DHA concentration of red blood cells is inversely associated with markers of lipid peroxidation in men taking DHA supplement

Mototada Shichiri,1,* Yuriko Adkins,2† Noriko Ishida,1 Aya Umeno,1 Yasushi Shigeri,1 Yasukazu Yoshida,1,* Dawn M. Fedor,2 Bruce E. Mackey,3 and Darshan S. Kelley2†

1Health Research Institute (HRI), National Institute of Advanced Industrial Science and Technology (AIST), 2217-14 Hayashi-cho, Takamatsu, Kagawa 761-0395, Japan
2Western Human Nutrition Research Center, ARS, USDA and Department of Nutrition, University of California Davis, CA 95616, USA
3Western Regional Research Center, ARS, USDA, Albany, CA 94710, USA

(Received 12 February, 2014; Accepted 22 June, 2014; Published online 9 September, 2014)

An increase in the proportion of fatty acids with higher numbers of double bonds is believed to increase lipid peroxidation, which augments the risk for many chronic diseases. (n-3) Polyunsaturated fatty acids provide various health benefits, but there is a concern that they might increase lipid peroxidation. We examined the effects of docosahexaenoic acid [22:6 (n-3)] supplementation on lipid peroxidation markers in plasma and red blood cells (RBC) and their associations with red blood cell and plasma fatty acids. Hypertriglyceridemic men (n = 17 per group) aged 39–66 years participated in a double-blind, randomized, placebo-controlled, parallel study. They received no supplements for the first 8 days and then received 7.5 g/day docosahexaenoic acid oil (3 g/day docosahexaenoic acid) or olive oil (placebo) for 90 days. Fasting blood samples were collected 0, 45, and 91 days after supplementation. Docosahexaenoic acid supplementation did not change plasma or RBC concentrations of lipid peroxidation markers (total hydroxyoctadecadienoic acid, total hydroxyeicosatetraenoic acid, total 8-isoprostaglandin F_2α, 7α-hydroxycholesterol, 7β-hydroxycholesterol) when pre- and post-supplement values were compared. However, the post-supplement docosahexaenoic acid (DHA) concentration was inversely associated with RBC concentrations of ZE-HODE, EE-HODE, t-HODE, and total 8-isoprostaglandin F_2α (p < 0.05). RBC concentration of hydroxycholesterol was also associated with DHA but it did not attain significance (p = 0.07). Our results suggest that increased concentration of DHA in RBC lipids reduced lipid peroxidation. This may be another health benefit of DHA in addition to its many other health promoting effects.

Key Words: antioxidant, docosahexaenoic acid, hypertriglyceridemia, lipid peroxidation, polyunsaturated fatty acid

Increased lipid peroxidation has been associated with the development and progression of a number of chronic human diseases, including cardiovascular disease and diabetes. It damages biological membranes, leading to changes in membrane fluidity and functions, including receptor activity and nutrient and ion transport. Peroxidation of low-density lipoprotein (LDL) renders it proatherogenic. Many lipid peroxidation products exert cytotoxic effects and alter cell signaling. Thus, control of lipid peroxidation plays a critical role in health maintenance and disease prevention.

Lipid peroxidation in biological systems is believed to increase with an increase in the proportion of fatty acids with higher numbers of double bonds in the fatty acid chain [polyunsaturated fatty acids (PUFAs)]. Long-chain PUFAs, particularly of the (n-3) type, provide a number of health benefits. People consuming diets rich in (n-3) PUFAs have decreased inflammation, platelet aggregation, cardiac arrhythmias, triglyceride levels, and number of total LDL and small dense LDL particles, as well as an increased omega-3 index [sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as % of total fatty acid content of red blood cells (RBCs)], endothelial relaxation, and atherosclerotic plaque stability. Thus, (n-3) PUFAs reduce the risk for a number of chronic diseases, but there is a concern that they may increase the risk for chronic diseases by increasing lipid peroxidation.

Results from human studies with (n-3) PUFAs regarding their effects on lipid peroxidation have been inconsistent; that is, no change, increase, or decrease in lipid peroxidation was observed. In another study, the effect of DHA ranged from protection to increased lipid peroxidation depending on its dose. Similarly, results from several in vitro studies have been inconsistent; (n-3) PUFAs decreased lipid peroxidation in some studies but increased it in other studies. Most human studies with (n-3) PUFAs have either examined Cu²⁺-catalyzed lipid peroxidation ex vivo or used surrogate markers of in vivo lipid peroxidation [e.g., F-2 isoprostanes, plasma total antioxidant activity, malondialdehyde (MDA), and thiobarbituric acid-reactive substances (TBARS)]. 8-Isoprostaglandin F_2α (8-isopGF_2α), one of the isoforms of F-2 isoprostanes, is formed from arachidonic acid [AA, 20:4 (n-6)]; it has been used as the gold standard for the assessment of in vivo oxidative injury. However, F-2 isoprostanes are only one of the several minor non-enzymatic products formed from AA. Similarly, there are limitations to the use of TBARS and MDA as markers for oxidative damage.

