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The effect of dietary menhaden fish oil on α-tocopherol status in rodents is both concentration and tissue dependent

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This study was designed to measure the dose and time dependent effect of menhaden fish oil (MFO) on immune cell α-tocopherol in the rat. The level of dietary fat and α-tocopherol (equalized in amount and chemical form) were constant across all experimental groups with increasing percentages of lard replaced with MFO. As the proportion of fish oil in the diet increased, plasma α-tocopherol decreased. Splenocyte α-tocopherol increased significantly with time with rats fed 4.5% MFO for 2 week having significantly higher α-tocopherol than rats on all other diets ($P < 0.05$*). Plasma arachidonic acid (AA) decreased and docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) increased as the percentage fish oil in the diet increased. Splenocyte fatty acid composition was significantly altered by diet, with inclusion of as little as 4.5% MFO sufficient to decrease AA from 25% to 12% of total fatty acids (*$P < 0.001$*) and increase EPA from 1% to 4% of the total fatty acids (*$P < 0.001$*). Additional MFO significantly increased splenocyte EPA and DHA to approximately 7% and 5% of the total fatty acids, respectively, without any further decline in splenocyte AA. Changes in splenocyte fatty acid composition occurred rapidly with approximately 85% of the total change occurring within the first week of dietary treatment. The rapid and dose dependent enrichment of immune cells with (n-3) fatty acids may compromise the antioxidant status of these cells. However, at the lowest level of dietary fish oil tested (4.5%), immune cells appeared to compensate for the higher (n-3) polyunsaturated fatty acid content with a significant accumulation of α-tocopherol. That changes in fatty acid composition preceded changes in vitamin E status of the immune cell suggests that the former affects the latter.* (J. Nutr. Biochem. 8:518–526, 1997) © *Elsevier Science Inc. 1997*

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Introduction

Vitamin E is the primary lipid-soluble antioxidant associated with cell membranes. Increased lipid peroxidation with subsequent impairment of immunological and neurological function is associated with vitamin E deficiency,[1,2] whereas vitamin E supplementation may enhance immune responses.[3–6] Vitamin E is absorbed and transported with other dietary lipid in association with lipoproteins. Delivery to tissues occurs by transfer with other lipids during lipoprotein lipase catalyzed chylomicron delipidation. Vitamin E remaining with the chylomicron remnant may also be transferred to high density lipoprotein (HDL) along with the excess surface coat or enter the liver for later redistribution as part of very low density lipoproteins (VLDL) released from the liver.[7–9]

It has become clear that vitamin E status, as evaluated by plasma vitamin E levels, is negatively affected by dietary polyunsaturated fatty acids (PUFA),[1] but the relationship between these two nutrients is not well defined. Both increased usage[10–12] and decreased absorption[13–15] of vita-

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min E have been cited as components of the PUFA-induced increase in vitamin E requirement. Further, PUFA of the omega-3 (n-3) family are more antagonistic to tocopherol status than those of the omega-6 (n-6) family,[16–19] with blunted responses to dietary supplementation with vitamin E noted in (n-3) fed animals.[19–21] Dietary fish oil, a rich source of (n-3) PUFA, decreases both postprandial lipemia[22,23] and VLDL secretion[24,25] as well as increases lipid peroxidation in rats[17,26–28] and humans,[29,30] further complicating delineation of the relationship.

Defining this relationship at the level of the immune cell continues to be very important because the immune system is sensitive to modulation by both dietary (n-3) PUFA and vitamin E.[31–34] Recently, we have shown that the effect of (n-3) PUFA on immune cell vitamin E status is a function of both fish oil source and the amount and chemical form of vitamin E provided in the experimental diets.[16] In fact, we have made the novel observation that in some cases, such as low plasma tocopherol levels, a mechanism exists whereby immune cell vitamin E concentration may be maintained at higher levels in rats fed fish oil as compared with rats fed a low PUFA fat.[35]

The research reported here was undertaken to further examine this phenomenon. By maintaining dietary fat levels at 20% across all experimental groups while increasing the percentage of the fat provided by fish oil we were able to examine a dose response to fish oil. This experimental design allows us to compare these data to previous work as well as to establish a model of fish oil consumption which is more applicable to human nutritional circumstances. Here, we report that inclusion of fish oil in the diet decreases plasma α-tocopherol in a dose-dependent fashion. In contrast, immune cell α-tocopherol concentration was not decreased by increasing levels of dietary fish oil. In fact, despite significantly lower plasma α-tocopherol concentration, inclusion of a moderate amount of fish oil in the diet (i.e., 4.5%) resulted in significantly higher immune cell α-tocopherol concentration as compared with rats fed the control diet containing no fish oil. These data are particularly important in light of our recent findings that show that increased PUFA content of immune cells without concomitant increases in vitamin E increases lipid peroxidation.[36]

Methods and materials

Animals and Diets

Ninety, 5- to 6-week-old female Sprague-Dawley rats (Sasco, St. Louis, MO) were housed individually in hanging *wire-mesh*, stainless steel cages. Temperature and humidity in the room were kept constant (i.e., 21 to 24°C and 45 to 50%, respectively) and a diurnal light cycle of 12 hr was maintained. Housing, handling and sample collection procedures conformed to policies and recommendations of the University of Missouri's Laboratory Animal Care Advisory Committee.

