Original Article

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

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(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 $F_{2\alpha}$, 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 $F_{2\alpha}$, ($p<0.05$). RBC concentration of hydroxycholesterol was also inversely 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.[1–4] 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.[5–8] 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.[9–11] 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,[12–21] increase,[22–28] or decrease in lipid peroxidation was observed.[29–32] In another study, the effect of DHA ranged from protection to increased lipid peroxidation depending on its dose.[33,34] 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.[35–44]

Most human studies with (*n*-3) PUFAs have either examined Cu^{2+}-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-Iso-prostagrandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), 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.[5,45] Similarly, there are limitations to the use of TBARS and MDA as markers for oxidative damage.[46]

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.[47] Within the same analysis, we can determine the concentration of total 8-iso-$PGF_{2\alpha}$ by LC-MS/MS and that of total 7-hydroxycholesterol (t7-OHCh) by gas chromatography–mass spectrometry (GC-MS). t-HODE and t7-OHCh assessed by our method accounted for much of the oxidized linoleates and cholesterol, respectively. Since the tissue concentra-

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doi: 10.3164/jcbn.14-22

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.[7]

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 t7-OHCh in RBC and plasma lipids in hypertriglyceridemic men. In addition, we measured the concentrations of individual isomers of HODE {13-hydroxy-9*Z*,11*E*-octadecadienoic acid [13-(*Z*,*E*)-HODE], 9-hydroxy-10*E*,12*Z*-octadecadienoic acid [9-(*Z*,*E*)-HODE], 13-hydroxy-9*E*,11*E*-octadecadienoic acid [13-(*E*,*E*)-HODE], 9-hydroxy-10*E*,12*E*-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 anti-oxidative 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-Iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$-d_4 (8-iso-$PGF_{2\alpha}$-d_4), 5-hydroxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid (5-HETE), 12-hydroxyeicosa-5*Z*,8*Z*,10*E*,14*Z*-tetraenoic acid (12-HETE), 15-hydroxyeicosa-5*Z*,8*Z*,11*Z*,13*E*-tetraenoic acid (15-HETE), 13-(*Z*,*E*)-HODE, 9-(*Z*,*E*)-HODE, and 13*S*-hydroxy-10*E*,12*Z*-octadecadienoic-9,10,12,13-d_4 acid (13-HODE-d_4) were obtained from Cayman Chemical Company (Ann Arbor, MI). 9-(*E*,*E*)-HODE, 13-(*E*,*E*)-HODE, 10-hydroxy-8*E*,12*Z*-octadecadienoic acid (10-(*Z*,*E*)-HODE), and 12-hydroxy-9*Z*,13*E*-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-25,26,26,26,27,27,27-d_7 (7α-OHCh-d_7) and 7β-hydroxycholesterol-25,26,26,26,27,27,27-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 agents, non-sulfonyl urea medications for diabetes mellitus, or drugs (i.e., fibrates and niacin) that alter serum triacylglycerol and high-density lipoprotein–cholesterol (HDL-C) levels were excluded. Consumers of illegal substances, more than 5 drinks of alcohol per week, more than one fish meal per week, and those taking supplements of fish oil, flaxseed oil, or vitamin C or E were excluded.

Clinical chemistry and hematology panels for all qualified participants were in normal ranges with the exception of blood lipids. All selected participants had serum C-reactive protein concentrations of 1–10 mg/L, fasting serum triglyceride concentrations of 150–400 mg/dl (1.70–4.53 mmol/L), total cholesterol level <300 mg/dl (7.78 mmol/L), LDL-C level <220 mg/dl (5.69 mmol/L), and body mass index between 22 and 35 kg/m^2.

Seventeen participants in each group completed the study. The study protocol was approved by the Institutional Review Boards of the University of California Davis and the Veterans Administration Medical Center, Mather, CA and the institutional review boards of the National Institute of Advanced Industrial Science and Technology. It is listed by the identifier NCT00728338 at http://clinicaltrials.gov.