We have recently developed a method for in vivo assessment of lipid peroxidation in which total hydroxyoctadecadienoic acid (t-HODE) is measured by liquid chromatography–mass spectrometry (LC-MS/MS) after reduction and saponification in plasma and RBC lipids. Within the same analysis, we can determine the concentration of total 8-isopGF_2α by LC-MS/MS and that of total 7-hydroxycholesterol (7-OHCh) by gas chromatography–mass spectrometry (GC-MS). t-HODE and 7-OHCh assessed by our method accounted for much of the oxidized linoelates and cholesterol, respectively. Since the tissue concentra-

To whom correspondence should be addressed.
E-mail: yoshida-ya@aist.go.jp (Yoshida Y), darshan.kelley@ars.usda.gov (Kelly DS)
*These authors contributed equally to this work.
tions of t-HODE are several-fold greater than those of F-2 isoprostanes, the t-HODE concentration is a more reliable indicator of lipid peroxidation than the F-2 isoprostane concentration. We have previously reported pathways for the enzymatic and non-enzymatic oxidation of octadecadienoic acid.17

To the best of our knowledge, no human studies have examined the effects of dietary (n-3) PUFAs on actual plasma or RBC concentrations of HODE and OHCh. Therefore, in this study, we examined the effect of DHA supplementation on t-HODE and 7-OHCh in RBC and plasma lipids in hypertriglyceridemic men. In addition, we measured the concentrations of individual isomers of HODE [13-hydroxy-9,11,14-octadecadienoic acid [13(Z,E)-HODE], 9-hydroxy-10E,12Z-octadecadienoic acid [9-(Z,E)-HODE], 13-hydroxy-9,11,14-octadecadienoic acid [13(E,E)-HODE], 9-hydroxy-10E,12Z-octadecadienoic acid [9(E,E)-HODE]], and OHCh (7α-OHCh and 7β-OHCh). Measurement of the individual isomers can provide information regarding the enzymatic versus non-enzymatic pathways: 9- and 13-(Z,E)-HODE are formed by both enzymatic and non-enzymatic oxidations, whereas 9- and 13-(E,E)-HODE are formed by a non-enzymatic free radical oxidation only. We also measured the concentrations of total hydroxyeicosatetraenoic acid (t-HETE) in both the plasma and RBC lipids. To assess the pro- and antioxidative effects of individual fatty acids, we determined the associations between the concentrations of individual fatty acids in the RBC and plasma lipids with the lipid peroxidation products within each sample type.

Materials and Methods

Materials. 8-IsopGF_2α, 8-isoprostaglandin F_2α-d_3 (8-isopGF_2α-d_3), 5-hydroxyeicos-α-e,8,12,14-octadecatrienoic acid (5-HETE), 12-hydroxyeicos-α-e,8,10,14Z-octadecatrienoic acid (12-HETE), 15-hydroxyeicos-α-e,8,11Z,14E-octadecatrienoic acid (15-HETE), 13-(Z,E)-HODE, 9-(Z,E)-HODE, and 13S-hydroxy-10E,12Z-octadecadienoic acid [13S-(10-Z,E)-HODE], 12-hydroxy-9Z,13E-octadecadienoic acid [12(Z,E)-HODE] were obtained from Cayman Chemical Company (Ann Arbor, MI). 9-(E,E)-HODE, 13(E,E)-HODE, 10-hydroxy-8E,12Z-octadecadienoic acid (10-(Z,E)-HODE), and 12-hydroxy-9Z,13E-octadecadienoic acid (12(Z,E)-HODE) were obtained from Larodan Fine Chemicals AB (Malmo, Sweden). 7α-OHCh and 7β-OHCh were obtained from Steraloids Inc. (Newport, RI), and their isotopes 7α-hydroxycholesterol-25,26,27,28,29,30,33-d_7 (7α-OHCh-d_7) and 7β-hydroxycholesterol-25,26,27,28,29,30,33-d_7 (7β-OHCh-d_7) were obtained from Medical Isotopes Inc. (Pelham, NH). Other materials were of the highest grade available commercially.