On receipt, 90 rats were randomly assigned as groups of 18 to one of five purified diets. Fourteen rats from the same shipment were sacrificed to obtain baseline (time zero) values. Experimental diets contained 2% by weight tocopherol-stripped corn oil (United States Biochemical, Cleveland OH) and 18% by weight of one of the following oil blends: 100, 75, 50, 25 or 0 (% by weight) menhaden fish oil (Zapata Protein Corp., Reedville, VA) with the remaining oil as tocopherol-stripped lard (United States Biochemical, Cleveland OH). The diet composition is shown in *Table 1* and the fatty acid composition of each mixed diet is shown in *Table 2*. Corn oil provided sufficient linoleic acid [18:2 (n-6)], an essential fatty acid, across treatments. Endogenous α-tocopherol concentration of each oil blend was determined, in duplicate, as described by Slover and Thompson[37] and equalized by addition of RRR-α-tocopherol (a gift from Eastman Kodak). Equalization of α-tocopherol across fat sources as a baseline was necessary because fish oils stripped of endogenous α-tocopherol could not be obtained. The only form of vitamin E detected in the fish oils was α-tocopherol.

Table 1 Composition of experimental diets containing increasing amounts of menhaden fish oil (MFO) substituted for lard (LRD) in a 20% fat diet[1]

Ingredient	Dietary treatment groups as % MFO (g/100 g mixed diet)				
	0	4.5	9.0	13.5	18.0
Casein	20	20	20	20	20
DL-methionine	0.3	0.3	0.3	0.3	0.3
Corn starch	20	20	20	20	20
Dextrose	29.3	29.3	29.3	29.3	29.3
Cellulose	5	5	5	5	5
AIN-76 mineral mix	4	4	4	4	4
AIN-76 vitamin mix	1.2	1.2	1.2	1.2	1.2
Choline bitartrate	0.2	0.2	0.2	0.2	0.2
Lard	18.0	13.5	9.0	4.5	0
Menhaden fish oil	0	4.5	9.0	13.5	18.0
Corn oil	2	2	2	2	2

[1]Weanling female Sprague-Dawley rats were fed semi-purified diets for 1, 2, or 4 weeks. Endogenous α-T in the lard and menhaden fish oil was measured and equalized with α-T (i.e., RRR-α-tocopherol).

Experimental diets were isocaloric and formulated according to AIN guidelines[38] with minor modifications (*Table 1*). Animals had free access to fresh diet each day with remaining diet discarded. Animals also had free access to distilled water. Auto-oxidation of the diets was prevented by adding 1.2 mM/L tertiary-butyl hydroquinone (Eastman Kodak Co.) to the oils upon receipt as described by Fritsche and Johnston.[39] Oils were mixed into the dry components of the diet in small batches, and stored at 4°C. Oxidative stability of these diets was assessed by leaving aliquots of each diet in feed bowls for 48 hr at room temperature. After ether extraction, the total lipids were gently saponified and analyzed for α-tocopherol by HPLC as described later. Under these conditions, recovery equaled 101 ± 4% of the α-tocopherol that was originally measured in freshly prepared diets. Furthermore, peroxide values of the diets did not increase under these conditions at any time during the study.

Sample collection

After 0, 1, 2, or 4 weeks of consuming the experimental diets, rats were anesthetized by intramuscular injection of Ketaset (Ketamine-HCl, Aveco Co., Inc., Fort Dodge, IA) and Rompun (Xylazine, Mobay Co., Animal Health Division, Shawnee, KS). The time zero animals were killed within 24 hr of receipt to establish baseline values. Rats were deprived of feed for 12 hr before sample collection. Samples of blood were collected by cardiac puncture into a syringe containing 50 units of heparin (Elkins-Sinn, Inc., Cherry Hill, NJ). Plasma was separated by centrifugation at 250 × g for 10 min and stored (−80°C) for later α-tocopherol and fatty acid determination. Livers were placed in polyethylene bags and immediately frozen in liquid nitrogen.