Study design. This was a double blind, placebo controlled, parallel study with 2 metabolic periods: baseline (first 8 day) and intervention (last 90 day). During the baseline period, participants did not receive supplements, while during the intervention period participants supplemented their diets with either placebo or DHA capsules. The DHA group received 7.5 g/day DHA oil (DHA 3.0 g/day, and no EPA), which is produced in the microalga Crypthecodinium cohinii (Martek Biosciences Corporation, Columbia, MD). The placebo group received 7.5 g/day extra-virgin olive oil. Olive oil was used as the placebo because of its minimal effects on blood lipids and markers of inflammation which were the primary response variables of interest. Both oils were provided as 15 capsules (0.5 g each) every day, of which 5 were taken with each meal. Ascorbyl palmitate and mixed tocopherol (250 ppm or 0.125 mg/capsule each) were added as antioxidants to both oils. The dose and sources of DHA and placebo oils were based on published reports and on our previous DHA study.[51]

Subjects continued to consume their regular diets and were instructed not to change their usual diets and activity levels throughout the study. Usual dietary intakes were estimated by 3 unannounced 24-h dietary recalls obtained by telephone using a multi-pass interview method during each of the metabolic periods. One of the recalls was on a weekend day and the other two were on weekdays. Dietary intake data were collected and analyzed using the Nutrition Data System for Research software (ver. 2005, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN).

To 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 consumption 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, monounsaturated fatty acids, and PUFA 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-methylphenol (BHT) and internal standards 8-iso-$PGF_{2\alpha}$-d_4 (100 μg/L), 13-HODE-d_4 (100 μg/L), 7α-OHCh-d_7 (36 μg/L), 7β-OHCh-d_7 (38 μg/L), and 16-hydroxyhexadecanoic acid (140 μg/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

Oxy-lipid (pmol/mg protein)	DHA group (n = 17)				Placebo group (n = 17)			
	Day 0		Day 91		Day 0		Day 91	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
ZE-HODE	3.00	1.04	3.03	0.94	2.79	0.63	3.56	0.91
EE-HODE	1.33	0.53	1.44	0.50	1.30	0.39	1.96	0.68
t-HODE	4.33	1.57	4.47	1.42	4.09	0.99	5.52	1.58
t-HETE	8.64	2.36	7.60	1.46	9.77	3.15	10.8	1.94
t8-iso-$PGF_{2\alpha}$	0.15	0.08	0.08	0.05	0.08	0.03	0.43	0.26
7α-OHCh	19.65	3.54	32.78	10.48	40.79	14.30	58.8	14.52
7β-OHCh	34.73	7.67	58.52	19.32	88.81	35.47	109.4	39.08
t7-OHCh	55.06	10.35	91.29	28.74	129.07	49.61	168.2	52.33

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 using a 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-$PGF_{2\alpha}$, t-HETE, t-HODE, and t7-OHCh. The analysis of t8-iso-$PGF_{2\alpha}$, t-HETE, and t-HODE by LC-MS/MS and of t7-OHCh by GC-MS were performed according to the methods previously reported.[52,53] Briefly, after an extraction with chloroform and ethyl acetate, t8-iso-$PGF_{2\alpha}$, 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-$PGF_{2\alpha}$; m/z = 357.0 and 196.5–197.5 at 29 eV for 8-iso-$PGF_{2\alpha}$-d_4; 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 24 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*,*E*)-HODE; and m/z = 299.0 and 197.6–198.6 at 26 eV for 13-HODE-d_4.