Study participants. Details regarding the study design and participants have been published previously.48-50 Moderately hyperlipidemic but otherwise healthy men (39–66 years old) participated in this study. Participants regularly taking anti-inflammatory medications (including steroids), antihypertensive medications, or smoking were excluded. Usual dietary intakes were estimated by 3 multi-pass interviews during each of the metabolic periods. Usual dietary intakes were estimated by 3

Provide uniformity in the composition of diets between the subjects and blood draw days, the metabolic kitchen provided all 3 meals the day before each blood draw. The energy intake was adjusted for the body height, body weight, age, and estimated physical activity of the subjects using the Mifflin–St. Jeor equation and appropriate activity factors. The reported consump- tion of the pretest diet that was weighed and served on the day before each blood draw did not differ in its composition or the total energy intake between the two groups (not shown). For this day, mean energy intake for the two groups was 10,450 ± 240 kJ, and mean intakes for fat, carbohydrates, and proteins were 82, 340, and 100 g/day, respectively. Saturated fatty acids, mono- and polyunsaturated fatty acids, and PUFAs provided 11.1%, 10.1%, and 8.8% of the total energy intake.

Sample preparation for lipid peroxidation products. Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) after overnight fasting on days 0, 45, and 91 after initiating supplements. The samples were placed on ice immediately after the collection. Plasma was obtained by centrifugation at 1,580 × g for 10 min at 4°C, and stored at −80°C until analyzed. The erythrocytes were washed twice with a 4-fold volume of saline and stored at −80°C. Before analysis of the lipid peroxidation products, erythrocyte samples were extracted by vortexing with a 4-fold volume of methanol containing 100 μmol/L 2,6-di-tert-butyl-4-methyl-phenol (BHT) and internal standards 8-iso-PGF_2α-d_3 (100 μg/L), 13-HODE-d_3 (100 μL/L), 7α-OHCh-d_3 (36 μL/L), 7β-OHCh-d_3 (38 μL/L), and 16-hydroxyhexadecanoic acid (140 μL/L), followed by centrifugation (20,400 × g at 4°C for 10 min). The analysis was then performed immediately.

The extracted methanol solution (500 μL) of erythrocytes and plasma (200 μL) was mixed with 300 μL saline. Subsequently, 500 μL methanol containing internal standards and BHT were added to the plasma samples. This was followed by the reduction of hydroperoxides by using an excess of triphenylphosphine (final concentration, 1 mmol/L) at room temperature for 30 min. Next,
Table 1. Effect of docosahexaenoic acid supplementation to hypertriglyceridemic men on the concentrations of markers of lipid peroxidation in RBC lipids

<table>
<thead>
<tr>
<th>Oxy-lipid (pmol/mg protein)</th>
<th>DHA group (n = 17)</th>
<th>Placebo group (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 91</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>ZE-HODE</td>
<td>3.00</td>
<td>1.04</td>
</tr>
<tr>
<td>EE-HODE</td>
<td>1.33</td>
<td>0.57</td>
</tr>
<tr>
<td>t-HODE</td>
<td>4.33</td>
<td>1.57</td>
</tr>
<tr>
<td>t-HETE</td>
<td>8.64</td>
<td>2.36</td>
</tr>
<tr>
<td>t8-iso-PGF2α</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>7α-OHCh</td>
<td>19.65</td>
<td>3.54</td>
</tr>
<tr>
<td>7β-OHCh</td>
<td>34.73</td>
<td>7.67</td>
</tr>
<tr>
<td>t7-OHCh</td>
<td>55.06</td>
<td>10.35</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (n = 17). None of the response variables tested was significantly different between days 0 and 91 within both the DHA and placebo groups (ANOVA-repeated measures, mixed model with single degree of freedom contrasts between days).

the reduced samples were mixed with 1 mol/L KOH in methanol (500 μL) under nitrogen and incubated on a shaker for 30 min in the dark at 40°C. The mixtures were cooled on ice and acidified with 10% acetic acid in water (2 ml) and then extracted with chloroform and ethyl acetate (chloroform/ethyl acetate = 4/1, v/v, 5 ml). The samples were mixed with an vortex mixer for 1 min and centrifuged at 1,500 × g for 5 min at 4°C. The chloroform and ethyl acetate layer was concentrated to 1 ml after the removal of the water layer and divided equally into two portions.  

