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# Relationship between tissue lipid peroxidation and peroxidizability index after α-linolenic, eicosapentaenoic, or docosahexaenoic acid intake in rats

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In a previous study, we found that the extent of dietary *n*-3 docosahexaenoic acid (DHA)-stimulated tissue lipid peroxidation was less than expected from the relative peroxidizability index of the total tissue lipids in rats with adequate vitamin E nutritional status. This suppression of lipid peroxidation was especially prominent in the liver. To elucidate whether this phenomenon was unique to DHA, we compared the peroxidation effects of *n*-3 α-linolenic acid (α-LN) and *n*-3 eicosapentaeonic acid (EPA) with those of DHA in rats. Either α-LN (8·6 % of total energy), EPA (8·2 %), or DHA (8·0 %) and one of two levels of dietary vitamin E (7·5 and 54 mg/kg diet) were fed to rats for 22 d. Levels of conjugated diene, chemiluminescence emission and thiobarbituric acid (TBA)-reactive substance in the liver, kidney, and testis were determined as indicators of lipid peroxidation. In rats fed the DHA diet deficient in vitamin E (7·5 mg/kg diet), TBA values in the liver, kidney, and testis correlated well with the tissues' relative peroxidizability indices. In rats fed the α-LN diet with an adequate level of vitamin E (54 mg/kg diet), a close association between relative peroxidizability indices and lipid peroxide levels was observed in all the tissues analysed. However, in rats fed either the EPA diet or the DHA diet with an adequate level of vitamin E, the extent of lipid peroxidation in each tissue was less than expected from the relative peroxidizability index. This suppression was particularly marked in the liver. We concluded that suppression of lipid peroxidation below the relative peroxidizability index was not unique to DHA, but was also seen with EPA, which has five double bonds, in rats with adequate vitamin E nutritional status, but not with α-LN, which has three double bonds.

**Docosahexaenoic acid: Eicosapentaeonic acid: Lipid peroxidation: Peroxidizability index**

*n*-3 Eicosapentaenoic acid (EPA; 20 : 5*n*-3) and docosahexaenoic acid (DHA; 22 : 6*n*-3) are the major polyunsaturated fatty acids (PUFA) in fish oils. Some vegetable oils such as flaxseed and perilla oils are rich in another *n*-3 fatty acid, α-linolenic acid (α-LN; 18 : 3*n*-3). Consumption of *n*-3 PUFA has been shown to be associated with a low incidence of atherosclerosis and cardiovascular diseases (Dyerberg, 1986; Herold & Kinsella, 1986; Harris, 1989; Simopoulos, 1991, 1999; Crawford *et al.* 2000). Studies of non-human primates and newborn infants have also shown that DHA is essential to the normal functional development of the retina and brain, especially in premature babies (Nestel, 1990; Simopoulos, 1991). Consequently, concentrates of *n*-3 PUFA, such as EPA and DHA from fish oils in particular, have been used as medicinal and/or nutritional supplements (Drevon, 1992; Lands, 1992).

However, the ingestion of purified DHA (Saito *et al.* 1996; Kubo *et al.* 1997, 1998), or fish oil (Hammer & Wills, 1978; Kobatake *et al.* 1983; Mouri *et al.* 1984; Hu *et al.* 1989; Song *et al.* 2000) enhanced the susceptibility of the liver and kidney to lipid peroxidation, whereas in the brain and testis lipid peroxide levels were not increased in rats with adequate vitamin E nutritional status (Kubo *et al.* 1998). Enhancement of lipid peroxidation was a function of the dietary DHA level and also increased the requirement for vitamin E, a lipophilic membrane antioxidant (Saito *et al.* 1996; Kubo *et al.* 1998). This is thought to be attributable to the partial substitution of membrane

**Abbreviations:** DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GSH, glutathione; LA, linoleic acid; α-LN, α-linolenic acid; PUFA, polyunsaturated fatty acids; SH, sulfhydryl; TBA, thiobarbituric acid.

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fatty acids with DHA, which is very susceptible to lipid peroxidation and thus potentially unstable (Kubo *et al.* 1998).

Nevertheless, in an earlier study, we found that in rats ingesting DHA along with an adequate level of vitamin E, the extent of tissue lipid peroxidation was less than expected from the relative peroxidizability index which was calculated from the fatty acid composition of the total tissue lipids (Kubo *et al.* 1998, 2000). This phenomenon was especially prominent in the liver and, to a lesser extent, in the testis (Kubo *et al.* 2000). However, the tissue lipid peroxide levels nearly coincided with the peroxidizability indices for total tissue lipids when a vitamin E-deficient diet was administered (Kubo *et al.* 2000). In the vitamin E-deficient state, tissue parenchymal cell injuries were also observed.

In the present study, therefore, we fed two other major *n*-3 PUFA, α-LN and EPA, to rats to elucidate whether this phenomenon of reduced tissue lipid peroxidation was unique to the administration of highly unsaturated DHA under adequate vitamin E nutritional status. In addition, we analysed changes in lipid peroxide scavenger levels to clarify the mechanism of the suppression of lipid peroxidation. To differentiate the effect of each *n*-3 PUFA, we employed purified EPA and DHA as a model, as well as perilla oil as a source of α-LN.

## Materials and methods

### *Animals and diets*

The experimental procedures used in the present study met the guidelines of the Animal Committee of the Independent Administrative Institution, National Institute of Health and Nutrition (Tokyo, Japan).