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 siloxane, 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.[49] Details regarding the methods pertaining to the plasma and RBC fatty acid analyses have already been published in the literature.[49]

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.[54] 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.[48,49]

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-$PGF_{2\alpha}$ (t8-iso-$PGF_{2\alpha}$), 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 (Table 2) 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-$PGF_{2\alpha}$, 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)

doi: 10.3164/jcbn.14-22

Table 2. Effect of docosahexaenoic acid supplementation to hypertriglyceridemic men on the concentrations of markers of lipid peroxidation in plasma lipids

Oxy-lipid (nmol/L)	DHA group (n = 17)				Placebo group (n = 17)			
	Day 0		Day 91		Day 0		Day 91	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
ZE-HODE	421.24	65.27	556.74	121.87	327.29[a]	72.76	454.21[b]	92.21
EE-HODE	227.70	55.15	446.33	203.67	177.50[a]	47.26	281.52[b]	73.82
t-HODE	648.84	112.48	1000.31	313.38	504.85[a]	116.99	735.73[b]	161.57
t-HETE	452.24	90.34	542.22	113.39	459.91[a]	124.45	696.90[b]	154.26
t8-iso-$PGF_{2\alpha}$	0.80	0.20	0.85	0.32	0.92	0.35	0.47	0.12
7α-OHCh	270.42	61.05	339.44	92.97	279.13	63.28	382.36	92.83
7β-OHCh	716.79	125.51	848.66	220.81	748.48	141.27	892.59	168.64
t7-OHCh	987.20	182.68	1188.10	309.73	1027.61	200.39	1274.94	249.17

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 [b] is greater than superscript [a].

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

Oxy-lipid	Fatty acid												
	14:0	15:0	16:0	18:0	20:0	18:1 (n-9)	18:2 (n-6)	20:2 (n-6)	20:4 (n-6)	18:3 (n-3)	20:5 (n-3)	22:5 (n-3)	22:6 (n-3)
ZE-HODE	0.41*	0.40*	0.25*	–0.21	–0.16	–0.18	0.13	–0.15	–0.01	0.00	–0.04	–0.12	–0.03
EE-HODE	0.39*	0.41*	0.24*	–0.21	–0.16	–0.18	0.14	–0.17	–0.03	–0.02	–0.05	–0.20*	0.03
t-HODE	0.42*	0.41*	0.25*	–0.20	–0.15	–0.18	0.15	–0.16	0.00	0.00	–0.04	–0.13	–0.02
t-HETE	0.24*	0.21*	0.17	–0.10	–0.08	–0.11	0.19	–0.19	–0.07	0.00	0.00	–0.13	0.02
t8-iso-$PGF_{2\alpha}$	0.31*	0.27*	0.18	–0.07	–0.12	–0.09	0.08	–0.10	0.08	–0.03	–0.12	–0.12	–0.06
7α-OHCh	–0.18	0.005	0.03	–0.06	0.08	–0.01	–0.03	0.00	–0.05	–0.07	–0.03	–0.24*	0.05
7β-OHCh	0.10	0.21*	0.18	–0.07	–0.01	–0.21	0.03	0.00	–0.05	–0.07	–0.01	–0.29*	0.10
t7-OHCh	–0.02	0.17	0.12	–0.07	0.02	–0.12	0.03	–0.06	–0.04	–0.06	–0.02	–0.30*	0.08

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 (*Tau*) between plasma fatty acids and markers of lipid peroxidation in hypertriglyceridemic men taking DHA or olive oil supplements

