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Enhanced level of n-3 fatty acid in membrane phospholipids induces lipid peroxidation in rats fed dietary docosahexaenoic acid oil

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Abstract

The effect of dietary docosahexaenoic acid (DHA, 22:6n-3) oil with different lipid types on lipid peroxidation was studied in rats. Each group of male Sprague–Dawley rats was pair fed 15% (w/w) of either DHA-triglycerides (DHA-TG), DHA-ethyl esters (DHA-EE) or DHA-phospholipids (DHA-PL) for up to 3 weeks. The palm oil (supplemented with 20% soybean oil) diet without DHA was fed as the control. Dietary DHA oils lowered plasma triglyceride concentrations in rats fed DHA-TG (by 30%), DHA-EE (by 45%) and DHA-PL (by 27%), compared to control. The incorporation of dietary DHA into plasma and liver phospholipids was more pronounced in the DHA-TG and DHA-EE group than in the DHA-PL group. However, DHA oil intake negatively influenced lipid peroxidation in both plasma and liver. Phospholipid peroxidation in plasma and liver was significantly higher than control in rats fed DHA-TG or DHA-EE, but not DHA-PL. These results are consistent with increased thiobarbituric acid reactive substances (TBARS) and decreased α-tocopherol levels in plasma and liver. In addition, liver microsomes from rats of each group were exposed to a mixture of chelated iron (Fe^{3+}/ADP) and NADPH to determine the rate of peroxidative damage. During NADPH-dependent peroxidation of microsomes, the accumulation of phospholipid hydroperoxides, as well as TBARS, were elevated and α-tocopherol levels were significantly exhausted in DHA-TG and DHA-EE groups. During microsomal lipid peroxidation, there was a greater loss of n-3 fatty acids (mainly DHA) than of n-6 fatty acids, including arachidonic acid (20:4n-6). These results indicate that polyunsaturation of n-3 fatty acids is the most important target for lipid peroxidation. This suggests that the ingestion of large amounts of DHA oil enhances lipid peroxidation in the target membranes where greater amounts of n-3 fatty acids are incorporated, thereby increasing the peroxidizability and possibly accelerating the atherosclerotic process.

Keywords: Atherosclerosis; Chemiluminescence; Hypotriglyceridemic effect; Peroxidizability index; Phospholipid peroxidation; Polyunsaturated fatty acid

1. Introduction

The health benefits of fish oils have been extensively studied since the observation that the incidence of cardiovascular disease was lower among populations that consumed large amounts of fish oil (fish) [1–4]. Fish oil contains a high proportion of n-3 fatty acids. Its ability to lower plasma triglycerides has been widely studied in animal and human models [4,5]. However, docosahexaenoic acid (DHA, 22:6n-3), a major polyunsaturated fatty acid (PUFA) of fish oil, is most susceptible to peroxidation because of its high degree of unsaturation [6–10]. When the n-3 fatty acids are ingested, the unsaturation of membrane fatty acids is increased as a result of the n-3 fatty acid incorporation. There may be an increased need for antioxidants (i.e. α-tocopherol) to prevent oxidative stress [11–13].

We have reported on oxidative stability of DHA-enriched oil during autoxidation [14,15]. Previous results have shown that DHA oil, in the form of phospholipids (PL), have a higher stability against autoxidation than in the form of triglycerides (TG) and ethyl esters (EE). This superior oxidative stability may be due to its abilities to chelate iron and react synergistically with

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α-tocopherol. To extend our in vitro findings, we have studied the more clinically relevant in vivo effect of different DHA oil ingestion on lipid peroxidation. The changes in lipid peroxidation in organs after ingestion of DHA-PL have not been established.

The objective was to determine the effects of DHA oils, in the form of TG, EE and PL on tissue lipid peroxidation. Male Sprague–Dawley rats were fed diets with mixed DHA oils that varied in lipid types with similar constituent fatty acid. Control rats were fed palm oil supplemented with 20% soybean oil. The changes in lipid peroxidation were measured by chemiluminescence-HPLC (CL-HPLC), thiobarbituric acid reactive substances (TBARS) and concentration of α-tocopherol (α-TOC) were determined by fluorescence-HPLC in plasma, liver and liver microsomes during NADPH-dependent lipid peroxidation in rats fed various dietary DHA oils.

2. Methods

2.1. Animals and diets

Male Sprague–Dawley rats (3-weeks-old; obtained from Funabashi Farm Co., Chiba, Japan) were divided into four groups (six rats each). During the 3 weeks, rats were pair fed semi-purified diets prepared daily in the laboratory, containing 15% oil by weight of different types of lipids (≈ 8.73 g total food mass/100 g rat mass containing 1.31 g fat including 0.14 g DHA) and maintained in an air-conditioned room (22°C with a 12:12 h light:dark cycle). The composition of the diets in weight percent was: vitamin-free casein 20, α-cornstarch 45, sucrose 10, cellulose 5, oil 15, mineral mixture (AIN-TM76) 3.5, vitamin mixture (AIN-TM76, α-tocopherol free) 1.2, and DL-methionine 0.3. All dietary components were purchased from Oriental Yeast Co. (Tokyo, Japan), except the DHA oils. DHA containing oils in the form of PL (Active DHA-10®, 3.1:1 in the ratio of phosphatidylcholine [PC]/phosphatidylethanolamine [PE]), TG and EE that were used in the present study were a gift from Bizen Chemical Co. (Okayama, Japan). DHA-PL was prepared from egg yolk lipids of laying hens that had been fed on a fish oil-supplemented diet. The fatty acid compositions of DHA oils in the form of TG, EE and PL were similar and the DHA contents were all similar with 10.7 mol.% of total fatty acids [15]. The control palm oil (supplemented with 20% soybean oil) contained no DHA. The amount of α-tocopherol was standardized to 20 mg per100 g dietary oil. Table 1 shows the fatty acid composition of dietary oils. Diets were stored at −20°C to prevent autoxidation and supplied everyday to ensure freshness. The rats were fasted for 20 h prior to dissection and then were sacrificed under light ether anesthesia. Heparinized blood was collected by cardiac puncture and centrifuged at low speed (1000 × *g* at 4°C for 10 min) to separate the plasma. The livers were perfused in situ with ice-cold 0.15 M saline containing 0.002% butylated hydroxytoluene (BHT; Sigma, Tokyo, Japan) and were then surgically dissected, weighed and immediately frozen for subsequent analyses.