Male Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan), 5 weeks of age and weighing 100–133 g, were housed individually in stainless steel wire-bottomed cages at a constant temperature of 22 ± 1°C and relative humidity of 50–60 % with a 12 h light–dark cycle. The composition of the experimental diets, based on the AIN-76 purified diet for rats (American Institute of Nutrition, 1977, 1980), is shown in Table 1. The lipid content of the diets was 100 g/kg diet and 21·6 % of the total energy. EPA ethyl esters (96 % pure) prepared from sardine oil and DHA ethyl esters (83 % pure) prepared from orbital fat of tuna were donated by Nippon Suisan Kaisha (Hachioji, Japan) and Maruha Corporation (Tsukuba, Japan), respectively. To prevent the auto-oxidation of DHA, EPA and α-LN in the diets, they were prepared beforehand without adding these PUFA and stored at −20°C. DHA and EPA were stored at −80°C and Perilla oil rich in α-LN was stored at −20°C. They were mixed with each diet every day immediately before feeding. The vitamin E concentrations as RRR-α-tocopherol equivalents of the control diet (linoleic acid (LA; 18 : 2*n*-6) group) and the test diets (α-LN, EPA and DHA groups) were 54 mg/kg. The vitamin E concentration of the DHA diet with low vitamin E (DHA + low vitamin E group) was 7·5 mg/kg. The RRR-α-, RRR-β-, RRR-γ- and RRR-δ-tocopherols biopotency ratio was 100 : 25 : 5 : 0·1, respectively, in the calculation of RRR-α-tocopherol equivalent (Mino *et al.* 1988). All-rac-α-tocopheryl acetate (>99 % pure) was used to adjust the vitamin E concentration in the diets.

The fatty acid composition (g/100 g fatty acids) of dietary lipids is also indicated in Table 1. The control lipid (LA group), devoid of EPA and DHA, contained 41 g LA/100 g, which is comparable to the α-LN (39·9 g/100 g), EPA (37·8 g/100 g) and DHA (37·2 g/100 g) levels of the test lipids. In addition, all the test lipids (α-LN, EPA, DHA and DHA + low vitamin E groups) were prepared to provide a constant amount of LA as essential *n*-6 PUFA at at least 2·1 % of total dietary energy, where the proportion was approximately 10 %. The degree of unsaturation of the dietary lipids is presented as the double-bond index (Pietrangelo *et al.* 1990) and the peroxidizability index (Hu *et al.* 1989). It has been reported that the relative reaction rate constants of peroxidation were 1, 2, 3, 4 and 5 against PUFA in which the number of methylene groups among the double bonds was one, two, three, four and five, respectively (Cosgrove *et al.* 1987). The peroxidizability index of the lipid, therefore, is calculated from its fatty acid composition (%, w/w) according to the following equation (Hu *et al.* 1989):

$$\begin{aligned}\text{peroxidizability index} = {} & (\%\ \text{dienoic} \times 1) + (\%\ \text{trienoic} \times 2) \\ & + (\%\ \text{tetraenoic} \times 3) \\ & + (\%\ \text{pentaenoic} \times 4) \\ & + (\%\ \text{hexaenoic} \times 5).\end{aligned}$$

After the rats were fed the basal diet containing 50 g olive oil/kg diet for 4 d, six or seven rats in each group were fed the experimental diets for 22 d. Food and water were consumed *ad libitum*. Each diet was made available to the rats in the evening and removed the next morning. After being deprived of food for 9 h, the rats were killed by cardiac puncture. The tissues were promptly excised, washed with isotonic saline and weighed. The liver was perfused with ice-cold isotonic saline via the portal vein. The liver, kidney and testis samples were stored at −80°C until needed for analysis. Serum was separated by centrifugation at 2700 ***g*** for 15 min at 4°C.

### *Conjugate diene and chemiluminescence intensity analyses*

Liver microsomes were prepared (Saito & Yamaguchi, 1988), and the conjugated dienes of the liver microsomes and tissues were determined by the method of Hu *et al.* (1989) and Rao & Recknagel (1968). The microsomal protein content was measured by the method of Lowry *et al.* (1951).

The liver, kidney and testis chemiluminescence intensities of the homogenates were determined according to the method of Miyazawa *et al.* (1984). The light emitted from the homogenates is due mainly to singlet molecular oxygen and/or excited carbonyl compounds resulting from the breakdown of lipid peroxyradicals (Boveris *et al.*

**Table 1.** Composition of experimental diets and fatty acid composition of dietary lipids given to rats*

| Diet... | LA | α-LN | EPA | DHA | DHA + lowVit E |
|---|---|---|---|---|---|
| *n*-3 Fatty acids (% total energy) | – | 8·6 | 8·2 | 8·0 | 8·0 |
| LA (% total energy) | 8·9 | 2·4 | 2·2 | 2·1 | 2·1 |
| Basic components† (g/kg diet) | 900·0 | 900·0 | 900·0 | 900·0 | 900·0 |
| Test lipids‡ (g/kg diet) | 100·0 | 100·0 | 100·0 | 100·0 | 100·0 |
| Olive oil | 50·0 | 36·0 | 48·5 | 37·2 | 37·2 |
| Safflower-seed oil | 50·0 | 0 | 8·2 | 9·0 | 9·0 |
| Perilla oil | 0 | 64·0 | 0 | 0 | 0 |
| EPA concentrate§ | 0 | 0 | 43·3 | 0 | 0 |
| DHA concentrate‖ | 0 | 0 | 0 | 53·8 | 53·8 |
| Fatty acid (g/100 g) | | | | | |
| 16:0 | 8·7 | 9·0 | 6·6 | 4·7 | 4·7 |
| 16:1*n*-7 | 0·6 | 0·6 | 0·5 | 0·4 | 0·4 |
| 18:0 | 2·8 | 2·1 | 1·8 | 1·5 | 1·5 |
| 18:1*n*-9 | 45·4 | 36·3 | 40·5 | 31·2 | 31·2 |
| 18:2*n*-6 LA | 41·0 | 11·3 | 10·1 | 9·8 | 9·8 |
| 18:3*n*-3 | 0·9 | 39·9 | 0·5 | 0 | 0 |
| 20:4*n*-6 AA | 0 | 0 | 0·9 | 0·4 | 0·4 |
| 20:5*n*-3 EPA | 0 | 0 | 37·8 | 1·5 | 1·5 |
| 22:1*n*-9 + 22:1*n*-11 | 0 | 0 | 0 | 1·8 | 1·8 |
| 22:3*n*-3 | 0 | 0 | 0 | 1·4 | 1·4 |
| 22:5*n*-3 | 0 | 0 | 0 | 3·0 | 3·0 |
| 22:6*n*-3 DHA | 0 | 0 | 0 | 37·2 | 37·2 |
| Others | 0·6 | 0·8 | 1·1 | 7·0 | 7·0 |
| Double-bond index¶ | 1·31 | 1·77 | 2·19 | 3·17 | 3·17 |
| Peroxidizability index** | 42·8 | 89·3 | 166·2 | 225·9 | 225·9 |

LA, linoleic acid; α-LN, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Vit E, vitamin E; AA, arachidonic acid.