Analysis of t8-iso-PGF2α, t-HETE, t-HODE, and t7-OHCh.  
The analysis of t8-iso-PGF2α, t-HETE, t-HODE, and t7-OHCh by LC-MS/MS was performed according to the methods previously reported. Briefly, after an extraction with chloroform and ethyl acetate, t8-iso-PGF2α, t-HETE, and t-HODE in plasma and RBCs were analyzed by LC-MS/MS. LC was carried out on an ODS column in a column oven set at 30°C. MS was carried out using a Thermo Finnigan TSQ Quantum Discovery Max system, a triple-quadrupole mass spectrometer (Thermo Fisher Scientific, CA) fitted with an electrospray ionization source. Electrospray ionization was carried out at a needle voltage of 4.2 kV. Nitrogen was used as the sheath gas (17 psi) and auxiliary gas (12 units). A specific precursor-to-product-ion transition was carried out by selected reaction monitoring after collision-induced dissociation in the negative mode. The precursor, product ions, and collision energy were determined after the optimization of MS/MS as follows: m/z = 353.5 and 192.6–193.6 at 29 eV for 8-iso-PGF2α; m/z = 357.0 and 196.5–197.5 at 29 eV for 8-iso-PGF2α-d4; m/z = 319.0 and 114.5–115.5 at 10 eV for 5-HETE; m/z = 319.3 and 162.8–163.8 at 13 eV for 12-HETE; m/z = 319.3 and 202.5–203.5 at 10 eV for 15-HETE; m/z = 295.0 and 194.6–195.6 at 21 eV for both 13-(Z,E)-HODE and 13-(E,E)-HODE; m/z = 295.0 and 170.5–171.5 at 10 eV for both 9-(E,Z)-HODE and 9-(E,E)-HODE; m/z = 295.0 and 182.6–183.6 at 22 eV for both 10-(Z,E)-HODE and 12-(Z,Z)-HODE; and m/z = 299.0 and 197.6–198.6 at 26 eV for 13-HODE-d3.

T7-OHCh in plasma and RBCs was analyzed by GC-MS. The other portion of the chloroform and ethyl acetate solution was subjected to this analysis. An aliquot of the silylated sample was injected into a gas chromatograph (GC 6890 N, Agilent Technologies, Palo Alto, CA) that was equipped with a quadrupole mass spectrometer (5973 Network, Agilent Technologies). A fused-silica capillary column (HP-5MS, 5% phenyl methyl silicone, 30 m × 0.25 mm, Agilent Technologies) was used.

Plasma and RBC fatty acid analysis. Plasma and RBC lipids were extracted from the 12 h fasted blood samples drawn on study days 0 and 91, and their fatty acid concentrations determined according to the methods previously published. Details regarding the methods pertaining to the plasma and RBC fatty acid analyses have already been published in the literature.

Statistical analysis. SAS ver. 9.2 was used for statistical analysis. The SAS proc mixed procedure was used to fit a repeated measures mixed model with a first-order autoregressive covariance structure among the repeated measures. Diet, time, and the interaction were the fixed effects, and subjects within diets was the random effect. Single degree of freedom contrasts were used to compare the baseline with the middle and end intervention means within diets using one-tailed tests; p values were adjusted using the Bonferroni method. Results shown are the mean ± SEM values. p <0.05 (p<0.016 after Bonferroni correction) was considered significant. Associations between RBC and plasma markers of lipid peroxidation with their respective fatty acid concentrations were determined by using the pooled data from both study groups (Table 3 and 4) to calculate the Kendall’s correlation coefficients (Tau). Data from the two groups were also analyzed individually and those results are shown in the supplemental tables, except day 91 for DHA group which are shown in Table 5, p<0.05 is considered significant.

Results

Data regarding participant characteristics, diets, and effects of DHA on plasma lipids and on plasma and RBC fatty acid compositions have been previously published. Effect of DHA on concentrations of RBC and plasma lipid peroxidation products. DHA supplementation for 45 days (data not shown) or 91 days did not alter the concentrations of ZE-HODE (13(Z,E)-HODE plus 9-(E,Z)-HODE), EE-HODE (13(E,E)-HODE plus 9-(E,E)-HODE), t-HODE, t-HETE, total 8-iso-PGF2α (t8-iso-PGF2α), 7α-OHCh, 7β-OHCh, or t7-OHCh in RBC lipids (Table 1). Concentrations of all these markers of lipid peroxidation did not change during the study in the RBC lipids from the placebo group either (Table 1).

Similar to the results for the RBC lipids, DHA supplementation for 45 days (data not shown) or 91 days did not alter the concentrations of the above listed markers of lipid peroxidation in plasma lipids. However, the concentrations of plasma ZE-HODE, EE-HODE, t-HODE, and t-HETE significantly increased after 91 days supplementation with olive oil; concentrations of these four markers in the placebo group were elevated even at day 45, but were not significantly different from day 0 or day 91 (data not shown). Concentrations of total t8-iso-PGF2α, 7α-OHCh, 7β-OHCh, and t7-OHCh did not change during the course of the study in the plasma lipids from the placebo group.