Table 1
Fatty acid composition of dietary oils

Fatty acid	Dietary oil (mol/100 mol)			
	Control[a]	DHA-TG	DHA-EE	DHA-PL
16:0	41.7	41.8	41.6	32.5
16:1	0.2	3.2	3.2	1.7
18:0	4.3	5.1	4.8	11.1
18:1n-9	35.8	22.4	22.8	27.7
18:2n-6	17.2	10.6	11.4	11.4
18:3n-3	0.8	0.6	0.6	0.2
20:4n-6	–	0.9	0.4	1.3
20:5n-3	–	3.1	3.0	1.5
22:5n-6	–	0.9	0.9	0.3
22:5n-3	–	0.7	0.7	1.5
22:6n-3	–	0.7	10.7	10.7
P:M:S	0.4:0.8:1	0.6:0.5:1	0.6:0.6:1	0.6:0.7:1
n-6/n-3	22.3	0.8	0.8	0.9
PI[b]	20.0	130.0	128.0	123.0

[a] Palm oil supplemented with 20% soybean oil.

[b] Peroxidizability index = (% monoenoic × 0.025) + (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 4) + (% pentaenoic × 6) + (% hexaenoic × 8). P:M:S denotes polyunsaturated:monounsaturated: saturated acids. The level of α-tocopherol was equalized in all dietary oils with DL-α-tocopherol (20 mg/100 g oil).

2.2. Lipid analysis

Total lipids were extracted from the plasma and liver in chloroform/methanol (2:1, v/v) containing 0.002% BHT, as previously described [16]. The chloroform phase containing the total lipid extract was collected and concentrated under a stream of nitrogen. The lipids were redissolved in chloroform/methanol (1:1, v/v) and analyzed by CL-HPLC for phospholipid hydroperoxides, which is the most accurate method of assessing the lipid peroxide formation in biological membranes [16].

Plasma and liver phospholipids were separated from total lipids by TLC (Silica gel 60, Merck, Germany) using chloroform/methanol/acetic acid/water (25:15:4:2, by vol) as the developing solvent [17].

Fatty acids methyl esters derived from total lipid and phospholipid extracts were catalyzed by incubation with 10% methanolic-HCl in sealed vials under 85°C for 1 h and were then extracted with hexane. For quantitative analysis, pentadecanoic acid (15:0) (Sigma) was added as the internal standard, assuming the response of the detector for all fatty acid methyl esters was identical. Fatty acids methyl esters were quantified

by a GC-380 gas chromatography (GL Sciences, Tokyo, Japan) equipped with a flame ionization detector and a CP-SIL 88 capillary column (0.25 mm × 50 m, Chrompack, Netherlands). The column temperature was programmed from 170 to 220°C at 2°C/min [15]. Fatty acid methyl esters were identified by comparing their retention times with those of standard methyl esters.

Total-cholesterol, free-cholesterol, triglycerides, HDL-cholesterol and phospholipids in plasma were analyzed enzymatically using the respective E-test (Wako Pure Chemical, Tokyo, Japan).

The activities of plasma glutamate oxalate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) were determined by the use of a commercial kit (TA-Lq, Dia-Iatron Co, Tokyo, Japan).

2.3. Phospholipid hydroperoxide determination

Both PCOOH and PEOOH in lipids were simultaneously determined with CL-HPLC, as described by Miyazawa et al. [16,18,19]. CL detection was carried out with a CLD-100 detector (Tohoku Electronic Industries Co., Sendai, Japan), which employs a cooled photomultiplier tube (PMT) to suppress PMT dark current and improve the S/N ratio of HPLC analysis. For hydroperoxide-specific post column chemiluminescence reagent, a mixture of luminol and cytochrome *c* in 50 mM borate buffer (pH 10.0) was used. Calibration was made with authentic PCOOH and PEOOH standards, as described previously [19].

2.4. TBARS assay

Thiobarbituric acid-reactive substances (TBARS), in the presence of 0.04% (w/v) BHT as antioxidant, were determined in plasma and tissue. Plasma TBARS were determined by the fluorometric measurement at 553 nm (with 515 nm excitation) [20] and those in liver and microsome were measured at 532 nm by the method of Buege and Aust [21] with minor modification. The concentrations of TBARS were calculated using tetraethoxy propane (Sigma, Tokyo, Japan) as a reference standard.

2.5. α-Tocopherol assay

α-Tocopherol was extracted from 0.2 ml of plasma, 2 ml liver homogenate (25% w/v) or 1 ml of microsome suspension with 5 ml of *n*-hexane after the addition of 2 ml of ethanol solution containing 2 μg of 2,2,5,7,8-pentamethyl-6-hydroxychroman (Wako Pure Chemical, Tokyo, Japan) as an internal standard. After vortexing for 1 min, the sample was centrifuged at 1000 × *g* for 15 min and the supernatant was collected. The solvent was evaporated under nitrogen and the residual fraction was redissolved in 50 μl of *n*-hexane/2-propanol (99.5:0.5, v/v) for HPLC analyses. HPLC separation was carried out with a JASCO Finepak SIL (5 μm, 250 × 4.6 mm, Japan). The mobile phase was *n*-hexane/2-propanol (99.5:0.5, v/v) at a flow rate of 1.3 ml/min using a JASCO 880-PU pump. α-Tocopherol was determined by JASCO 821-FP Spectrofluorometer (Ex = 298 nm, Em = 325 nm).