* The energy density of all the diets was 17·4 MJ/kg diet (4160 kcal/kg) using the Atwater energy factors for the energy calculation (Atwater, 1910). The vitamin E content as RRR-α-tocopherol equivalent of the LA, α-LN, EPA and DHA diets was 54 mg/kg and that of the DHA + lowVit E diet was 7·5 mg/kg, respectively.

† The basic components of the diet given to all the groups were as follows: casein, 200·0 g; DL-methionine, 3·0 g; maize starch, 150·0 g; sucrose, 225·0 g; glucose, 225·0 g; cellulose powder, 50·0 g; AIN-76 vitamin mixture (America Institute of Nutrition, 1977, 1980), 10·0 g; AIN-76 mineral mixture (America Institute of Nutrition, 1977, 1980), 35·0 g; choline bitartrate, 2·0 g.

‡ Fat energy percentage was 21·6 % of total energy.

§ EPA ethyl esters prepared from fatty acid ethyl esters of sardine oil were used and the purity was 96 %.

‖ DHA ethyl esters prepared from fatty acid ethyl esters of orbital fat of tuna were used and the purity was 83 %. Major fatty acids of the remaining 17 % were as follows: 20:4*n*-6, 0·7; 20:5*n*-3, 2·3; 22:1*n*-11, 1·2; 22:1*n*-9, 0·8; 22:3*n*-3, 4·6; 22:5*n*-3, 4·0; others, 3·4 %.

¶ Double-bond index expresses mean double bond number and is the sum of the fraction of each fatty acid multiplied by the number of double bonds in that acid (Pietrangelo *et al.* 1990).

** Peroxidizability index (Hu *et al.* 1989) is calculated as follows: peroxidizability index = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

1981; Miyazawa *et al.* 1981), which are produced in the early stage of peroxidation.

### *Thiobarbituric acid value and fluorescent substance analyses*

The serum thiobarbituric acid (TBA) value was determined by the method of Yagi (1976). Butylated hydroxytoluene (BHT) as an antioxidant was added to the reaction mixture at a final concentration of 0·36 mmol/l. The tissue TBA values were measured according to the method of Ohkawa *et al.* (1979) with a minor modification, in which BHT was added to the reaction mixture at a final concentration of 0·45 mmol/l. TBA values are expressed in terms of the malondialdehyde equivalent.

Serum water-soluble fluorescent substances were analysed by the method of Tsuchida *et al.* (1985). Liver microsomal lipofuscin concentration was determined by the method of Fletcher *et al.* (1973).

### *Vitamin E, total ascorbic acid and non-protein sulfhydryl assays*

The vitamin E levels in the test lipids, serum and tissues were analysed by HPLC as previously described (Saito *et al.* 1992).

Total ascorbic acid (Roe *et al.* 1948) and non-protein sulfhydryl (SH) (Beutler *et al.* 1963) levels in the tissues were measured. The non-protein SH component consists mostly of glutathione (GSH).

### *Selenium-dependent glutathione peroxidase assay*

Se-dependent glutathione peroxidase (*EC* 1.11.1.9) activity was determined according to the method of Noguchi *et al.* (1973) with a minor modification as previously described (Saito, 1990).

**Table 2.** Thiobarbituric acid reactive substance (TBA value), water-soluble fluorescent substance level, α-tocopherol concentration and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum of rats fed different types of polyunsaturated fatty acids*
(Mean values with their standard deviations for six or seven rats)

| Diet | *n*-3 Fatty acid (% energy) | LA (% energy) | TBA value (μmol malondialdehyde/l) | | Water-soluble fluorescent substance† (relative fluorescence intensity) | | α-Tocopherol (μmol/l) | | AST (μkat/l) | | ALT (μkat/l) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| LA (*n* 6) | – | 8·9 | 2·21$^{a}$ | 0·65 | 2·77$^{a}$ | 0·18 | 18·5$^{a}$ | 4·1 | 0·55$^{ab}$ | 0·11 | 0·18$^{a}$ | 0·07 |
| α-LN (*n* 7) | 8·6 | 2·4 | 2·60$^{a}$ | 0·36 | 3·05$^{a}$ | 0·14 | 15·0$^{b}$ | 3·1 | 0·50$^{a}$ | 0·04 | 0·16$^{a}$ | 0·02 |
| EPA (*n* 7) | 8·2 | 2·2 | 5·53$^{a}$ | 1·88 | 2·95$^{a}$ | 0·39 | 10·2$^{c}$ | 1·6 | 0·61$^{ab}$ | 0·17 | 0·19$^{a}$ | 0·03 |
| DHA (*n* 7) | 8·0 | 2·1 | 12·65$^{b}$ | 5·29 | 3·22$^{a}$ | 0·39 | 6·9$^{d}$ | 1·0 | 0·54$^{ab}$ | 0·04 | 0·19$^{a}$ | 0·03 |
| DHA+lowVit E‡ (*n* 7) | 8·0 | 2·1 | 10·55$^{b}$ | 4·60 | 5·73$^{b}$ | 1·57 | 1·8$^{e}$ | 0·3 | 0·74$^{b}$ | 0·34 | 0·27$^{a}$ | 0·19 |

LA, linoleic acid; α-LN, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Vit E, vitamin E.
$^{a,b,c,d,e}$Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).
* For details of diets and procedures, see Table 1 and p. 20.
† The instrument was calibrated to read 100 relative fluorescence units against a quinine sulfate solution (0·128 μmol/l of 50 mmol sulfuric acid/l solution).
‡ The vitamin E content as RRR-α-tocopherol equivalent of the LA, α-LN, EPA and DHA diets was 54 mg/kg and that of the DHA+lowVit E diet was 7·5 mg/kg, respectively.