Associations between markers of lipid peroxidation and RBC and plasma fatty acids. Among the saturated fatty acids in RBC and plasma lipids, the proportion of 14:0 was positively associated (p<0.05) with the concentrations of four markers of lipid peroxidation (ZE-HODE, EE-HODE, t-HODE, and t-HETE).
significant association with products of lipid peroxidation, (Table 3 and 4). In contrast to the positive associations of C14-16 t-HODE, and t-HODE in RBC and plasma lipids. 

(Table 3 and 4). Associations between products of lipid peroxidation and the weight % ratio of 15:0 in RBC lipids were similar to those of 14:0, but 15:0 also had a significant positive association with the concentration of 7β-OHCh. The ratio of 16:0 showed significant association with products of lipid peroxidation, ZE-HODE, EE-HODE, and t-HODE in RBC and plasma lipids (Table 3 and 4). In contrast to the positive associations of C14-16 fatty acids with markers of lipid peroxidation, 20:0 was negatively associated with the concentrations of ZE-HODE, EE-HODE, and t-HODE in both RBC and plasma lipids.

The relative amounts of 22:5 (n-3) in RBC lipids were negatively associated with the concentrations of EE-HODE, 7α-OHCh, 7β-OHCh, and t7-OHCh (Table 3). Relative proportions of 18:0, 20:0, 20:2 (n-6), 20:4 (n-6), and 18:3 (n-3) were not related to concentrations of all markers of lipid peroxidation tested both in the RBC and plasma lipids (Table 3 and 4).

Data are mean ± SEM (n = 17). None of the response variables tested was significantly different between days 0 and 91 within the DHA group, but concentrations of ZE-HODE, EE-HODE, t-HODE, and t-HETE increased significantly (p<0.05) at day 91 compared to the corresponding values at day 0 in the placebo group (ANOVA-repeated measures, mixed model with single degree of freedom contrasts between days). Superscript a is greater than superscript b.

### Table 3. Kendall’s Correlation Coefficients (τa) between RBC fatty acids and markers of lipid peroxidation in hypertriglyceridemic men taking DHA or olive oil supplements

<table>
<thead>
<tr>
<th>Oxy-lipid (nmol/L)</th>
<th>14:0</th>
<th>15:0</th>
<th>16:0</th>
<th>18:0</th>
<th>20:0</th>
<th>18:1 (n-9)</th>
<th>18:2 (n-6)</th>
<th>20:2 (n-6)</th>
<th>20:4 (n-6)</th>
<th>18:3 (n-3)</th>
<th>20:5 (n-3)</th>
<th>22:5 (n-3)</th>
<th>22:6 (n-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZE-HODE</td>
<td>0.41*</td>
<td>0.40*</td>
<td>0.25*</td>
<td>-0.21</td>
<td>-0.16</td>
<td>-0.18</td>
<td>0.13</td>
<td>-0.15</td>
<td>-0.13</td>
<td>0.00</td>
<td>-0.04</td>
<td>-0.12</td>
<td>-0.03</td>
</tr>
<tr>
<td>EE-HODE</td>
<td>0.39*</td>
<td>0.41*</td>
<td>0.24*</td>
<td>-0.21</td>
<td>-0.16</td>
<td>-0.18</td>
<td>0.14</td>
<td>-0.17</td>
<td>-0.03</td>
<td>-0.05</td>
<td>-0.20</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>t-HODE</td>
<td>0.42*</td>
<td>0.41*</td>
<td>0.25*</td>
<td>-0.20</td>
<td>-0.15</td>
<td>-0.18</td>
<td>0.15</td>
<td>-0.16</td>
<td>0.00</td>
<td>0.00</td>
<td>-0.04</td>
<td>-0.13</td>
<td>-0.02</td>
</tr>
<tr>
<td>t-HETE</td>
<td>0.24*</td>
<td>0.21*</td>
<td>0.17</td>
<td>-0.10</td>
<td>-0.08</td>
<td>-0.11</td>
<td>0.19</td>
<td>-0.19</td>
<td>-0.07</td>
<td>0.00</td>
<td>-0.13</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>t8-iso-PGF2α</td>
<td>0.31*</td>
<td>0.27*</td>
<td>0.18</td>
<td>-0.07</td>
<td>-0.12</td>
<td>-0.09</td>
<td>0.08</td>
<td>-0.10</td>
<td>0.08</td>
<td>-0.03</td>
<td>-0.12</td>
<td>-0.12</td>
<td>-0.06</td>
</tr>
<tr>
<td>7α-OHCh</td>
<td>-0.18</td>
<td>0.04</td>
<td>0.05</td>
<td>0.06</td>
<td>0.00</td>
<td>-0.01</td>
<td>-0.03</td>
<td>0.00</td>
<td>-0.05</td>
<td>-0.07</td>
<td>-0.03</td>
<td>-0.24*</td>
<td>0.05</td>
</tr>
<tr>
<td>7β-OHCh</td>
<td>0.10</td>
<td>0.21*</td>
<td>0.18</td>
<td>-0.07</td>
<td>-0.01</td>
<td>-0.21</td>
<td>0.03</td>
<td>0.00</td>
<td>-0.05</td>
<td>-0.07</td>
<td>-0.01</td>
<td>-0.29*</td>
<td>0.10</td>
</tr>
<tr>
<td>t7-OHCh</td>
<td>-0.02</td>
<td>0.17</td>
<td>0.12</td>
<td>-0.07</td>
<td>0.02</td>
<td>-0.12</td>
<td>0.03</td>
<td>-0.06</td>
<td>-0.04</td>
<td>-0.06</td>
<td>-0.02</td>
<td>-0.30*</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Correlation coefficients were calculated between concentrations of lipid peroxidation markers and weight % proportion of RBC fatty acids using data from both groups for study days 0 and 91 (n = 43 with 2 observations/participant). Tau values bearing * represent significant associations with p<0.05.