2.6. Microsome preparation

Liver was homogenized in 5 vol of ice cold 250 mM sucrose containing 50 mM KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl buffer (pH 7.4) [22,23]. The liver homogenate was centrifuged for 15 min at 12 000 × *g* at 4°C and the supernatant collected. After ultracentrifugation of the supernatant at 105 000 × *g* for 60 min, microsomes were collected from the pellet. The microsomes were washed with recentrifugation at 105 000 × *g* for 60 min in 0.15 M KCl. The washed microsomes were suspended in 50 mM Tris–HCl buffer (pH 7.4) and stored at −70°C until used. Microsomal protein content was determined by the method of Lowry et al. [24]. To test the peroxidation of microsomes, a solution containing both ADP (22 mM) and $FeCl_3$ (1.3 mM) was prepared in Tris–HCl buffer (50 mM, pH 7.4). A solution of NADPH (1 mM) was prepared in the same buffer. These solutions were mixed with the microsomes to initiate lipid peroxidation [22,25]. The reaction mixture (NADPH/Fe^{3+}/ADP) contained 2-mg microsome protein per mililitre, along with 1.0 mM ADP, 0.06 mM Fe^{3+} and 0.05 mM NADPH, in a total reaction volume of 2.2 ml. The incubation mixture was shaken in the dark at 37°C, under air atmosphere. To 1 ml of microsomal suspension, 4 ml of chloroform/methanol (2:1, v/v) containing 90 nM butylated hydroxytoluene (BHT, as antioxidant) was added and mixed vigorously for 1 min. The mixture was centrifuged at 1000 × *g* for 15 min. The lower chloroform layer was collected and evaporated to dryness under a nitrogen stream. The microsomal total lipids were redissolved in an appropriate amount of chloroform/methanol (2:1, v/v) and analyzed by CL-HPLC for phospholipid hydroperoxides. Microsomal fatty acid methyl esters were extracted and assayed as described in Section 2.2.

2.7. Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Bonferroni's *t*-test for multiple comparisons. Student's *t*-test was used when two groups were compared. Results are expressed as mean ± S.E.M. values and $P < 0.05$ was regarded as significant.

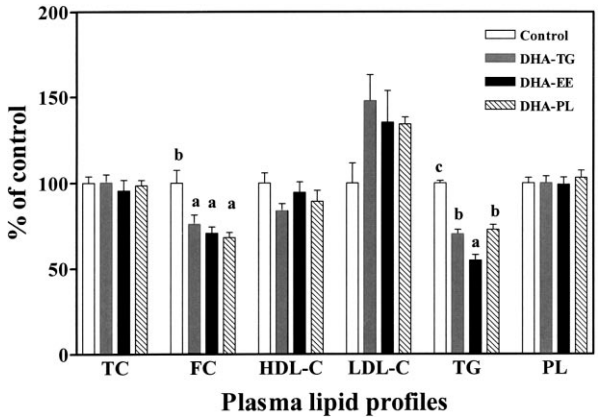

Fig. 1. Plasma lipid profiles in rats fed DHA oils for 3 weeks. DHA groups were compared with controls by ANOVA with a post hoc Bonferroni test. Each vertical bar represents the mean of six rats ± S.E.M. Means with different letters are significantly different at $P < 0.05$. TC, total-cholesterol; FC, free-cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglyceride; PL, phospholipid.

3. Results

3.1. Effect of dietary DHA oils on body weight and liver weight

To investigate the dietary effect of DHA oils on 3-week-old rats (body weight, 175–185 g), the rats were pair-fed with one of three DHA oil diets, which differed in the lipid types of DHA oils (the oil contributed 15% by weight of the diet and the total fat energy of the diets was 30.6 en%), compared with control (palm oil supplemented 20% soybean oil). 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. No significant differences in final body weight gains among the dietary groups (288–301 g) were observed. No significant differences in the weight gains of liver were seen in rats fed the various oil diets during 3 weeks (4.4–4.7 g/100 g body weight). No significant differences in plasma enzymes GOT and GPT activities were seen in any of the diet groups for 3 weeks of feeding (GOT; 57-78 Karmen Unit, GPT; 17-19 Karmen Unit).

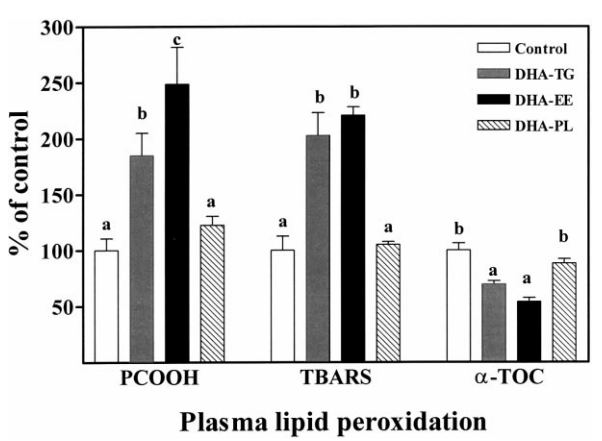

Fig. 2. Plasma PCOOH, TBARS and α-tocopherol (α-TOC) in DHA oils-fed and control oil-fed rats after 3 weeks of feeding. DHA groups were compared with controls by ANOVA with a post hoc Bonferroni test. Each vertical bar represents the mean of six rats ± S.E.M. Means with different letters are significantly different at $P < 0.05$.

3.2. Effect of dietary DHA oils on plasma lipid profile

The plasma total-cholesterol, free-cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and phospholipid was assayed in rats fed DHA oils, in the form of TG, EE and PL and then compared with control rats (Fig. 1). The plasma triglyceride was lowered by 45% in DHA-EE, by 30% in DHA-TG and by 27% in DHA-PL, compared with control (130.9 ± 1.8 mg/dl). Compared to control (24.3 ± 1.8 mg/dl), the plasma free-cholesterol was significantly lowered by 24–32% in all DHA groups. However, no significant differences in plasma total-cholesterol (85–89 mg/dl), high density lipoprotein-cholesterol (HDL-C) (27–33 mg/dl), low density lipoprotein-cholesterol (LDL-C) (30–44 mg/dl) and phospholipid (109–114 mg/dl) were seen in rats fed the various oil diets. DHA oil feeding with all types tested decreased plasma triglyceride levels, but this hypotriglyceridemic effect was most effective in EE-type DHA oil-fed rats.