Table 2 Fatty acid composition of experimental diets containing increasing amounts of menhaden fish oil (MFO) substituted for lard (LRD) in a 20% fat diet[1]

	Dietary treatment groups as % MFO (g/100 g total fatty acids)				
Fatty acid	0	4.5	9.0	13.5	18.0
14:0	0.8	2.5	2.3	5.5	6.5
16:0	22.1	21.9	19.6	19.3	17.3
16:1 (n-7)	1.9	4.3	6.1	9.1	11.6
18:0	20.0	12.8	10.0	6.6	5.7
18:1 (n-7) + (n-9)	38.3	36.2	30.6	22.4	12.3
18:2 (n-6)	15.4	13.5	12.6	10.7	8.8
18:3 (n-3)	0.7	0.9	1.2	1.4	1.6
20:4 (n-6)	0.1	0.3	0.5	0.6	0.8
20:5 (n-3)	0.0	3.4	8.0	11.9	16.7
22:5 (n-3)	0.0	0.5	1.2	1.7	2.4
22:6 (n-3)	0.0	2.3	5.4	8.0	11.4
Σ SFA	42.9	37.2	32.0	31.3	29.5
Σ MONO	40.2	40.5	36.7	31.5	23.9
Σ PUFA	16.9	22.4	31.4	37.3	46.6
Σ (n-6) PUFA	16.0	14.5	13.6	11.7	10.8
Σ (n-3) PUFA	1.0	7.9	17.7	25.6	35.7
(n-6)/(n-3)	16.0	1.84	0.77	0.46	0.30

[1]Fatty acids are denoted by the number of carbons: the number of double bonds, followed by the position of the first double bond relative to the methyl-end ("n-"). SFA, saturated fatty acids; MONO, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Single-cell suspensions of splenocytes were obtained by forcing the spleen through a sieve (Sigma Chemical, St. Louis, MO) equipped with a 80-mesh stainless steel screen into RPMI 1640 culture medium with 10 mM HEPES. Using a 10 mL syringe without a needle, cell clumps were dispersed by several gentle washings through the sieve. A single-cell suspension was obtained by allowing cell clumps to sediment out at room temperature for 10 min. Splenocytes were isolated from the crude cell suspension by density gradient centrifugation over Histopaque 1.077 (Sigma). The mononuclear leukocytes at the interface were collected and washed twice.[16] Immune cell recovery was determined electronically using a Coulter Counter, Model ZBI (Coulter Electronics, Hialeah, FL). Cells samples were pelleted and stored in 1 mL of 10 mM EDTA at −80°C for α-tocopherol analyses.

Alpha-tocopherol determination by HPLC

Alpha-tocopherol concentration of plasma and isolated immune cells was determined by HPLC as previously described.[16,40,41] Briefly, 100 μL of δ-tocopherol in absolute ethanol was introduced into each plasma sample (100 μL) as an internal standard (25 mM). Tocopherols were extracted by the addition of heptane (200 μL) followed by vigorous mixing with a vortex mixer. For isolated splenocytes ($0.6–2 \times 10^8$ cells in 1 mL of 10 mM EDTA) 1.45 mL of ethanol (Quantum Chemical Corporation, Tuscola, IL) and 50 μL internal standard (25 mM) were added; followed by 2 mL of heptane with intermittent vigorous mixing on a vortex mixer (45 sec). Plasma and immune cell samples were centrifuged to separate the phases and the organic (top) phase was transferred into a glass test tube. After removal of the organic phase, each sample was extracted a second time. The organic phase from the second extraction was pooled with the first and the extracts were evaporated to dryness under a stream of N_2 gas, then resuspended in methanol (150 μL).

Liver α-tocopherol determination was carried out in duplicate following a procedure adapted from Taylor et al.[41] Briefly, frozen liver tissue samples (500 mg) were weighed then homogenized (15 sec) with a Tissumizer (Tekmar, Cincinnati, OH) in 10 volumes of 10 mM EDTA which contained 100 μL of 0.054 M BHT in absolute ethanol. One mL of liver homogenate was then added to 2 mL of 0.057 M ascorbic acid to which 100 μL of internal standard, δ-tocopherol (25 mM) had previously been added. Tubes were vortexed and briefly heated (2 min) at 70°C in a covered waterbath. Saponification was accomplished by addition of 300 μL saturated KOH to the solution, which was then heated for 30 min at 70°C in a covered waterbath. At the end of the saponification period, samples were rapidly cooled on ice and tocopherols were extracted with 4 mL hexane, 1 mL water, and vigorous shaking. The homogenate was centrifuged (1300 × g for 5 min) and the top layer was removed and transferred into a glass test tube. The lower layer was then re-extracted with 4 mL of hexane, vortexed well, and centrifuged. The combined extracts were evaporated to dryness under a stream of N_2, then resuspended in 1 mL of methanol.