### Table 4. Kendall’s Correlation Coefficients (τa) between plasma fatty acids and markers of lipid peroxidation in hypertriglyceridemic men taking DHA or olive oil supplements

<table>
<thead>
<tr>
<th>Oxy-lipid (nmol/L)</th>
<th>14:0</th>
<th>15:0</th>
<th>16:0</th>
<th>18:0</th>
<th>20:0</th>
<th>18:1 (n-9)</th>
<th>18:2 (n-6)</th>
<th>20:2 (n-6)</th>
<th>20:4 (n-6)</th>
<th>18:3 (n-3)</th>
<th>20:5 (n-3)</th>
<th>22:5 (n-3)</th>
<th>22:6 (n-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZE-HODE</td>
<td>0.23*</td>
<td>0.00</td>
<td>0.35*</td>
<td>0.02</td>
<td>-0.02</td>
<td>-0.36*</td>
<td>0.11</td>
<td>-0.11</td>
<td>-0.20</td>
<td>0.15</td>
<td>0.10</td>
<td>-0.22*</td>
<td>0.24*</td>
</tr>
<tr>
<td>EE-HODE</td>
<td>0.23*</td>
<td>0.00</td>
<td>0.37*</td>
<td>0.00</td>
<td>-0.03</td>
<td>-0.38*</td>
<td>0.08</td>
<td>-0.17</td>
<td>-0.14</td>
<td>0.16</td>
<td>0.10</td>
<td>-0.13</td>
<td>0.19</td>
</tr>
<tr>
<td>t-HODE</td>
<td>0.23*</td>
<td>0.00</td>
<td>0.37*</td>
<td>0.02</td>
<td>-0.03</td>
<td>-0.36*</td>
<td>0.08</td>
<td>-0.13</td>
<td>-0.19</td>
<td>0.14</td>
<td>0.08</td>
<td>-0.19</td>
<td>0.22*</td>
</tr>
<tr>
<td>t-HETE</td>
<td>0.24*</td>
<td>0.16</td>
<td>0.17</td>
<td>0.13</td>
<td>0.05</td>
<td>-0.27*</td>
<td>-0.01</td>
<td>-0.06</td>
<td>-0.02</td>
<td>0.18</td>
<td>0.02</td>
<td>-0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>t8-iso-PGF2α</td>
<td>0.02</td>
<td>0.14</td>
<td>0.07</td>
<td>-0.03</td>
<td>0.00</td>
<td>-0.21</td>
<td>0.22*</td>
<td>-0.15</td>
<td>-0.07</td>
<td>0.11</td>
<td>-0.01</td>
<td>-0.19</td>
<td>0.03</td>
</tr>
<tr>
<td>7α-OHCh</td>
<td>0.16</td>
<td>-0.04</td>
<td>0.20</td>
<td>0.01</td>
<td>0.08</td>
<td>-0.18</td>
<td>0.06</td>
<td>-0.17</td>
<td>-0.12</td>
<td>0.00</td>
<td>0.07</td>
<td>-0.11</td>
<td>0.18</td>
</tr>
<tr>
<td>7β-OHCh</td>
<td>0.23*</td>
<td>-0.02</td>
<td>0.23*</td>
<td>0.13</td>
<td>0.06</td>
<td>-0.33</td>
<td>-0.02</td>
<td>-0.11</td>
<td>-0.06</td>
<td>0.05</td>
<td>0.03</td>
<td>-0.12</td>
<td>0.16</td>
</tr>
<tr>
<td>t7-OHCh</td>
<td>0.26*</td>
<td>-0.06</td>
<td>0.28*</td>
<td>0.07</td>
<td>0.10</td>
<td>-0.33*</td>
<td>0.01</td>
<td>-0.14</td>
<td>-0.13</td>
<td>-0.24</td>
<td>0.09</td>
<td>-0.16</td>
<td>0.22*</td>
</tr>
</tbody>
</table>