Oxy-lipid	Fatty acid												
	14:0	15:0	16:0	18:0	20:0	18:1 (n-9)	18:2 (n-6)	20:2 (n-6)	20:4 (n-6)	18:3 (n-3)	20:5 (n-3)	22:5 (n-3)	22:6 (n-3)
ZE-HODE	0.23*	0.00	0.35*	0.02	–0.02	–0.36*	0.11	–0.11	–0.20	0.15	0.10	–0.22*	0.24*
EE-HODE	0.23*	0.00	0.37*	0.00	–0.03	–0.38*	0.08	–0.17	–0.14	0.16	0.10	–0.13	0.19
t-HODE	0.23*	–0.02	0.37*	0.02	–0.03	–0.36*	0.08	–0.13	–0.19	0.14	0.08	–0.19	0.22*
t-HETE	0.24*	0.11	0.16	0.13	0.05	–0.27*	–0.01	–0.06	–0.02	0.18	0.02	–0.13	0.12
t8-iso-$PGF_{2\alpha}$	0.02	0.14	0.07	–0.03	0.00	–0.21	0.22*	–0.15	–0.07	0.11	–0.01	–0.19	0.03
7α-OHCh	0.16	–0.04	0.20	0.01	0.08	–0.18	0.06	–0.17	–0.12	0.00	0.07	–0.11	0.18
7β-OHCh	0.23*	–0.02	0.23*	0.13	0.06	–0.33	–0.02	–0.11	–0.06	0.05	0.03	–0.12	0.16
t7-OHCh	0.26*	–0.06	0.28*	0.07	0.10	–0.33*	0.01	–0.14	–0.13	–0.24	0.09	–0.16	0.22*

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 associtions with p<0.05.

(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).

Table 5. Kendall's Correlation Coefficients (*Tau*) between RBC fatty acids and markers of lipid peroxidation in hypertriglyceridemic men taking DHA supplements for 91 days

Oxy-lipid	Fatty acid												
	14:0	15:0	16:0	18:0	20:0	18:1 (*n*-9)	18:2 (*n*-6)	20:2 (*n*-6)	20:4 (*n*-6)	18:3 (*n*-3)	20:5 (*n*-3)	22:5 (*n*-3)	22:6 (*n*-3)
ZE-HODE	0.57*	0.47*	0.25	–0.15	–0.41*	–0.13	0.10	0.06	0.22	–0.28	–0.18	–0.13	–0.59*
EE-HODE	0.50*	0.57*	0.18	–0.10	–0.28	–0.21	0.15	0.07	0.26	–0.29	–0.19	–0.21	–0.54*
t-HODE	0.57*	0.53*	0.22	–0.12	–0.35*	–0.16	0.10	0.03	0.22	–0.31	–0.18	–0.13	–0.56*
t-HETE	0.41*	0.37*	0.18	–0.04	–0.16	–0.12	–0.03	0.01	0.00	–0.21	0.10	0.15	–0.25
t8-iso-$PGF_{2\alpha}$	0.53*	0.49*	0.26	–0.07	–0.34	–0.03	0.00	0.16	0.21	–0.26	–0.19	–0.21	–0.54*
7α-OHCh	–0.09	0.04	0.18	–0.22	0.04	–0.12	0.00	0.07	0.09	–0.15	–0.10	–0.35*	–0.07
7β-OHCh	0.16	0.32	0.16	–0.06	–0.09	–0.37*	0.01	0.09	0.22	–0.34	–0.09	–0.16	–0.32
t7-OHCh	0.04	0.21	0.16	–0.12	–0.09	–0.28	0.04	0.09	0.22	–0.28	–0.15	–0.31	–0.24

Correlation coefficients were calculated between concentrations of lipid peroxidation markers and weight % proportion of RBC fatty acids using data from study day 91 after DHA supplementation (n = 17). *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*-HODE, *EE*-HODE, and t-HODE; similar positive associations were observed for 15:0 (Supplemental Table 1A*). Same data showed inverse associations of 18:0 with *ZE*-HODE, 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 also 15:0 with *ZE*-HODE, *EE*-HODE, t-HODE, t-HETE, and t8-iso-$PGF_{2\alpha}$ (Table 5). There were significant inverse associations of 20:0 with *ZE*-HODE and t-HODE; 18:1 (*n*-9) with 7β-OHCh; 22:6 (*n*-3) with *ZE*-HODE, *EE*-HODE, t-HODE, and t8-iso-$PGF_{2\alpha}$ (Table 5). Inverse associations of DHA with 7β-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 associaitions 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 2A, B, and C*. Day 0 revealed significant positive associations between 15:0 with t8-iso-$PGF_{2\alpha}$; 16:0 with *ZE*-HODE, *EE*-HODE, and t-HODE; 18:2 with t8-iso-$PGF_{2\alpha}$ (Supplemental Table 2A*). There were significant inverse associations between 18:1 and *ZE*-HODE, *EE*-HODE, t-HETE, and t-HODE, t8-iso-$PGF_{2\alpha}$ (Supplemental Table 2A*). Day 91, DHA group, there were no significant associations between any of the fatty acids and markers of lipid peroxidation (Supplemental Table 2B*). Day 91, placebo group, there significant positive associations of 16:0 with *ZE*-HODE and t-HODE; negative significant associations of 18:1 (*n*-9) with 7β-OHCh and t7-OHCh, 22:2 (*n*-6) with *ZE*-HODE, *EE*-HODE, t-HODE and t8-iso-$PGF_{2\alpha}$ (Supplemental Table 2C*).