A 100 μL aliquot was injected into a high performance liquid chromatograph (Gilson HPLC) equipped with a C-18 reverse-phase column (15 cm × 4.6 mm; 3 mm; Supelco Inc., Bellefonte, PA). The mobile phase used consisted of methanol and water (98:2, by vol) with a flow rate of 1.5 mL/min. The effluent was monitored at a UV wavelength of 292 nm (Gilson UV detector 117). Typical retention times for standards were (min): 4.0, δ-tocopherol; 4.7, γ-tocopherol; 5.8, α-tocopherol. Sample α-tocopherol concentration was calculated from peak area responses using a standard curve that was established from known amounts of pure α-tocopherol and δ-tocopherol. Values were corrected for losses during processing by following the recovery of the internal standards, which generally exceeded 90%.

Fatty acid and lipid determination

Plasma samples (0.5 mL) were diluted with 1.5 ml Tris/EDTA/sucrose buffer (50 mM Trizma-HCl; 1 mM EDTA; 0.32 mol/L sucrose; pH 7.4). Two mL of the liver homogenate, aliquoted and flash frozen when prepared for α-tocopherol analysis, was thawed in hot water. Splenocyte pellets in 1 mL 10 mM EDTA were quickly thawed before use. Lipids in the diluted plasma, splenocytes or liver homogenate (2 mL) were extracted with 4 volumes of chloroform and methanol (2:1, by vol). The organic phase

Table 3 Body, liver, and spleen weights of female rats fed increasing amounts of menhaden fish oil (MFO) substituted for lard (LRD) in a 20% fat diet for 1, 2 or 4 wk[1–3]

	Dietary treatment groups (% MFO)					
	0.0	4.5	9.0	13.5	18.0	*P* value
Week one						
Body weight (g)	121 ± 5.0	120 ± 6.3	124 ± 5.0	126 ± 5.9	121 ± 4.4	ns
Liver (g)	3.7 ± 0.2	3.7 ± 0.3	4.3 ± 0.3	4.0 ± 0.2	4.0 ± 0.2	ns
Spleen (g)	0.32 ± 0.02	0.33 ± 0.04	0.33 ± 0.03	0.32 ± 0.03	0.32 ± 0.03	ns
Splenocyte (10^7)	1.1 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	1.6 ± 0.3	1.0 ± 0.3	ns
Week two						
Body weight (g)	145 ± 7.7	145 ± 6.4	142 ± 4.3	150 ± 7.1	143 ± 5.8	ns
Liver (g)	4.8 ± 0.4	4.6 ± 0.2	5.4 ± 0.3	5.4 ± 0.3	5.0 ± 0.2	ns
Spleen (g)	0.39 ± 0.02	0.39 ± 0.03	0.47 ± 0.01	0.48 ± 0.04	0.45 ± 0.03	ns
Splenocyte (10^7)	2.0 ± 0.5	2.1 ± 0.4	3.0 ± 0.2	3.1 ± 0.5	1.9 ± 0.3	ns
Week four						
Body weight (g)	175 ± 12.5	196 ± 7.4	195 ± 6.4	190 ± 6.9	187 ± 4.0	ns
Liver (g)	4.7a ± 0.4	5.1 ± 0.4	5.7ab ± 0.3	5.6ab ± 0.2	6.6b ± 0.5	0.03
Spleen (g)	0.45 ± 0.03	0.47 ± 0.02	0.51 ± 0.03	0.47 ± 0.03	0.52 ± 0.03	ns
Splenocyte (10^7)	1.9 ± 1.9	1.8 ± 0.2	1.8 ± 0.2	1.7 ± 0.3	2.8 ± 0.6	ns

[1]Weanling female Sprague-Dawley rats were fed semipurified diets for 1, 2, or 4 weeks.
[2]Values represent the means ($n = 6$) ± standard error of the mean.
[3]Values with different letters within a row are significantly different at the *P* value listed within that row, "ns" is not significant ($P > 0.05$).

containing the lipid extract was removed and the aqueous phase was re-extracted with two volumes of chloroform/methanol (4:1). Organic layers were pooled, filtered, then reduced in volume under N_2. Plasma fatty acid methyl esters were made using 0.7 M methanolic sulfuric acid and splenocytes methyl esterification was accomplished with sodium hydroxide in methanol. Lipid extract from the liver was further separated by one dimensional thin layer chromatography (TLC) with 80 mL hexane, 20 mL diethyl ether, and 2 mL glacial acetic acid as the developing solvent.[42] Triglyceride and phospholipid methyl esters were made individually with sodium hydroxide in methanol (0.5 mol/L).