### *Glutathione reductase and glucose-6-phosphate dehydrogenase assays*

The activities of glutathione reductase (*EC* 1.6.4.2) and glucose-6-phosphate dehydrogenase (*EC* 1·1·1·49) in the liver 12000 ***g*** supernatant fraction were analysed by the methods of Goldberg & Spooner (1983) and Bergmeyer *et al.* (1983), respectively.

### *Serum aspartate aminotransferase and alanine aminotransferase assays*

The activities of aspartate aminotransferase (*EC* 2·6·1·1) and alanine aminotransferase (*EC* 2·6·1·2) in the serum were determined with a clinical enzyme assay kit (Wako Pure Chemical, Osaka, Japan) by the method of Reitman & Frankel (1957).

### *Fatty acid analysis*

Tissue total lipids were extracted from each tissue according to the method of Folch *et al.* (1957). The fatty acid compositions of the dietary lipids and total tissue lipids were analysed by GLC with dual-flame ionization detectors (Hitachi 263–30 gas-liquid chromatograph; Tokyo, Japan) by using a 50 m × 0·25 mm internal diameter glass capillary column coated with CP-Sil-88 (Saito *et al.* 1990; Kubo *et al.* 2000). The column temperature was programmed from 160°C to 210°C. $N_2$ was employed as the carrier gas.

### *Statistical analysis*

After confirming the normality of data and the homogeneity of variance of data for the treatment groups (the latter being evaluated by the Bartlett test), the significance of differences between mean values was assessed by ANOVA coupled with Duncan's multiple-range test at the 1 or 5 % level of significance (Duncan, 1957).

## Results

The rats consumed 14·3–14·8 g food/d and gained 5·4–5·9 g/d over the 22 d experiment. There were no significant differences in food intake and body-weight gain between any of the treatment groups. The weights of the liver, kidney, and testis in the treatment groups changed within small ranges, and the differences were not significant (data not shown).

Serum TBA values were significantly higher in the two DHA groups fed diets with 7·5 or 54 mg vitamin E/kg than in the LA (control), α-LN, and EPA groups (Table 2), but the value in the DHA + low vitamin E group was no higher than in the DHA group. The water-soluble fluorescent substance level of the DHA + low vitamin E group was significantly higher than those of the other groups. Serum α-tocopherol concentrations were significantly lower in all of the *n*-3 PUFA groups than in the control LA group, and became lower with increasing degrees of unsaturation of dietary PUFA. Serum aspartate aminotransferase activity tended to be higher in the

**Table 3.** Conjugated diene level, chemiluminescence intensity, thiobarbituric acid reactive substance (TBA value) and microsomal lipofuscin level in the liver, kidney and testis of rats fed different types of polyunsaturated fatty acids*

(Mean values with their standard deviations for six or seven rats)

| Diet... | LA (*n* 6) | | α-LN (*n* 7) | | EPA (*n* 7) | | DHA (*n* 7) | | DHA + lowVit E† (*n* 7) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| *n*-3 Fatty acids (% energy) | – | | 8·6 | | 8·2 | | 8·0 | | 8·0 | |
| LA (% energy) | 8·9 | | 2·4 | | 2·2 | | 2·1 | | 2·1 | |
| Liver | | | | | | | | | | |
| Conjugated diene (μmol/g) | 0·97[a] | 0·09 | 0·95[a] | 0·06 | 1·01[a] | 0·13 | 1·24[b] | 0·10 | 1·31[b] | 0·16 |
| Microsomal conjugated diene (nmol/mg protein) | 13·2[a] | 2·2 | 13·9[ab] | 1·7 | 13·8[ab] | 2·1 | 15·9[b] | 1·1 | 15·0[ab] | 2·1 |
| Chemiluminescence intensity (count/30 s) | 80·1[a] | 34·1 | 89·3[a] | 41·7 | 104·2[a] | 77·6 | 110·6[ab] | 48·7 | 181·4[b] | 98·5 |
| TBA value (nmol malondialdehyde/g) | 124·8[a] | 16·5 | 149·9[b] | 13·9 | 149·2[b] | 8·2 | 193·6[c] | 23·6 | 209·2[c] | 21·7 |
| Microsomal lipofuscin‡ (ng/mg protein) | 2·8[a] | 0·4 | 2·7[a] | 0·3 | 2·6[a] | 0·3 | 2·9[a] | 0·4 | 2·6[a] | 0·4 |
| Kidney | | | | | | | | | | |
| Conjugated diene (μmol/g) | 0·67[a] | 0·11 | 0·64[a] | 0·07 | 0·69[ab] | 0·06 | 0·78[b] | 0·06 | 0·72[ab] | 0·09 |
| Chemiluminescence intensity (count/30 s) | 64·2[a] | 28·1 | 73·2[a] | 73·7 | 93·3[a] | 119·1 | 115·1[a] | 125·9 | 167·1[a] | 149·0 |
| TBA value (nmol malondialdehyde/g) | 132·3[a] | 4·0 | 125·8[a] | 9·8 | 129·5[a] | 14·4 | 148·2[b] | 4·5 | 157·5[b] | 7·3 |
| Testis | | | | | | | | | | |
| Conjugated diene (μmol/g) | 0·21[a] | 0·03 | 0·23[ab] | 0·05 | 0·22[a] | 0·03 | 0·27[b] | 0·01 | 0·24[ab] | 0·03 |
| Chemiluminescence intensity (count/30 s) | 52·0[a] | 19·5 | 47·4[a] | 26·0 | 40·6[a] | 15·5 | 35·7[a] | 9·7 | 44·0[a] | 12·0 |
| TBA value (nmol malondialdehyde/g) | 29·5[ab] | 1·6 | 27·1[a] | 2·5 | 25·0[a] | 2·5 | 24·9[a] | 1·8 | 32·9[b] | 9·5 |

LA, linoleic acid; α-LN, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Vit E, vitamin E.