3.3. Effect of dietary DHA oils on plasma and liver lipid peroxidation

To determine the effect of dietary DHA oil on lipid peroxidation in rats, phospholipid hydroperoxides (PLOOH), mainly phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH), TBARS production and α-tocopherol were examined in plasma and liver of rats fed DHA oils or control diets. The level of PCOOH in plasma was much higher in DHA-TG (185 ± 20%) and DHA-EE (248 ± 33%) than in DHA-PL (122 ± 8%), compared with control (100 ± 11%, 59.4 ± 6.5 pmol PCOOH/ml plasma) (Fig. 2). The plasma TBARS was much higher in DHA-TG (203 ± 21%) and DHA-EE (221 ± 8%) than in DHA-PL (105 ± 3%), compared with control (100 ± 13%; 3.9 ± 0.5 nmol MDA/ml plasma) (Fig. 2). The plasma α-tocopherol concentration was significantly lower in DHA-TG (70 ± 3%) and DHA-EE (54 ± 4%) than those of DHA-PL (88 ± 4%) and control (100 ± 6%; 8.5 ± 0.6 μg α-TOC/ml plasma) (Fig. 2). The level of PCOOH and PEOOH in liver was much higher in DHA-TG (179 ± 12 and 145 ± 8%) and DHA-EE (195 ± 12 and 156 ± 9%) than in DHA-PL (137 ± 10 and 104 ± 7%), with control values of 453 ± 30 pmol PCOOH/100 mg protein (100 ± 7%) and

346 ± 32 pmol PEOOH/100 mg protein (100 ± 9%) (Fig. 3). The liver TBARS were much higher in DHA-TG (140 ± 14%), DHA-EE (148 ± 8%) than in DHA-PL (114 ± 9%), compared with the control group (100 ± 6%; 376 ± 23 nmol MDA/mg protein) (Fig. 3). The liver α-tocopherol content was significantly lowered in DHA-EE (62 ± 5%) and DHA-TG (81 ± 7%) than that of DHA-PL (107 ± 7%) and control (100 ± 3%; 23.2 ± 0.8 μg α-TOC/100 mg protein) (Fig. 3). These results demonstrate that lipid peroxidation products increased together with an increase in polyunsaturation, while α-tocopherol level was reduced in response to the increase in peroxidation products.

3.4. Effect of dietary DHA oils on plasma and liver fatty acid composition

Rats receiving dietary DHA oil treatment showed changes in fatty acid composition of plasma and liver phospholipids (Table 2). The n-3 fatty acids content in plasma and liver phospholipid was much higher in DHA-EE ($P < 0.05$) and DHA-TG ($P < 0.05$) than in DHA-PL, compared with levels in control rats, while the n-6 fatty acids content was significantly lower in all DHA oil groups. DHA (22:6n-3) and arachidonic acid (20:4n-6), the principal n-3 and n-6 fatty acids, were apparently changed in plasma and liver phospholipids of rats fed DHA oil diets. The values of peroxidizability index (PI) of fatty acids, which indicates the relative susceptibilities to peroxidation, were significantly higher in the DHA-TG and DHA-EE than DHA-PL as a result of differences in DHA incorporation into tissue membranes. These results indicated that high levels of DHA ingestion with different lipid types, DHA-TG, DHA-EE and DHA-PL differently affected fatty acid compositions of plasma and liver phospholipids.

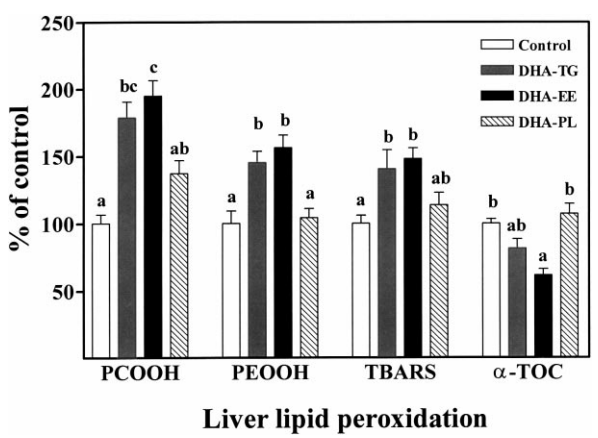

Fig. 3. Liver PCOOH, PEOOH, TBARS and α-tocopherol (α-TOC) in DHA oils-fed and control oil-fed rats after 3 weeks of feeding. DHA groups were compared with controls by ANOVA with a post hoc Bonferroni test. Each vertical bar represents the mean of six rats ± S.E.M. Means with different letters are significantly different at $P < 0.05$.

Table 2
Fatty acid composition of plasma and liver phospholipids in rats fed dietary DHA oils for 3 weeks[a]