Correlation coefficients were calculated between concentrations of lipid peroxidation markers and weight % proportion of plasma fatty acids using data from both groups for study days 0 and 91 (n = 40 with 2 observations/participant). Tau values bearing * represent significant associations with p<0.05.
When the data were analyzed individually for each study day, results for study day 0 for the pooled RBC data from both groups showed significant positive associations between 14:0 and ZE-ODE, EE-ODE, and t-HODE; similar positive associations were observed for 15:0 (Supplemental Table 1A*). Same data showed inverse associations of 18:0 with ZE-ODE, and t-HODE, and also between 22:5 (n-3) and t-HETE, t8-iso-PGF\(_2\alpha\), and t7-OHCh (Supplemental Table 1A*). Day 91 for the DHA group RBC data analysis showed significant positive associations of 14:0 and 15:0 with ZE-ODE, EE-ODE, t-HODE, t-HETE, and t8-iso-PGF\(_2\alpha\) (Table 5). There were significant inverse associations of 20:0 with ZE-ODE and t-HODE; 18:1 (n-9) with 7\(\beta\)-OHCh; 22:6 (n-3) with ZE-ODE, EE-ODE, t-HODE, and t8-iso-PGF\(_2\alpha\) (Table 5). Inverse associations of DHA with 7\(\beta\)-OHCh and t7-OHCh did not attain statistical significance (\(p = 0.07\) for both). For the olive oil group, day 91, there were no significant associations between any of the RBC fatty acids and markers of lipid peroxidation (Supplemental Table 1B*).

Associations between plasma fatty acids and markers of lipid peroxidation for each of days individually are shown in Supplemental Table 1A*. Same data showed inverse associations of 18:0 with ZE-ODE, t-HODE, and also between 22:5 (n-3) and t-HETE, t8-iso-PGF\(_2\alpha\), and t7-OHCh (Supplemental Table 1B*). Day 91 for the DHA group RBC data analysis showed significant positive associations between 14:0 and also 15:0 with ZE-ODE, EE-ODE, t-HODE, t-HETE, and t8-iso-PGF\(_2\alpha\) (Table 5). These results suggest that DHA, when supplemented along with antioxidants (vitamins C and E), decreased lipid peroxidation in RBC. DHA supplementation increased the weight % ratio of DHA in RBC lipids from 2.91% to 8.12%.(39) Even if DHA concentrations in RBC increased almost 3-fold, it did not increase lipid peroxidation (Table 3). Our results showing no increase in \(in\ _{vivo}\) lipid peroxidation after DHA supplementation agree with those of several studies with fish oils or individual (n-3) PUFAs in which either \(ex\ _{vivo}\) lipid peroxidation or plasma or urinary isoprostane concentrations were measured.(12–20)

Our results differ from those of studies showing an increase or decrease in \(ex\ _{vivo}\) markers of oxidation after supplementation with (n-3) PUFAs.(22–23) Our findings with DHA also differ from those of \(in\ _{vitro}\) studies in which DHA increased lipid peroxidation when used at concentrations of 50–200 \(\mu\)mol/L,(30,42,43) but not at 25 \(\mu\)mol/L. A dose-response study with DHA conducted in healthy men showed that DHA can act both as a pro- and as an antioxidant, depending on its concentration.(33,44) In that study, the urinary 15-F2 isoprostane concentration was decreased by a DHA supplement of 200 mg/day; it did not change with supplements of 400 and 800 mg/day, and it increased with a supplement of 1,600 mg/day.(44) Similarly, DHA supplementations of 200–800 mg/day decreased the plasma concentration of MDA and increased the lag time for LDL oxidation.(53) We used a similar DHA preparation in our study, except that the concentrations of vitamins E and C supplemented in our study were twice those supplemented in the study by Guillot et al.(34) Lipid peroxidation in tissues results from an imbalance between the production of reactive oxygen species and their detoxification; this balance is determined by a number of factors, including the amount and source of (n-3) and total PUFAs, amount and type of antioxidants in the diet, methods used, age, and health status of the study participants. Difference in the concentrations of the antioxidant nutrients supplemented between our study and that of Guillot et al. may be the reason for the discrepancy between the results.