Discussion

Our study with hypertriglyceridemic men showed that DHA supplementation did not alter the concentration of eight markers of lipid peroxidation tested both for RBC and plasma lipids (Table 1 and 2). Furthermore, the weight % proportion of DHA did not show any positive association with markers of lipid peroxidation tested in RBC lipids when the data from two groups were pooled (Table 3). However, when the post-supplemental data from the DHA group were analyzed separately, RBC DHA concentration was inversely associated with RBC concentrations of several markers of lipid peroxidation (Table 5). The lack of positive or negative associations of DHA with markers of lipid peroxidation in the pooled data may be due the large variance in both the concentrations of DHA and markers of lipid peroxidation. However, DHA supplementation markedly increased the RBC DHA concentrations and reduced the variance which showed significant inverse of DHA with several markers of lipid peroxidation (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* LDL 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.[22–32] 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 μmol/L,[40,42,43] but not at 25 μmol/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.[33,34] 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.[34] Similarly, DHA supplementations of 200–800 mg/day decreased the plasma concentration of MDA and increased the lag time for LDL oxidation.[33] 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) PUFA 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,

*See online. https://www.jstage.jst.go.jp/article/jcbn/55/3/55_14-22/_article/supplement

doi: 10.3164/jcbn.14-22

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, *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 nutrients as added to the DHA oil and it did not alter the relative proportion of any of the fatty acids in either plasma or RBC lipids.[48,49] 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.

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 there seems to be no specific reasons for this to occur. Fatty acids of different carbon chain lengths are known to have different physiological effects, but we are not aware of any report where different saturated or *n*-3 PUFAs have been reported to have different associations with *in vivo* markers of lipid peroxidation; this needs to be addressed in future studies.