Fatty acid	Control	DHA-TG	DHA-EE	DHA-PL
Plasma (mol/100 mol)				
16:0	32.7 ± 0.7	32.3 ± 0.6	33.9 ± 1.8	37.0 ± 1.9
16:1	0.9 ± 0.2[a]	0.6 ± 0.7[a]	0.5 ± 0.0[a]	1.9 ± 0.2[b]
18:0	14.6 ± 0.5[a]	19.0 ± 1.2[b]	18.5 ± 1.0[b]	19.1 ± 0.5[b]
18:1n-9	13.7 ± 0.4[b]	10.4 ± 0.4[a]	10.2 ± 0.5[a]	14.3 ± 0.3[b]
18:2n-6	10.3 ± 0.2	9.0 ± 0.4	8.6 ± 1.1	10.1 ± 0.3
18:3n-3	0.2 ± 0.1[a]	2.0 ± 0.9[b]	1.6 ± 0.3[b]	0.3 ± 0.1[a]
20:4n-6	23.5 ± 0.7[b]	10.9 ± 2.3[a]	11.6 ± 1.1[a]	9.7 ± 1.2[a]
20:5n-3	0.6 ± 0.0[a]	4.9 ± 0.6[c]	4.8 ± 0.3[c]	2.3 ± 0.4[b]
22:5n-6	0.6 ± 0.4	0.9 ± 0.5	0.4 ± 0.1	0.4 ± 0.3
22:5n-3	0.3 ± 0.1[a]	1.0 ± 0.2[b]	1.0 ± 0.1[b]	1.3 ± 0.3[b]
22:6n-3	2.3 ± 0.1[a]	8.8 ± 0.6[c]	8.6 ± 0.8[c]	3.6 ± 0.5[b]
n-6	34.5 ± 0.6[b]	20.8 ± 2.0[a]	20.6 ± 2.2[a]	20.2 ± 1.4[a]
n-3	3.3 ± 0.1[a]	16.7 ± 1.2[c]	16.0 ± 0.8[c]	7.5 ± 0.7[b]
n-6/n-3	10.4 ± 0.2[c]	1.4 ± 0.2[a]	1.3 ± 0.2[a]	2.7 ± 0.1[b]
PI	130.9 ± 3.0[a]	168.0 ± 7.0[b]	164.6 ± 6.7[b]	102.5 ± 9.9[a]
Liver (mol/100 mol)				
16:0	20.1 ± 0.4	22.2 ± 0.3	22.4 ± 0.4	20.5 ± 0.2
16:1	0.1 ± 0.1	0.5 ± 0.2	1.1 ± 0.2	0.9 ± 0.2
18:0	25.9 ± 0.2	23.3 ± 0.1	22.7 ± 0.2	23.8 ± 0.8
18:1n-9	6.2 ± 0.5	5.6 ± 0.4	4.1 ± 0.6[a]	7.4 ± 0.7[b]
18:2n-6	6.9 ± 0.1[a]	8.2 ± 0.3[ab]	7.2 ± 0.2[ab]	9.3 ± 0.1[b]
18:3n-3	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
20:4n-6	28.5 ± 0.4[b]	19.9 ± 0.2[a]	20.0 ± 0.2[a]	20.2 ± 0.4[a]
20:5n-3	0.7 ± 0.0[a]	2.0 ± 0.2[b]	2.3 ± 0.1[b]	3.4 ± 0.5[b]
22:5n-6	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.1
22:5n-3	1.0 ± 0.5	1.4 ± 0.3	1.6 ± 0.3	1.5 ± 0.3
22:6n-3	9.8 ± 0.3[a]	16.3 ± 0.2[c]	18.1 ± 0.4[c]	12.7 ± 0.4[b]
n-6	35.9 ± 0.5[b]	28.5 ± 0.4[a]	27.6 ± 0.3[a]	29.6 ± 4.9[a]
n-3	11.7 ± 0.3[a]	19.9 ± 0.3[c]	22.2 ± 0.4[c]	17.8 ± 0.4[b]
n-6/n-3	3.1 ± 0.1[c]	1.4 ± 0.1[a]	1.2 ± 0.1[a]	1.7 ± 0.1[b]
PI	213.6 ± 2.4[a]	241.7 ± 1.3[b]	258.6 ± 3.4[b]	221.9 ± 4.9[a]

[a] Values are means ± S.E.M. of six rats in each group. Superscripts a–c, means in the horizontal row with different superscripts are significantly different at $P < 0.05$ to each other.

3.5. Effect of dietary DHA oils on microsomal lipid peroxidation

The microsomal total lipids were extracted from the liver of DHA oil-fed rats and control oil-fed rats (Table 3). A rapid accumulation of both PCOOH and PEOOH was observed in microsomes incubated with NADPH/Fe^{3+}/ADP, as shown in Fig. 4. PCOOH (Fig. 4A) and PEOOH (Fig. 4B) production during microsomal peroxidation was higher in DHA-TG and DHA-EE groups than in DHA-PL and control groups with time dependent incubation. Examination of phospholipid hydroperoxides (PCOOH and PEOOH) and TBARS in liver homogenate and liver microsomal preparations indicate higher levels of these oxidative stress markers in DHA-EE and DHA-TG groups relative to controls. This may reflect a higher basal rate of oxidative stress possibly related to these groups' lower concentrations

Table 3
Fatty acid composition of liver microsomal phospholipids in rats fed dietary DHA oils for 3 weeks[a]

Fatty acid	Microsomal group (mol/100 mol)			
	Control	DHA-TG	DHA-EE	DHA-PL
16:0	26.8 ± 0.6	25.8 ± 1.4	26.9 ± 2.0	24.5 ± 0.7
16:1	1.7 ± 0.1[a]	1.2 ± 0.1[a]	1.7 ± 0.5[a]	4.6 ± 0.8[b]
18:0	11.3 ± 0.6[b]	12.6 ± 1.7[b]	12.1 ± 0.9[b]	8.8 ± 1.2[a]
18:1n-9	27.5 ± 0.8[c]	12.8 ± 1.4[a]	12.8 ± 1.0[a]	24.0 ± 1.4[b]
18:2n-6	11.4 ± 1.0	10.3 ± 0.7	10.1 ± 1.0	9.8 ± 0.5
18:3n-3	0.5 ± 0.2	0.3 ± 0.2	0.3 ± 0.1	0.5 ± 0.1
20:4n-6	14.2 ± 0.7[c]	11.8 ± 1.3[b]	11.4 ± 1.7[b]	7.5 ± 1.0[a]
20:5n-3	0.4 ± 0.0	2.7 ± 0.1	3.0 ± 0.3	3.5 ± 0.2
22:5n-6	0.5 ± 0.1	0.2 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
22:5n-3	0.7 ± 0.0[a]	2.8 ± 0.4[b]	2.8 ± 0.4[b]	2.8 ± 0.3[b]
22:6n-3	5.0 ± 0.4[a]	19.7 ± 1.8[c]	18.7 ± 1.7[c]	13.9 ± 0.9[b]
n-6/n-3	4.0 ± 0.4[b]	0.9 ± 0.1[a]	0.9 ± 0.1[a]	0.8 ± 0.1[a]
PI	119.4 ± 3.6[a]	249.5 ± 13.5[c]	241.7 ± 19.7[c]	191.2 ± 9.7[b]

[a] Values are means ± S.E.M. of six rats in each group. Superscripts a–c, means in the horizontal row with different superscripts are significantly different at $P<0.05$ to each other.

of α-tocopherol. For all samples, no significant change in TBARS was observed during the first 10-min incubation (Fig. 5A). The level of TBARS in DHA-TG and DHA-EE groups increased 2- to 3-fold over DHA-PL and control groups at later times (Fig. 5A). Endogenous α-tocopherol content of microsomes was found to decrease during peroxidation initiated by a combination of NADPH/Fe^{3+}/ADP (Fig. 5B). In DHA-TG and DHA-EE groups, ≈80% of endogenous α-tocopherol was degraded during 10 min incubation and α-tocopherol was almost exhausted by 40 min incubation. In DHA-PL and control groups, some endogenous α-tocopherol was still present in the microsomes at the end of 40 min of peroxidation.