The fatty acid composition of lipid extracts was determined as described elsewhere.[43] Fatty acid methyl esters were analyzed using a gas-liquid chromatograph (Model 5890, Hewlett-Packard, Avondale, PA) equipped with a 30 m × 0.25 mm id. fused silica capillary column with 25 mm film thickness (SUPELCOWAX 10; Supelco Bellefonte PA). Results, expressed as percent of total fatty acids, were determined by electronic integration (Hewlett-Packard 3380A integrator).

Statistical analyses

Data were subjected to two-way analysis of variance (ANOVA) to test for the effect of dietary fat source and time. When significant differences occurred ($P < 0.05$), individual ANOVA were computed at each time point and treatment mean differences were identified by Fisher's Least Significant Difference (LSD) test. All analyses were conducted using a Macintosh II computer with version 1.03 of StatView II (Abacus Concepts, Inc., Berkeley, CA).

Results

Body, liver and spleen weights

Dietary fat source did not influence the final body weight, spleen weight or splenocyte yield (*Table 3*). After 4 weeks, rats fed the 18% MFO diet, had significantly heavier livers than the 4.5% or 0% MFO-fed rats ($P < 0.05$).

Plasma, liver, and splenocyte α-tocopherol

Plasma α-tocopherol concentration was significantly influenced by fat source ($P < 0.0001$), but not by time over the period of dietary treatment ($P > 0.3$). After 1 week on diet, plasma α-tocopherol concentration decreased as the percentage of MFO increased in the diet. Liver α-tocopherol (*Table 4*) concentration was not different over time or by dietary treatment ($P > 0.05$) although rats fed diets with greater percentages of MFO had numerically lower liver α-tocopherol values.

Splenocytes isolated from rats on day zero of this study contained 17.4 nM α-tocopherol per 10^9 cells. Immune cell α-tocopherol concentration was significantly influenced by time such that α-tocopherol increased with age ($P < 0.001$). Immune cell α-tocopherol was also significantly influenced by dietary fat source (i.e., % of MFO, $P < 0.001$). Specifically, inclusion of 4.5% MFO in the diet was found to numerically increase immune cell α-tocopherol at all time periods, although the increase was only significant after 4 weeks of feeding experimental diets (*Figure 1*). No interaction of the two main effects, time and diet were noted ($P > 0.05$). These main effects are therefore presented separately.

Plasma, liver, and splenocyte FAME

Analysis of plasma fatty acids showed that total PUFA and saturated and monounsaturated fatty acid levels were not significantly affected by diet or time (data not shown). *Figure 2* illustrates the effect of diet on the overall changes in selected fatty acids in the plasma. Arachidonic acid was significantly affected by diet ($P < 0.0001$), but not by time, with a decrease in plasma AA observed as the percentage of MFO in the diet was increased from 0 to 4.5% and again at 9% MFO. Plasma EPA levels were significantly increased over time (data not shown) and as the

Table 4 Plasma and liver α-tocopherol of female rats fed increasing amounts of menhaden fish oil (MFO) substituted for lard (LRD) in a 20% fat diet for 1, 2, or 4 weeks[1–4]

	Dietary treatment groups (% MFO)					
	0.0	4.5	9.0	13.5	18.0	*P* value
μM α-tocopherol/L plasma						
Week 1	$12.2^{a} \pm 0.9$	$8.6^{b} \pm 0.7$	$7.6^{b} \pm 0.6$	$6.4^{bc} \pm 0.3$	$5.5^{c} \pm 0.4$	0.0001
Week 2	$12.6^{a} \pm 1.9$	$9.5^{ab} \pm 1.2$	$7.9^{b} \pm 1.4$	6.5 ± 0.5	$5.7^{b} \pm 0.7$	0.005
Week 4	$12.1^{a} \pm 1.5$	$7.6^{b} \pm 0.7$	$7.5^{b} \pm 1.1$	$5.5^{bc} \pm 0.6$	$4.8^{c} \pm 0.4$	0.0001
nmol α-tocopherol/g liver						
Week 1	115 ± 16.1	102 ± 11.1	80.8 ± 4.0	85.8 ± 14.7	81.4 ± 7.7	ns
Week 2	97.0 ± 9.7	85.4 ± 8.9	73.9 ± 7.8	86.3 ± 10.1	74.0 ± 9.1	ns
Week 4	93.1 ± 19.9	76.5 ± 9.3	81.1 ± 20.8	82.1 ± 24.5	73.7 ± 6.7	ns

[1]Weanling female Sprague-Dawley rats were fed semi-purified diets for 1, 2, or 4 weeks.
[2]Values represent the means ($n = 6$) ± standard error of the mean.
[3]Values with different letters within a row are significantly different at the P value listed within that row, "ns" is not significant ($P > 0.05$).
[4]Initial plasma α-tocopherol was 6.6 ± 0.5 μmol/L plasma, initial liver α-tocopherol was 29.5 ± 8.7 nmol/g.

percentage of fish oil increased in the diet ($P < 0.0001$). There was no significant effect of time on plasma DHA levels, but as the percentage of fish oil in the diet increased, so did plasma DHA. Greater than 75% of the change in these three fatty acids occurred during the first week of feeding.