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, 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 alleviate 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).[48–50] 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*: hydroxyoctadecadienoic acid and hydroxycholesterol. *Biofactors* 2006; **27**: 195–202.
2 Kaikkonen J, Tuomainen TP, Nyyssönen K, Morrow JD, Salonen JT. C18 hydroxy fatty acids as markers of lipid peroxidation *ex vivo* and *in vivo*. *Scand J Clin Lab Invest* 2004; **64**: 457–468.
3 Spiteller G. The relation of lipid peroxidation processes with atherogenesis: a new theory on atherogenesis. *Mol Nutr Food Res* 2005; **49**: 999–1013.
4 Spiteller G. The important role of lipid peroxidation processes in aging and age dependent diseases. *Mol Biotechnol* 2007; **37**: 5–12.
5 Yoshida Y, Saito Y, Hayakawa M, *et al*. Levels of lipid peroxidation in human plasma and erythrocytes: comparison between fatty acids and cholesterol. *Lipids* 2007; **42**: 439–449.
6 Leonarduzzi G, Arkan MC, Başağa H, Chiarpotto E, Sevanian A, Poli G. Lipid oxidation products in cell signaling. *Free Radic Biol Med* 2000; **28**: 1370–1378.
7 Niki E, Yoshida Y, Saito Y, Noguchi N. Lipid peroxidation: mechanisms, inhibition, and biological effects. *Biochem Biophys Res Commun* 2005; **338**: 668–676.
8 Tang DG, La E, Kern J, Kehrer JP. Fatty acid oxidation and signaling in apoptosis. *Biol Chem* 2002; **383**: 425–442.
9 Harris WS, Von Schacky C. The Omega-3 Index: a new risk factor for death from coronary heart disease? *Prev Med* 2004; **39**: 212–220.
10 Calder PC. *n*-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. *Clin Sci* 2004; **107**: 1–11.
11 Breslow JL. *n*-3 fatty acids and cardiovascular disease. *Am J Clin Nutr* 2006; **83** (6 Suppl): 1477S–1482S.
12 Shoji H, Franke C, Campoy C, Rivero M, Demmelmair H, Koletzko B. Effect of docosahexaenoic acid and eicosapentaenoic acid supplementation on oxidative stress levels during pregnancy. *Free Radic Res* 2006; **40**: 379–384.
13 Nenseter MS, Rustan AC, Lund-Katz S, *et al*. Effect of dietary supplementation with *n*-3 polyunsaturated fatty acids on physical properties and metabolism of low density lipoprotein in humans. *Arterioscler Thromb* 1992; **12**: 369–379.
14 Frankel EN, Parks EJ, Xu R, Schneeman BO, Davis PA, German JB. Effect of *n*-3 fatty acid-rich fish oil supplementation on the oxidation of low density lipoproteins. *Lipids* 1994; **29**: 233–236.
15 Bonanome A, Biasia F, De Luca M, *et al*. *n*-3 fatty acids do not enhance LDL susceptibility to oxidation in hypertriacylglycerolemic hemodialyzed subjects. *Am J Clin Nutr* 1996; **63**: 261–266.
16 Brude IR, Drevon CA, Hjermann I, *et al*. Peroxidation of LDL from combined-hyperlipidemic male smokers supplied with omega-3 fatty acids and antioxidants. *Arterioscler Thromb Vasc Biol* 1997; **17**: 2576–2588.
17 Sørensen NS, Marckmann P, Hoy CE, van Duyvenvoorde W, Princen HM. Effect of fish-oil-enriched margarine on plasma lipids, low-density-lipoprotein particle composition, size, and susceptibility to oxidation. *Am J Clin Nutr* 1998; **68**: 235–241.
18 Higdon JV, Liu J, Du SH, Morrow JD, Ames BN, Wander RC. Supplementation of postmenopausal women with fish oil rich in eicosapentaenoic acid and docosahexaenoic acid is not associated with greater *in vivo* lipid peroxidation compared with oils rich in oleate and linoleate as assessed by plasma malondialdehyde and F(2)-isoprostanes. *Am J Clin Nutr* 2000; **72**: 714–722.
19 Higdon JV, Du SH, Lee YS, Wu T, Wander RC. Supplementation of postmenopausal women with fish oil does not increase overall oxidation of LDL *ex vivo* compared to dietary oils rich in oleate and linoleate. *J Lipid Res* 2001; **42**: 407–418.
20 Malaguti M, Baldini M, Angeloni C, Biagi P, Hrelia S. High-protein-PUFA supplementation, red blood cell membranes, and plasma antioxidant activity in volleyball athletes. *Int J Sport Nutr Exerc Metab* 2008; **18**: 301–312.
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