3.6. Loss of major microsomal PUFA during lipid peroxidation

Fig. 6 shows the loss of n-3 and n-6 PUFA in microsomes during peroxidation initiated by a combination of NADPH/Fe^{3+}/ADP. Both DHA (22:6n-3) and arachidonic acid (20:4n-6) were consumed during the 40 min incubation. Substantial amounts of DHA remained in the microsomes at the end of the 40 min of incubation: 31% (DHA-TG), 26% (DHA-EE) and 55% (DHA-PL) of the initial value (Fig. 6A). The amount of

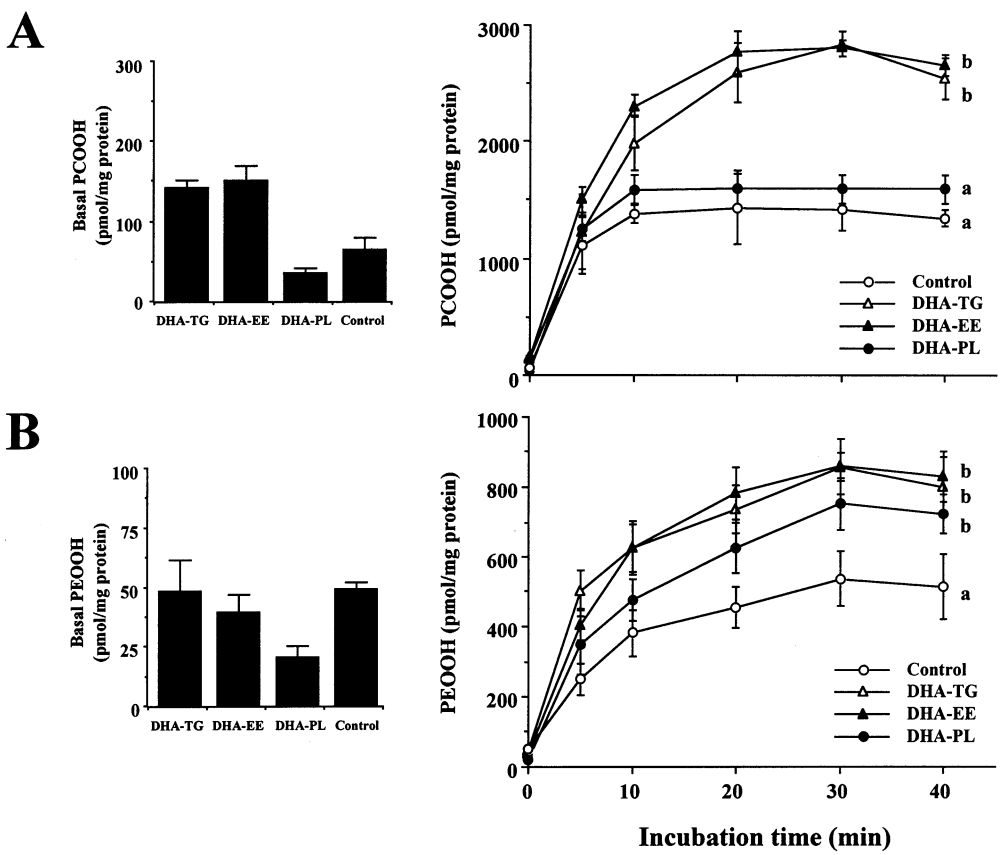

Fig. 4. Effects of feeding DHA oils to rats on the PCOOH (upper panel) and PEOOH (lower panel) in the NADPH-dependent liver microsomal peroxidation system. The complete microsome system (total volume of 2.2 ml:2.1 mg microsomal protein/ml, 1.0 mM ADP, 0.06 mM Fe^{3+}, 0.05 mM NADPH in 50 mM Tris–HCl buffer at pH 7.4) was incubated at 37°C. Each point represents the mean ± S.E.M. of six experiments. Means with different letters are significantly different at $P<0.05$.