Several markers of lipid peroxidation were positively associated with the relative proportions of saturated fatty acids with a chain length of C14–C16 and negatively associated with the proportion of 20:0. One potential mechanism by which saturated fatty acids may increase lipid peroxidation and (n-3) PUFAs may decrease it is through their effects on toll-like receptors (TLR) and the downstream NF-κB pathways. Saturated fatty acids activated TLR-2 and TLR-4, while the long-chain n-3 PUFAs suppressed its activation in RAW 264.7 cells.(55) Activation of TLR-4 also increases NADPH oxidase activity, which increases production of reactive oxygen species.(56) A positive association between plasma saturated fatty acids and markers of lipid peroxidation could also result from an increase in the consumption of saturated fat, which may be linked with low intake of antioxidant nutrients. However,
this is unlikely because the dietary records collected at the start and end of our study showed no change in the amounts of different types of dietary fats and antioxidant nutrients consumed. (n-3) PUFAs may decrease oxidative stress by inhibiting TLRs or NADPH oxidase, or through other mechanisms.

Placebo oil (olive) supplementation did not alter any of the RBC markers, but it significantly increased concentrations of ZE-HODE, 5-EE-HODE, t-HODE, and t-HETE in plasma lipids (Table 1). This was unexpected and we have no explanation for it. The placebo oil contained the same amount of antioxidant (Table 1). This was unexpected and we have no explanation for it. Some component other than the fatty acids in the olive oil may have increased oxidative stress.

The strengths of our study include the use of DHA in the absence of EPA, good compliance as determined by the change in fatty acid profiles, the use of the latest techniques to evaluate in vivo lipid peroxidation, and concurrence for the associations between several fatty acids and markers of lipid peroxidation between plasma and RBC lipids. The study had several limitations. The most significant one is the large variation in the concentrations of the markers of lipid peroxidation among different subjects and the small number of subjects in each group. Some discrepancies may have arisen from sample handling, but these seem to be no specific reasons for this to occur. Fatty acids of different carbon chain lengths are known to have different physio-

The placebo oil contained the same amount of an- proportion of any of the fatty acids in either plasma or RBC lipids. Using our present data, it cannot be determined whether these are random effects of the placebo oil or whether some component other than the fatty acids in the olive oil may have increased oxidative stress.

In summary, DHA supplementation for 91 day (3 g/day) did not significantly decrease the concentrations of a number of markers of lipid peroxidation in plasma and RBC lipids. Despite the lack of significant difference between the pre- and post-DHA supplementation concentrations of lipid peroxidation markers, concentra-

of significant difference between the pre- and post-DHA supplementation concentrations of lipid peroxidation markers, concentrations of several markers of lipid peroxidation were inversely associated with RBC post supplementation concentrations of DHA. In general, markers of lipid peroxidation were positively associated with the concentrations of saturated fatty acids of C14–C16. Further human studies are needed to confirm these findings and to determine the effects of fish oil and EPA on the in vivo lipid peroxidation.

The fact that DHA supplementation did not increase lipid peroxidation under our experimental conditions should allievate concerns regarding an increase in lipid peroxidation with DHA supplementation when it is taken in moderate amounts and is co-supplemented with adequate amounts of antioxidant nutrients. DHA supplementation in this study decreased several risk factors for cardiovascular disease (fasting and postprandial triglycerides, number of atherogenic small dense LDL and chylomicron remnant particles, markers of inflammation, blood pressure, and heart rate). Negative associations between RBC DHA concentrations and makers of lipid peroxidation may be an added benefit of DHA.

Acknowledgments

We are most appreciative of the study participants for their time and commitment. We thank Drs. Ellen Bonnel, Leslie Woodhouse, and their staff at WHNRC in the coordination of the study and analysis of blood samples. We are also grateful to Dr. Etsuo Niki of AIST, Japan for his critical review of the manuscript and to Martek Biosciences for donating the DHA capsules and for RBC fatty acid analysis (Edward Nelson and Eileen Bailey).

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Yoshida Y, Niki E. Bio-markers of lipid peroxidation in vivo: hydroxyocta-
13 Nenster MS, Rustain AC, Lund-Katz S, et al. Effect of dietary supplementa-
tion with n-3 polynsaturated fatty acids on physical properties and metabo-

©2014 JCBN

21 Hanwell HE, Kay CD, Lampe JW, Holub BJ, Duncan AM. Acute fish oil and soy isoflavone supplementation increase postprandial serum (n-3) polyunsaturated fatty acids and isoflavones but do not affect triacylglycerols or


38 Shimazawa M, Nakajima Y, Mashima Y, Har A. Docosahexaenoic acid (DHA) has neuroprotective effects against oxidative stress in retinal ganglion cells. *Brain Res* 2009; 1251: 269–275.