The inclusion of as little as 4.5% MFO in the diet significantly increased EPA and DHA and decreased AA in the liver triglyceride and phospholipids fractions at all time periods (*Figures 3* and *4*, $P < 0.0001$). In general, greater than 80% of the diet-induced change in AA, EPA, and DHA occurred during the first week, thus data presented represent the overall means for each dietary treatment group, irrespective of length of time on diet. As in plasma, total saturated, polyunsaturated, and monounsaturated fatty acid profiles of liver phospholipids were not affected by dietary treatment (data not shown). In contrast, increasing the percentage of dietary fat coming from fish oil significantly increased total PUFA and decreased total saturated and monounsaturated fatty acids within the liver triglyceride fraction (data not shown).

At the start of the experiment, splenocytes had 18.8, 0.9, and 2.7 mol % AA, EPA, and DHA, respectively. Inclusion of 4.5% fish oil in the 20% fat diet was sufficient to significantly decrease AA and increase EPA in isolated splenocytes (*Figure 5*) with further increase of EPA noted at the 13.5% level of fish oil. DHA was not affected by diet until fish oil replaced three-quarters of the lard (i.e., 13.5% MFO), at which point, it was significantly increased as compared with the diets containing 0 or 4.5% fish oil. Changes in splenocyte AA, EPA, and DHA were found to

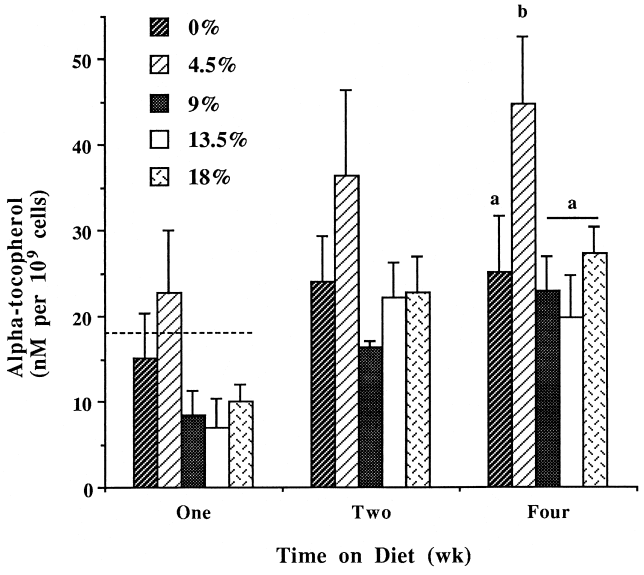

Figure 1 Splenocyte α-tocopherol concentrations from female rats fed different levels of menhaden fish oil in a 20% fat diet for 1, 2, and 4 weeks. Values given are means ± SEM, $n = 2$ to 6 for each group. Values within time 4, not sharing a common superscript letter are significantly different, $P < 0.05$. Dotted lines refer to basal values from rats sacrificed at the beginning of the study. Two-way ANOVA indicate significant effects of both time and diet on α-tocopherol. No interactions were noted.

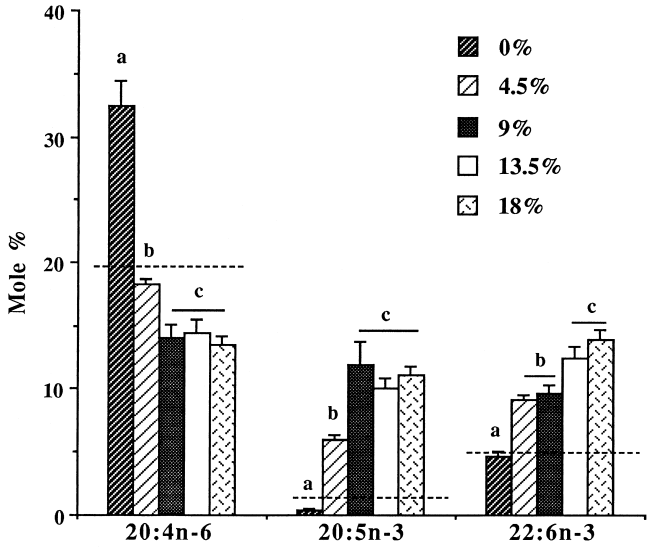

Figure 2 Selected plasma fatty acids from female rats fed different levels of menhaden fish oil in a 20% fat diet. Values given are means ± SEM, $n = 9$ to 12 for each group. Values not sharing a common superscript letter are significantly different, $P < 0.05$. Dotted lines refer to basal values from rats sacrificed at the beginning of the study.