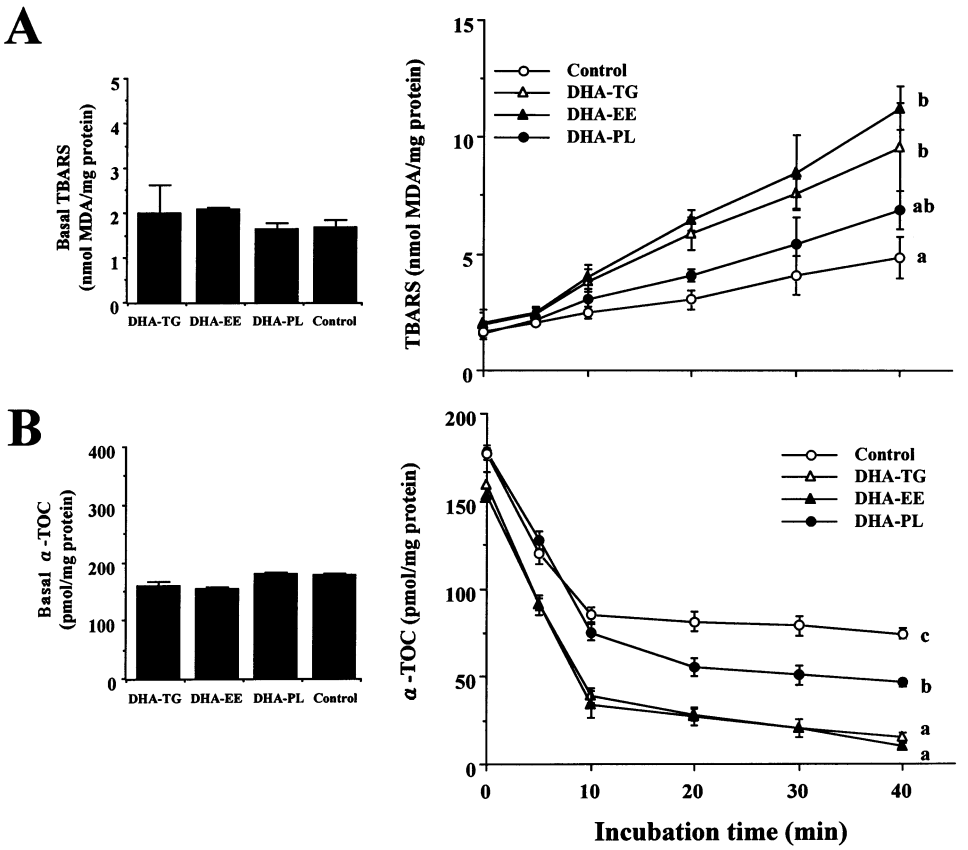

Fig. 5. Effects of feeding DHA oils to rats on the TBARS formation (upper panel) and endogeneous α-tocopherol (α-TOC) (lower panel) in the NADPH-dependent liver microsomal peroxidation system. The reaction conditions were the same as those given in Fig. 4. Each point represents the mean ± S.E.M. of six experiments. Means with different letters are significantly different at $P < 0.05$.

arachidonic acid (20:4n-6) remaining in the microsomes at the end of the 40-min of incubation was: 42% (DHA-TG), 32% (DHA-EE) and 53% (DHA-PL) of the initial value (Fig. 6B). The change in the loss of PUFA levels was bigger in the n-3 fatty acid than in the n-6 fatty acid after exposure of microsomes to oxidative stress (Fig. 6C,D).

4. Discussion

A number of studies have demonstrated that dietary DHA lowers the level of plasma triglyceride in rats [26–29]. Our results clearly showed that dietary DHA oils fed to rats lowered plasma triglycerides. Among the three DHA groups, EE-type DHA oil was more effective than TG- and PL-DHA oils in lowering plasma triglycerides (Fig. 1). In view of the relationship of hypertriglyceridemia and vascular disease [30], reduction of plasma triglycerides and free cholesterol by DHA oil is an important intervention in reducing the risk of atherosclerosis. Despite the prospect that DHA oils may be part of interesting therapeutic approaches for the prevention of this disease, there is the potential to enhance the lipid peroxidation in mammals. Because of the increased presence of polyunsaturated fatty acids in tissue membranes, these membranes are more susceptible to damage by lipid peroxidation. DHA taken into the body is mostly delivered to the liver through plasma lipoprotein [31], thus, both plasma and liver may be the primary targets for oxidative stress induced damage in oil-fed rats. Recently, several investigators have reported the effect of fish oil PUFA, especially DHA, on lipid peroxidation in tissues after being ingested by mammals [10,32,33]. It is, therefore, of interest to investigate whether these effects of n-3 fatty acids including DHA are offset by increased susceptibility to lipid peroxidation. In order to further pursue our in vitro observations, which showed the differential stability of PL-type DHA oil against autoxidation, we compared the plasma and liver lipid peroxidation in rats fed DHA oils in the form of TG, EE and PL. To evaluate lipid peroxidation, we designed CL-HPLC system in which a mixture of Cytochrome *c* and luminol was utilized as post column chemiluminescence cocktail [18,19]. It is well known that the PLOOH, such as PCOOH and PEOOH formed during oxidative stress to the membrane [34–36], can lead to an abnormality of membrane functions, and further perturbation of cellular signaling pathways [37].

DHA oil ingestion in the form of TG and EE results in elevated lipid hydroperoxide levels. Alternatively, DHA oils given in the form of PL which reflects the difference in unsaturation of their constituent fatty acids in membrane phospholipids had only a modest effect compared to control. Compared with the DHA-PL group, both DHA-TG and DHA-EE groups significantly elevated the accumulation of TBARS and PLOOH (both PCOOH and PEOOH) (Figs. 2 and 3). The amount of PLOOH accumulation is in agreement with the TBARS content.

The present study showed an inverse relationship between peroxidized lipid products (PLOOH and TBARS) and α-tocopherol content in both plasma and liver of rats fed the dietary oils. α-Tocopherol is the most potent antioxidant in biological systems which scavenges peroxyl radicals. A reduction of α-tocopherol levels has been shown to be involved in the susceptibility to lipid peroxidation [38,39]. The reduction in plasma and liver α-tocopherol levels observed with TG- and EE-types DHA oil feeding, in this study, is in keeping with the observation that more tissue α-tocopherol is required to protect the tissue lipids against oxidative stress. DHA-TG and DHA-EE groups show a higher level of lipid peroxidation than DHA-PL due to the difference in unsaturation of their constituent fatty acids in membrane phospholipids. The results of this study showed that dietary DHA oil feeding significantly alters tissue membrane phospholipids by modifying fatty acid composition. DHA oil in the diets led to a marked incorporation of n-3 fatty acids into membrane phospholipids. With dietary DHA oil in the form of PL, lower DHA levels were found in plasma and liver phospholipids than in both DHA-TG and DHA-EE which have similar fatty acid compositions. This demonstrates that feeding with DHA oil of PL type was sufficient to change tissue membrane lipid content, even though DHA-PL supplementation had little effect on the tissue DHA content unlike that of rats fed DHA-TG and DHA-EE oils diets. Our results clearly showed that DHA oils feeding significantly lowered the proportion of arachidonic acid (ARA, 20:4n-6) in plasma and liver phospholipids. Dietary DHA-PL feeding results in a smaller increase in tissue n-3 PUFA (especially, DHA) compared with other dietary DHA oils feeding, but the effect on the decrease of n-6 PUFA (especially, arachidonic acid) was no different among the three different types of DHA oils feeding. The ratio of n-6/n-3 fatty acids in DHA groups was much lower than that of control group, in plasma and liver. DHA-TG and DHA-EE was more effective than DHA-PL. The differences in lipid peroxidation in the present study can be attributed directly to dietary DHA because the three DHA oils contain comparable amounts of total PUFA.