[a,b,c]Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* For details of diets and procedures, see Table 1 and p. 20.

† The vitamin E content as RRR-α-tocopherol equivalent of the LA, α-LN, EPA and DHA diets was 54 mg/kg and that of the DHA + lowVit E diet was 7·5 mg/kg, respectively.

‡ Quinine sulfate at a concentration of 0·128 μmol/l of 50 mmol sulfuric acid/l solution was used as a standard for fluorescence intensity and wave-length calibration. The level of lipofuscin was expressed in terms of the quinine sulfate equivalent.

DHA + low vitamin E group than in the control LA group, but the difference was not significant (Table 2). A similar tendency was also observed for alanine aminotransferase activity.

Conjugated diene levels in liver homogenates were significantly higher in the two DHA-fed groups than in the other groups, but the value in the DHA + low vitamin E group was no higher than that in the DHA group (Table 3). A similar trend was also observed in liver microsomal conjugated diene concentration. Liver chemiluminescence intensity was significantly greater than in the control only in the DHA + low vitamin E group. Liver TBA values were significantly higher in all *n*-3 PUFA groups than in the control group, and values in the two DHA-fed groups were greater than in the α-LN and EPA groups, although there was no significant difference between the two DHA groups. Liver microsomal lipofuscin concentration did not differ among any of the treatment groups.

The conjugated diene level in the kidney homogenates was significantly higher in the DHA group than in the control LA group, but there was no significant difference among the other LA, α-LN, EPA, and DHA + low vitamin E groups (Table 3). The chemiluminescence intensities tended to be higher in the two DHA groups, but no significant difference was noticed in the values among any of the treatment groups, owing to considerable scattering of the values. The kidney TBA values in the two DHA groups were significantly greater than in the control group, but the value in the DHA + low vitamin E group was no higher than in the DHA group.

The conjugated diene level in the testis homogenates was significantly higher in the DHA group than in the control LA group (Table 3). No significant difference was recognized in chemiluminescence intensity among any of the treatment groups. The TBA value was significantly higher in the DHA + low vitamin E group than in the other treatment groups, except for the control.

Liver α-tocopherol levels showed a similar trend to serum level (Table 4). Liver ascorbic acid levels were significantly higher in all the *n*-3 PUFA groups than in the control LA group, but there was no significant difference among the *n*-3 PUFA groups. A similar trend was observed in non-protein SH concentrations, with the exception of the DHA + low vitamin E group, which showed no significant difference from the control LA group. Glutathione peroxidase activity in the two DHA groups was significantly lower than in the control, irrespective of the fact that the tissue lipid peroxide levels were higher in the two DHA groups. Glutathione reductase activity in the treatment groups changed within a small range, and the differences were insignificant. Glucose-6-phosphate dehydrogenase activity was significantly lower in all of the *n*-3 PUFA groups than in the control LA group, and became lower with increasing degrees of unsaturation of dietary PUFA.

Kidney α-tocopherol levels in the two DHA groups were significantly lower than in the control, but those of the α-LN and EPA groups did not decrease (Table 4). The ascorbic acid concentration was significantly higher or tended to be higher in all of the *n*-3 PUFA groups compared with the control. Non-protein SH levels were significantly higher than in the control only in the two DHA

**Table 4.** Lipid peroxide scavengers in the liver, kidney and testis of rats fed different types of polyunsaturated fatty acids*
(Mean values with their standard deviations for six or seven rats)

| Diet... | LA (*n* 6) | | α-LN (*n* 7) | | EPA (*n* 7) | | DHA (*n* 7) | | DHA+lowVit E† (*n* 7) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| *n*-3 Fatty acid (% energy) | – | | 8·6 | | 8·2 | | 8·0 | | 8·0 | |
| LA (% energy) | 8·9 | | 2·4 | | 2·2 | | 2·1 | | 2·1 | |
| Liver | | | | | | | | | | |
| α-tocopherol (nmol/g) | 51·4$^{a}$ | 7·0 | 40·6$^{b}$ | 8·8 | 33·0$^{c}$ | 4·9 | 23·7$^{d}$ | 4·9 | 4·9$^{e}$ | 0·7 |
| Ascorbic acid (μmol/g) | 0·93$^{a}$ | 0·13 | 1·15$^{b}$ | 0·14 | 1·17$^{b}$ | 0·20 | 1·20$^{b}$ | 0·15 | 1·19$^{b}$ | 0·11 |
| Non-protein SH (μmol/g) | 4·26$^{a}$ | 0·68 | 5·27$^{b}$ | 0·91 | 5·40$^{b}$ | 0·78 | 5·79$^{b}$ | 1·01 | 4·95$^{ab}$ | 0·72 |
| GSHPx (unit/mg protein) | 208·5$^{a}$ | 25·9 | 202·8$^{a}$ | 23·4 | 199·5$^{ab}$ | 7·0 | 177·5$^{bc}$ | 25·2 | 173·0$^{c}$ | 20·0 |
| GR (nmol/min per mg protein) | 76·0$^{ac}$ | 4·0 | 70·1$^{a}$ | 9·0 | 74·9$^{ac}$ | 6·1 | 79·4$^{bc}$ | 4·5 | 80·7$^{c}$ | 6·2 |
| G6PDH (nmol/min per mg protein) | 69·6$^{a}$ | 13·7 | 44·5$^{b}$ | 13·0 | 28·5$^{c}$ | 4·6 | 20·8$^{cd}$ | 4·6 | 18·2$^{d}$ | 4·4 |
| Kidney | | | | | | | | | | |
| α-Tocopherol (nmol/g) | 28·3$^{a}$ | 9·8 | 29·0$^{a}$ | 10·7 | 27·9$^{a}$ | 4·2 | 18·1$^{b}$ | 2·3 | 7·59$^{c}$ | 0·9 |
| Ascorbic acid (μmol/g) | 0·56$^{a}$ | 0·12 | 0·64$^{ab}$ | 0·11 | 0·73$^{b}$ | 0·10 | 0·71$^{ab}$ | 0·15 | 0·68$^{ab}$ | 0·06 |
| Non-protein SH (μmol/g) | 3·12$^{a}$ | 0·29 | 3·03$^{a}$ | 0·29 | 3·12$^{a}$ | 0·23 | 3·64$^{b}$ | 0·29 | 4·59$^{c}$ | 0·49 |
| GSHPx (unit/mg protein) | 175·4$^{a}$ | 35·0 | 176·9$^{a}$ | 17·4 | 168·9$^{a}$ | 14·1 | 168·3$^{a}$ | 19·3 | 172·6$^{a}$ | 19·5 |
| Testis | | | | | | | | | | |
| α-Tocopherol (nmol/g) | 39·2$^{a}$ | 1·2 | 39·2$^{a}$ | 4·6 | 30·0$^{b}$ | 3·7 | 24·8$^{c}$ | 2·1 | 15·8$^{d}$ | 2·6 |
| Ascorbic acid (μmol/g) | 1·40$^{ab}$ | 0·08 | 1·52$^{a}$ | 0·13 | 1·42$^{ab}$ | 0·13 | 1·44$^{ab}$ | 0·06 | 1·39$^{b}$ | 0·08 |
| Non-protein SH (μmol/g) | 3·22$^{a}$ | 0·23 | 3·32$^{a}$ | 0·23 | 3·32$^{a}$ | 0·13 | 3·29$^{a}$ | 0·23 | 3·32$^{a}$ | 0·16 |
| GSHPx (unit/mg protein) | 15·8$^{a}$ | 3·2 | 17·2$^{a}$ | 1·2 | 17·3$^{a}$ | 1·1 | 16·3$^{a}$ | 1·5 | 17·8$^{a}$ | 1·6 |