biomarkers of oxidative stress in overweight and obese hypertriglyceridemic men. *J Nutr* 2009; **139**: 1128–1134.
22 Harats D, Dabach Y, Hollander G, *et al*. Fish oil ingestion in smokers and nonsmokers enhances peroxidation of plasma lipoproteins. *Atherosclerosis* 1991; **90**: 127–139.
23 Meydani M, Natiello F, Goldin B, *et al*. Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women. *J Nutr* 1991; **121**: 484–491.
24 Suzukawa M, Abbey M, Howe PR, Nestel PJ. Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages. *J Lipid Res* 1995; **36**: 473–484.
25 Palozza P, Sgarlata E, Luberto C, *et al*. *n*-3 fatty acids induce oxidative modifications in human erythrocytes depending on dose and duration of dietary supplementation. *Am J Clin Nutr* 1996; **64**: 297–304.
26 Wu WH, Lu SC, Wang TF, Jou HJ, Wang TA. Effects of docosahexaenoic acid supplementation on blood lipids, estrogen metabolism, and *in vivo* oxidative stress in postmenopausal vegetarian women. *Eur J Clin Nutr* 2006; **60**: 386–392.
27 Parra D, Bandarra NM, Kiely M, Thorsdottir I, Martínez JA. Impact of fish intake on oxidative stress when included into a moderate energy-restricted program to treat obesity. *Eur J Nutr* 2007; **46**: 460–467.
28 Egert S, Somoza V, Kannenberg F, *et al*. Influence of three rapeseed oil-rich diets, fortified with alpha-linolenic acid, eicosapentaenoic acid or docosahexaenoic acid on the composition and oxidizability of low-density lipoproteins: results of a controlled study in healthy volunteers. *Eur J Clin Nutr* 2007; **61**: 314–325.
29 Mori TA, Dunstan DW, Burke V, *et al*. Effect of dietary fish and exercise training on urinary F2-isoprostane excretion in non-insulin-dependent diabetic patients. *Metabolism* 1999; **48**: 1402–1408.
30 Mori TA, Woodman RJ, Burke V, Puddey IB, Croft KD, Beilin LJ. Effect of eicosapentaenoic acid and docosahexaenoic acid on oxidative stress and inflammatory markers in treated-hypertensive type 2 diabetic subjects. *Free Radic Biol Med* 2003; **35**: 772–781.
31 Nälsén C, Vessby B, Berglund L, *et al*. Dietary (*n*-3) fatty acids reduce plasma F2-isoprostanes but not prostaglandin F2alpha in healthy humans. *J Nutr* 2006; **136**: 1222–1228.
32 Kim YJ, Chung HY. Antioxidative and anti-inflammatory actions of docosahexaenoic acid and eicosapentaenoic acid in renal epithelial cells and macrophages. *J Med Food* 2007; **10**: 225–231.
33 Calzada C, Colas R, Guillot N, *et al*. Subgram daily supplementation with docosahexaenoic acid protects low-density lipoproteins from oxidation in healthy men. *Atherosclerosis* 2010; **208**: 467–472.
34 Guillot N, Caillet E, Laville M, Calzada C, Lagarde M, Véricel E. Increasing intakes of the long-chain omega-3 docosahexaenoic acid: effects on platelet functions and redox status in healthy men. *FASEB J* 2009; **23**: 2909–2916.
35 Bechoua S, Dubois M, Dominguez Z, *et al*. Protective effect of docosahexaenoic acid against hydrogen peroxide-induced oxidative stress in human lymphocytes. *Biochem Pharmacol* 1999; **57**: 1021–1030.
36 Richard D, Kefi K, Barbe U, Bausero P, Visioli F. Polyunsaturated fatty acids as antioxidants. *Pharmacol Res* 2008; **57**: 451–455.
37 Brand A, Schonfeld E, Isharel I, Yavin E. Docosahexaenoic acid-dependent iron accumulation in oligodendroglia cells protects from hydrogen peroxide-induced damage. *J Neurochem* 2008; **105**: 1325–1335.
38 Shimazawa M, Nakajima Y, Mashima Y, Hara H. Docosahexaenoic acid (DHA) has neuroprotective effects against oxidative stress in retinal ganglion cells. *Brain Res* 2009; **1251**: 269–275.