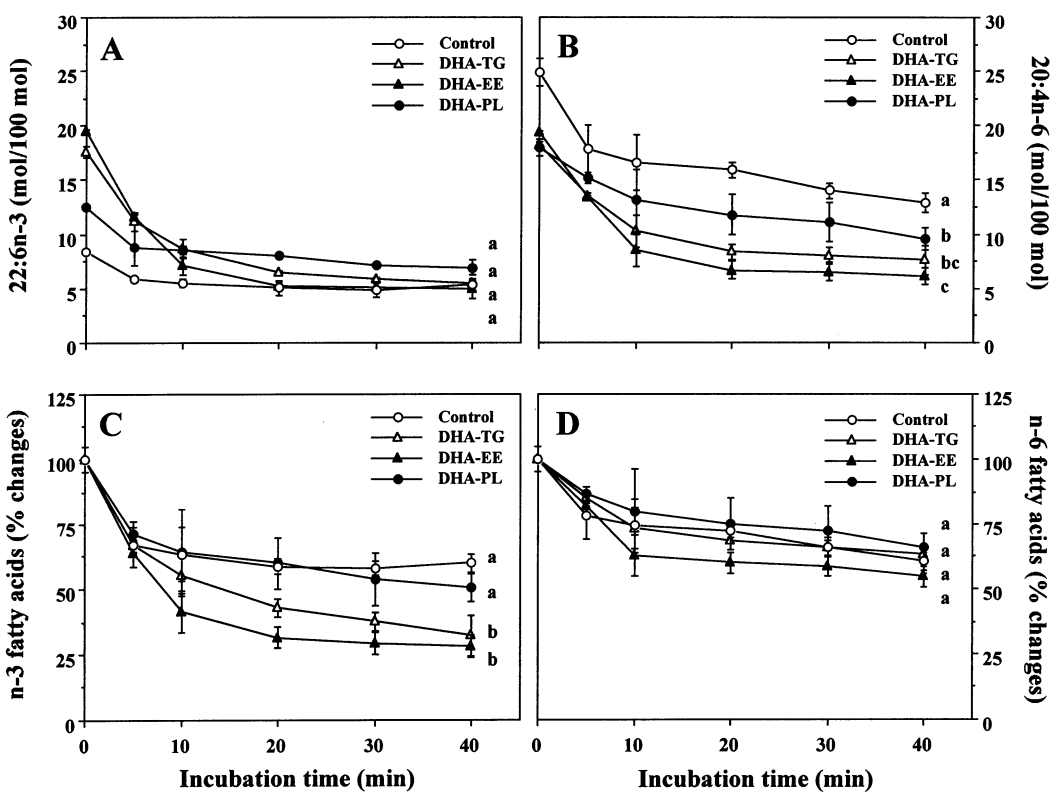

Fig. 6. Loss of microsomal major PUFA during NADPH-dependent lipid peroxidation. The reaction conditions were the same as in Fig. 4. Each point represents the mean ± S.E.M. of six experiments. Means with different letters are significantly different at $P < 0.05$. (A) Loss of 22:6n-3, docosahexaenoic acid; (B) loss of 20:4n-6, arachidonic acid; (C) loss of n-3 fatty acids; (D) loss of n-6 fatty acids.

These results show changes in lipid peroxidation during NADPH-dependent microsomal lipid peroxidation. During NADPH-dependent peroxidation of microsomes, both PCOOH and PEOOH accumulated over a 40-min incubation (Fig. 4A,B). In microsomal phospholipid peroxidation, we found that the accumulation of phospholipid peroxides was 4.5-fold greater in DHA-TG and DHA-EE groups than DHA-PL group.

Compared with DHA-PL group, both DHA-TG and DHA-EE groups had significantly elevated accumulations of TBARS similar to PLOOH (PCOOH and PEOOH) (Figs. 4 and 5A), while the endogenous α-tocopherol content was rapidly consumed during NADPH-dependent peroxidation of microsomes (Fig. 5B). Consistent with the low content of PI, the control group showed that α-tocopherol was not fully eliminated after 40 min incubation. Our results from microsomal study showed that increased oxidative stress resulted from an increase in polyunsaturation in microsomal membranes. The major PUFA composition during microsomal peroxidation was changed to reduce PUFA levels in DHA feeding groups. Interestingly, n-3 PUFA is a more likely target for peroxidation than n-6 PUFA (Fig. 6A,B). Therefore, the higher changes in n-3 PUFA but not n-6 PUFA during NADPH-dependent microsomal lipid peroxidation can be explained by an increase of membrane n-3 fatty acids resulting an increased susceptibility to an oxidative stress (Fig. 6C,D).

We observed that DHA oil feeding has shown preventive value in relation to cardiovascular disease by a reduction in plasma triglyceride levels and it can possibly inhibit platelet function by reducing arachidonic acid levels in membrane phospholipids. However, the consumption of DHA oil resulted in increased phospholipid hydroperoxides and TBARS of plasma, liver and microsomal membranes. This elevated lipid peroxidation was seen in conjunction with increased phospholipid n-3 fatty acid incorporation and decreased levels of α-tocopherol. Our results suggest that the ingestion of DHA oil enhances the lipid peroxidation in the target membranes where high amounts of n-3 fatty acids are incorporated, thereby increasing the peroxidizability and possibly accelerating the atherosclerotic process [40].

In summary, the present results indicate that dietary DHA oil feeding enhances the susceptibility of the plasma and liver to lipid peroxidation concomitant with higher levels of n-3 fatty acid (especially, DHA) in the fatty acid composition of membrane phospholipid.

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