, affected serum, LDL and erythrocyte susceptibility to lipid peroxidation by increasing levels of (n-3) fatty acids and by decreasing the tocopherol content in these organs (Korpela et al. 1999, Palozza et al. 1996).

Although dietary supplementation with DHA oil has

shown preventive value in relation to cardiovascular disease by a reduction in plasma TG levels and possibly by inhibiting platelet function by reducing arachidonic acid levels in tissue lipids, it may also be a health risk because it substantially increases lipid peroxidation. This elevated lipid peroxidation was seen in conjunction with increased DHA incorporation and decreased levels of  $\alpha$ -TOC. Further studies will be required to clarify the effects of long-term administration of DHA oil on lipid metabolism and peroxidation. We conclude, therefore, that DHA is able to exert a beneficial effect with regard to reducing the risk of atherosclerosis, only if antioxidative protection against oxidative stress is sufficient. Thus, our results suggest that DHA oil interventions should be coupled with antioxidant therapy to minimize the peroxidative damage of tissue lipids.

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