39 Shoji H, Franke C, Demmelmair H, Koletzko B. Effect of docosahexaenoic acid on oxidative stress in placental trophoblast cells. *Early Hum Dev* 2009; **85**: 433–437.
40 Leonardi F, Attorri L, Benedetto RD, *et al*. Docosahexaenoic acid supplementation induces dose and time dependent oxidative changes in C6 glioma cells. *Free Radic Res* 2007; **41**: 748–756.
41 Li M, Kong ZM, Liu ZL. Antioxidant enzyme activities and lipid peroxidation induced by eicosapentaenoic acid (EPA) in PC12 cells. *Cell Biol Toxicol* 2006; **22**: 331–337.
42 Merendino N, Loppi B, D'Aquino M, *et al*. Docosahexaenoic acid induces apoptosis in the human PaCa-44 pancreatic cancer cell line by active reduced glutathione extrusion and lipid peroxidation. *Nutr Cancer* 2005; **52**: 225–233.
43 Roig-Pérez S, Guardiola F, Moretó M, Ferrer R. Lipid peroxidation induced by DHA enrichment modifies paracellular permeability in Caco-2 cells: protective role of taurine. *J Lipid Res* 2004; **45**: 1418–1428.
44 Ng Y, Barhoumi R, Tjalkens RB, *et al*. The role of docosahexaenoic acid in mediating mitochondrial membrane lipid oxidation and apoptosis in colonocytes. *Carcinogenesis* 2005; **26**: 1914–1921.
45 Yoshida Y, Niki E. Detection of lipid peroxidation *in vivo*: total hydroxyoctadecadienoic acid and 7-hydroxycholesterol as oxidative stress marker. *Free Radic Res* 2004; **38**: 787–794.
46 Kojima T, Kikugawa K, Kosugi H. Is the thiobarbituric acid-reactivity of blood plasma specific to lipid peroxidation? *Chem Pharm Bull (Tokyo)* 1990; **38**: 3414–3418.
47 Yoshida Y, Niki E. Relative susceptibilities of linoleates and cholesterol to oxidation assessed by total hydroxyoctadecadienoic acid and 7-hydroxycholesterol. *J Oleo Sci* 2008; **57**: 407–414.
48 Kelley DS, Siegel D, Vemuri M, Mackey BE. Docosahexaenoic acid supplementation improves fasting and postprandial lipid profiles in hypertriglyceridemic men. *Am J Clin Nutr* 2007; **86**: 324–333.
49 Kelley DS, Siegel D, Vemuri M, Chung GH, Mackey BE. Docosahexaenoic acid supplementation decreases remnant-like particle-cholesterol and increases the (*n*-3) index in hypertriglyceridemic men. *J Nutr* 2008; **138**: 30–35.
50 Kelley DS, Siegel D, Fedor DM, Adkins Y, Mackey BE. DHA supplementation decreases serum C-reactive protein and other markers of inflammation in hypertriglyceridemic men. *J Nutr* 2009; **139**: 495–501.
51 Kelley DS, Taylor PC, Nelson GJ, *et al*. Docosahexaenoic acid ingestion inhibits natural killer cell activity and production of inflammatory mediators in young healthy men. *Lipids* 1999; **34**: 317–324.
52 Yoshida Y, Kodai S, Takemura S, Minamiyama Y, Niki E. Simultaneous measurement of F2-isoprostane, hydroxyoctadecadienoic acid, hydroxyeicosatetraenoic acid, and hydroxycholesterols from physiological samples. *Anal Biochem* 2008; **379**: 105–115.
53 Umeno A, Shichiri M, Ishida N, *et al*. Singlet oxygen induced products of linoleates, 10- and 12-(*Z,E*)-hydroxyoctadecadienoic acids (HODE), can be potential biomarkers for early detection of type 2 diabetes. *PLoS One* 2013; **8**: e63542.
54 Littell RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O. *SAS for Mixed Models, Second Edition.* Cary: SAS Institude inc, 2006.
55 Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J Biol Chem* 2001; **276**: 16683–16689.
56 Park HS, Jung HY, Park EY, Kim J, Lee WJ, Bae YS. Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-kappa B. *J Immunol* 2004; **173**: 3589–3593.

doi: 10.3164/jcbn.14-22