LA, linoleic acid; α-LN, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Vit E, vitamin E; SH, sulfhydryl; GSHPx, glutathione peroxidase; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase.

$^{a,b,c,d,e}$Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* For details of diets and procedure, see Table 1 and p. 20.

† The vitamin E content as RRR-α-tocopherol equivalent of the LA, α-LN, EPA and DHA diets was 54 mg/kg and that of the DHA+lowVit E diet was 7·5 mg/kg, respectively.

groups; the level in the DHA + low vitamin E group was significantly greater than that in the DHA group. No significant difference in glutathione peroxidase activity was found among any of the treatment groups.

Testis α-tocopherol levels of the EPA and DHA groups were significantly lower than that of the control, and the level in the DHA group was significantly lower than that of the EPA group (Table 4). Ascorbic acid and non-protein SH concentrations and glutathione peroxidase activity did not change significantly.

The liver peroxidizability indices were 85·3 (SD 9·6), 95·0 (SD 14·4), 139·3 (SD 5·3), and 160·6 (SD 9·3) for the control LA, α-LN, EPA and DHA groups, respectively. The control LA group peroxidizability index was significantly different ($P<0·01$) from those of the EPA and DHA groups, that of the α-LN group was also significantly different ($P<0·01$) from the EPA and DHA groups, that of the EPA group was significantly different to all other groups ($P<0·01$), and that of the DHA group was also significantly different to all other groups ($P<0·01$). As no significant influence of dietary vitamin E levels on the fatty acid composition of the liver was reported (Kaasgaard *et al.* 1992; Kubo *et al.* 2000), a pooled sample from seven rats in the DHA + low vitamin E group was analysed to calculate the peroxidizability index, which was 152·1. Because there were inadequate amounts of tissue in the kidney and testis samples, the fatty acid compositions of these organs were analysed by using pooled samples from six rats from the LA group and seven rats each from the α-LN, EPA, DHA, and DHA + low vitamin E groups. The kidney peroxidizability indices were 98·8, 110·1, 129·9, 132·9 and 128·6 for the LA, α-LN, EPA, DHA, and DHA + low vitamin E groups, respectively. Those of the testis were 113·2, 111·2, 119·2, 118·7 and 122·9 for the same groups, respectively.

Fig. 1 shows the relationship between peroxidizability indices and lipid peroxide levels in the liver, kidney, and testis, expressed relative to the control values. In the liver, the relative peroxidizability indices of total lipids increased as the degrees of unsaturation of dietary PUFA increased (Fig. 1 (A)). The relative chemiluminescence intensity for the DHA + low vitamin E group was 2·3 times the control value, and higher than the relative peroxidizability index. The relative TBA value for the same group coincided with the relative peroxidizability index. On the other hand, the relative values of lipid peroxides, as assessed by levels of conjugated diene, chemiluminescence emission, and TBA-reactive substances in the EPA and DHA groups, were much lower than the respective relative peroxidizability index. However, the relative values of lipid peroxides in the α-LN group were closely associated with the peroxidizability index.

In the kidney, the relative peroxidizability indices of the total lipids increased as the degrees of unsaturation of dietary PUFA increased, but to a lesser extent than in the liver (Fig. 1 (B)). The relative values of lipid peroxides in the α-LN group seemed to be associated with the relative peroxidizability index. The relative values of lipid peroxides, except for the chemiluminescence intensities, in the EPA and DHA groups appeared to be lower than the respective