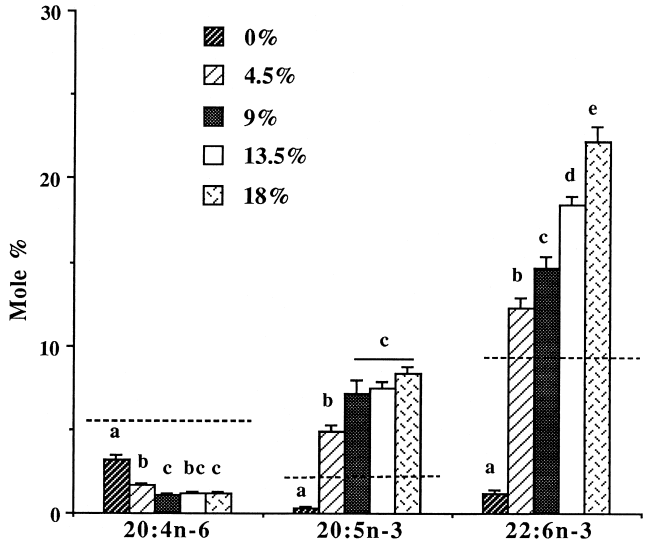

Figure 3 Selected fatty acids from the liver triglyceride fraction of female rats fed different levels of menhaden fish oil in a 20% fat diet. Values given are means ± SEM, $n = 12$ to 15 for each group. Values not sharing a common superscript letter are significantly different from other values, $P < 0.05$. Dotted lines refer to basal values from rats sacrificed at the beginning of the study.

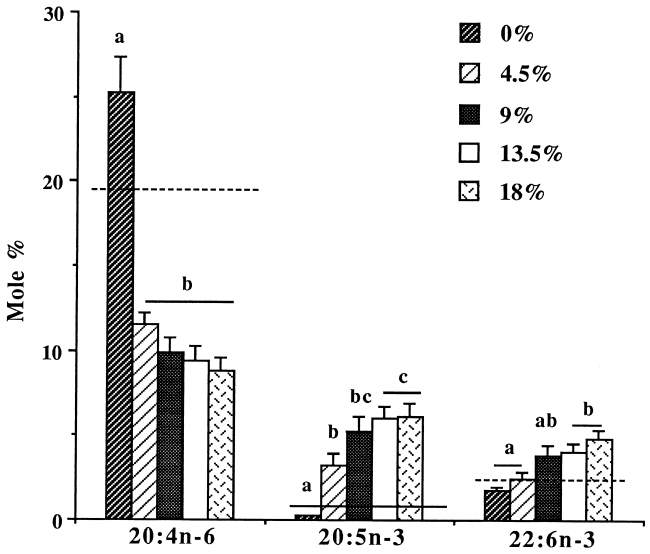

Figure 5 Selected fatty acids from isolated splenocytes of female rats fed different levels of menhaden fish oil in a 20% fat diet. Values given are means ± SEM, $n = 3$ to 5 for each group. Values not sharing a common superscript letter are significantly different, $P < 0.05$. Dotted lines refer to basal values from rats sacrificed at the beginning of the study.

be 85% complete by the end of the first week of feeding. There were no significant differences by time or treatment in the total PUFA, saturated fatty acids or monounsaturated fatty acids of these splenocytes. The basal (n-6)/(n-3) ratio of splenocytes was 6.0. The (n-6)/(n-3) ratio of the LRD diet was 13.6. Inclusion of fish oil in the diet significantly decreased this ratio to 2.9, 2.1, 1.6, and 1.4 for the 4.5%, 9%, 13.5% and 18% fish oil groups, respectively. By the end of 4 weeks of feeding, overall fatty acid composition of splenocytes from each treatment group was found to reflect the individual fatty acid composition of the diet.