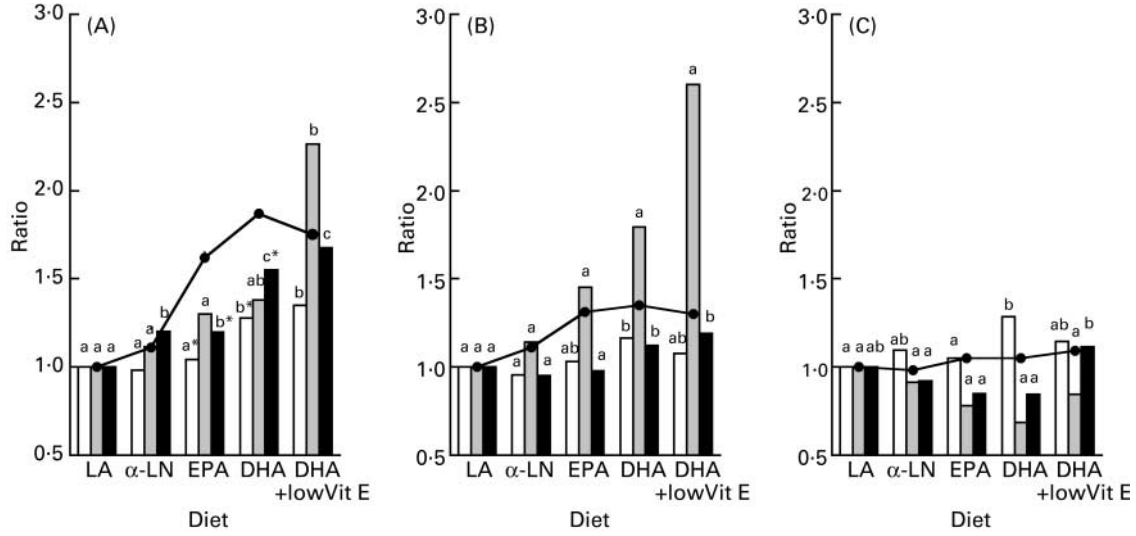


**Fig. 1.** Lipid peroxide levels (□, ■, ■) and peroxidizability indices of the total lipids (−○−) expressed relative (as a ratio) to the control values (linoleic acid (LA) group) in the liver (A), kidney (B) and testis (C) of rats fed different *n*-3 polyunsaturated fatty acids. The peroxidizability index was used in the present study as an indicator to determine the vulnerability of tissue lipids to oxidation. The peroxidizability index of lipids was calculated according to the following equation: peroxidizability index = (dienoic % × 1) + (trienoic % × 2) + (tetraenoic % × 3) + (pentaenoic % × 4) + (hexaenoic % × 5). (□), Conjugated diene levels; (■), chemiluminescence intensities; (■), thiobarbituric acid values. Mean values are shown for six rats fed the LA diet and for seven rats fed the other *n*-3 polyunsaturated fatty acids diets. [a,b,c]Mean values for the same analytical item in each tissue with unlike letters were significantly different by ANOVA coupled with the Duncan's multiple-range test ($P<0·05$). *Significant difference by Student's *t* test between the relative increase of peroxidizability index and that of each lipid peroxide in the liver except for the docosahexaenoic acid (DHA) + low vitamin E (Vit E) group ($P<0·01$). The fatty acid analysis to calculate the peroxidizability index of the DHA + lowVit E group was carried out using a pooled sample from seven rats. The fatty acid analyses for the kidney and testis were also conducted using pooled samples from six rats of the LA group and from seven rats of the α-linolenic acid (α-LN), eicosapentaenoic acid (EPA), DHA and DHA + lowVit E groups.

relative peroxidizability indices. The relative TBA value of the DHA + low vitamin E group in the kidney seemed to be correlated well with the relative peroxidizability index. The chemiluminescence intensities in the two DHA groups were higher than their relative peroxidizability indices, although the relative intensities were not significantly different among any of the treatment groups.

In the testis, the relative peroxidizability indices of the total lipids in the *n*-3 PUFA groups were 1·0 to 1·1 times the control value (Fig. 1 (C)). The relative conjugated diene levels in the treatment groups, except for the DHA group, seemed to be associated with each relative peroxidizability index. The TBA value for the DHA + low vitamin E group and the chemiluminescence intensity and TBA value for the α-LN group also appeared to coincide with their relative peroxidizability indices. The relative chemiluminescence intensities and TBA values for the EPA and DHA groups were 0·7 to 0·85 times the control values, and the levels seemed to be lower than their relative peroxidizability indices.

## Discussion

The potentially harmful influences of fish oils and fish-oil products rich in EPA and DHA have been overlooked because of the health benefits conferred by *n*-3 PUFA. Therefore, we considered that a study to clarify the mechanisms suppressing dietary *n*-3 PUFA-induced lipid peroxidation below the relative peroxidizability index was important for reasons of safety, and also to define a means of efficiently enhancing the physiological efficacy of *n*-3 PUFA.

We chose in the present study three typical tissues and dietary *n*-3 PUFA; that is, liver, kidney, and testis, and α-LN, EPA, and DHA, for the reasons described earlier (p. 000). The peroxidizability indices ratio of the dietary lipids was approximately 1 : 2 : 4 : 5 for the control LA, α-LN, EPA and DHA diets, respectively (Table 1).

Our results show that serum lipid peroxidation, as assessed by the level of TBA-reactive substances, and the antioxidant α-tocopherol were correlated inversely with increasing degrees of unsaturation of dietary PUFA (Table 2). Additionally, under a very low supply of vitamin E (DHA + low vitamin E group), the water-soluble fluorescent substance level and aspartate aminotransferase and alanine aminotransferase activities were higher than those of the control, indicating the potential for tissue parenchymal cell injuries in rats given as little as 7·5 mg vitamin E/kg diet. In this vitamin E-deficient nutritional state, tissue parenchymal cell injuries are thought to be promoted through enhanced lipid peroxidation, and thus the endproducts of lipid peroxidation, i.e. water-soluble fluorescent substances, appear in the blood. Farwer *et al.* (1994) reported that when fish oil was administered to rats, yellow fat was recognized in the storage fat in which lipofuscin pigments were accumulated, especially with an insufficient supply of dietary vitamin E. Hence, we thought that lipid peroxidation was not stimulated to induce tissue parenchymal cell injuries under adequate vitamin E nutritional status, even after large amounts of highly unsaturated DHA were given to the rats.

In the liver, lipid peroxidation, as assessed by the conjugated diene, chemiluminescence emission and TBA-reactive substances, together with the relative peroxidizability indices, became higher (Table 3, Fig. 1 (A)), and α-tocopherol levels inversely lower (Table 4) with increasing degrees of unsaturation of dietary PUFA. When a deficient level of vitamin E was supplied (DHA + low vitamin E group), levels of relative TBA-reactive substances nearly coincided with the relative peroxidizability index. However, the relative chemiluminescence intensity for the DHA + low vitamin E group was higher than the relative peroxidizability index. In chemiluminescence analysis, light is emitted mainly from singlet molecular oxygen and/or excited carbonyl compounds yielded by the breakdown of lipid peroxyradicals (Boveris *et al.* 1981; Miyazawa *et al.* 1981). With low dietary vitamin E, which approximates to vitamin E deficiency, the chain reaction of lipid peroxidation to form peroxyradicals of fatty acids such as highly unsaturated DHA may not be suppressed easily. In the lipid peroxides analysed, TBA-reactive substances which were formed in a relatively late stage of lipid peroxidation compared with the early-stage products such as conjugated diene and chemiluminescence emission, reflected most of the respective dietary *n*-3 PUFA-induced lipid peroxidation. Similar results were also observed *in vitro* by Visioli *et al.* (1998). We noticed a close association between the relative peroxidizability index and lipid peroxidation in the α-LN group (Fig. 1 (A)). However, the extent of lipid peroxidation in the EPA and DHA groups was far less than expected from their relative peroxidizability indices. Therefore, this phenomenon is not unique to DHA, which has six double bonds, but also occurs with EPA, which has five double bonds, in the liver under adequate vitamin E nutritional status.

In the kidney, the changes and relationships between the relative peroxidizability indices for the total lipids and the extent of lipid peroxidation were similar to those observed in the liver, i.e. both the indices and the lipid peroxidation increased as the degrees of unsaturation of dietary PUFA increased, but to a lesser extent than in the liver, even in the EPA and DHA groups (Fig. 1 (B)).

In the testis, almost no increase was observed in the relative peroxidizability indices for total lipids, even after a high intake of DHA or EPA (Fig. 1 (C)). Therefore, the extent of lipid peroxidation also did not increase, on the whole, in the testis, irrespective of the degree of unsaturation of dietary PUFA and the vitamin E level (Table 3, Fig. 1 (C)). Accordingly, the susceptibility to lipid peroxidation is lower in the testis than in the liver and kidney, as was observed previously (Kubo *et al.* 1998, 2000).

Our results show a characteristic pattern of changes, in both the extent of lipid peroxidation and the relative peroxidizability indices in each tissue. Accordingly, to clarify the mechanisms of the suppression of lipid peroxidation, variations in the lipid peroxide scavengers were further investigated.

The α-tocopherol concentration in the liver decreased remarkably with increasing degrees of unsaturation of dietary PUFA, but the levels of both ascorbic acid and GSH increased significantly in all of the *n*-3 PUFA groups,

probably to compensate for the decrease in α-tocopherol (Table 4). It has been suggested that three antioxidants, vitamin E, ascorbic acid, and GSH, are interrelated in the maintenance of tissue vitamin E levels. Reductive recycling by ascorbic acid of α-tocopheroxyl radicals is known to occur (Tappel, 1962; Meister, 1992; Wells *et al.* 1995), and the enzymic and non-enzymic reductive regeneration of ascorbic acid from oxidized ascorbic acid by GSH is also known (Winkler *et al.* 1994). Thus the antioxidant efficacy of α-tocopherol is potentiated by the action of ascorbic acid and GSH. The activity of glutathione reductase in reproducing ascorbic acid from oxidized ascorbic acid via the GSH-dependent system in the liver did not change significantly, and that of glucose-6-phosphate dehydrogenase in supplying NADPH for the GSH-dependent system decreased with increasing degrees of unsaturation of dietary PUFA (Table 4). Hence, the biosynthesis of ascorbic acid and GSH, not the reproduction of reduced forms of ascorbic acid and GSH from their oxidized forms, may be promoted as the lipid peroxidation-induced increase in the requirement for vitamin E is enhanced in the liver. This probable potentiation of the antioxidant system may explain, at least in part, the observation that the extent of lipid peroxidation in the EPA and DHA groups fed diets with adequate level of vitamin E was far less than expected from the relative peroxidizability indices of the total liver lipids.

In the kidney, the levels of lipid peroxide scavengers were lower, on the whole, than those of the liver and testis (Table 4). However, the increase in the relative peroxidizability index of total lipids was also moderate or low, even after highly unsaturated DHA was given to the animals (Fig. 1 (B)). Accordingly, in the kidney the extent of lipid peroxidation may be closer to the relative peroxidizability indices, even in the EPA and DHA groups, than in the liver.

The testis was insensitive to lipid peroxidation under adequate vitamin E nutritional status, even after a high intake of highly unsaturated DHA or EPA (Fig. 1 (C)). Presumably this phenomenon was ascribable primarily to the negligible increase in the relative peroxidizability index and to the higher levels of α-tocopherol than those of other tissues, even in the low vitamin E (7·5 mg/kg) group (Table 4). Moreover, the ascorbic acid level of the testis was retained at a higher level (about 2·5 times that of the kidney) (Table 4), suggesting effective α-tocopherol recycling mediated by ascorbic acid and the GSH-dependent system. Therefore, the testis probably contains enough lipid peroxide scavengers to suppress lipid peroxidation, even after a high DHA or EPA intake.

Because DHA and EPA are the major PUFA in fish oils, the phenomena seen here with DHA and EPA intakes may also be applicable to fish oil and fish-oil supplement intakes.

In conclusion, our results suggest that antioxidative suppression of lipid peroxidation below the relative peroxidizability index is not unique to DHA, which has six double bonds, but also occurs with EPA, which has five double bonds, under adequate vitamin E nutritional status. However, it does not occur with α-LN, which has three double bonds.

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