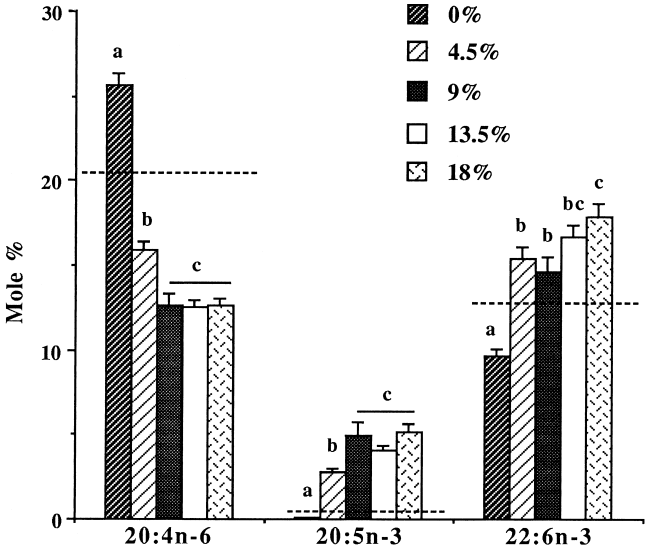

Figure 4 Selected fatty acids from the liver phospholipid fraction of female rats fed different levels of menhaden fish oil in a 20% fat diet. Values given are means ± SEM, $n = 9$ to 12 for each group. Values not sharing a common superscript letter are significantly different $P < 0.05$. At each time point, values not sharing a common superscript letter are significantly different from other values within that time point, $P < 0.05$. Dotted lines refer to basal values from rats sacrificed at the beginning of the study.

Approximately 75% of the changes in plasma fatty acid profile occurred within the first week whereas only 60% of the plasma vitamin E decrease in the 18% MFO diet occurred within that period. During the first week, all other diets either maintained or increased plasma vitamin E, with only the 18% and 13.5% MFO diets having numerically lower plasma vitamin E levels at 4 weeks than did basal animals.

Discussion

Plasma α-tocopherol concentration, a generally accepted measure of vitamin E status in both animals and humans, was significantly reduced by the substitution of menhaden fish oil for lard. As the percentage of fish oil in the diet increased, plasma vitamin E decreased in a dose-dependent manner. These data are in agreement with those of Meydani et al.[19] and others.[16–18] More interesting and novel is our finding that rats fed a modest amount of fish oil (i.e., 4.5%) had significantly higher levels of α-tocopherol in their immune cells (i.e., splenocytes) compared to rats fed lard. However, as the proportion of (n-3) PUFA in the dietary fat mixture was increased by substituting fish oil for lard, splenocyte α-tocopherol levels did not continue to increase. Surprisingly, despite the progressive decline in plasma α-tocopherol concentration as the percentage of fish oil in the diet increased, splenocyte α-tocopherol remained at the same level as that in lard-fed rats.

This is not the first such report of a tissue being enriched in vitamin E when (n-3) fatty acids are present in the diet.

For example, when 15 g/day of fish oil was added to the diet of normal human subjects, α-tocopherol content of erythrocyte membranes was significantly increased although plasma α-tocopherol was reduced.[44,45] Similarly, Chautan et al.[46] examined the effect of changing the dietary (n-6)/(n-3) PUFA ratio on plasma and tissue α-tocopherol concentrations in rats. The authors held the ratio of PUFA/SFA constant as well as total fat and cholesterol content, whereas the (n-6)/(n-3) PUFA ratio was changed from 50 to 5, 2, and 0.8. Although plasma and liver α-tocopherol decreased significantly as the (n-3) PUFA increased, heart tissue was found to be enriched in vitamin E. In a study by Croset et al.,[47] elderly humans were supplemented with a small amount of EPA (200 mg). No reduction in plasma vitamin E was noted, although platelets from EPA supplemented individuals were found to have significantly more α-tocopherol than those of the placebo group.

Our demonstration of tissue enrichment with α-tocopherol in response to (n-3) PUFA, as well as the others reports cited above, are in contrast with those of Meydani et al.[19] who found no significant difference between α-tocopherol content of kidney and lung of fish oil-fed mice compared with those fed coconut or corn oil. Similarly, Javouhey-Donzel et al.[17] did not observe an increase, but rather a significant decrease in rat heart α-tocopherol concentration per gram of wet tissue when diets contained long chain (n-3) fatty acids, but not 18:3 (n-3). However, when heart α-tocopherol was expressed on a lipid basis, significant differences were abrogated. These authors also noted no effect of n-3 PUFA intake on brain, liver, or adipose α-tocopherol concentration.

At this time it is not clear why such discrepancies exist in the literature. Further, it is unclear how dietary n-3 PUFA could lead to certain tissues becoming enriched with α-tocopherol, particularly at a time when circulating α-tocopherol may be declining. Evidence from the literature suggests three plausible mechanisms by which this interaction between dietary (n-3) PUFA and tissue α-tocopherol may occur: membrane stabilization, redistribution of α-tocopherol within lipoproteins, and cellular adaptation to oxidative stress.

This first mechanism is based on the concept that the enrichment of membrane phospholipids with long-chain n-3 PUFA may create a lipid environment suitable for a given cell to retain more α-tocopherol. This hypothesis is consistent with the suggested role of α-tocopherol in the stabilization of membranes.[48] Further support for a specific effect of membrane-bound (n-3) PUFA was provided by Chautan et al.[46] They found a significant, positive correlation between heart α-tocopherol and DHA ($r = 0.86$) as well as the sum of DHA + EPA ($r = 0.84$). One major weakness with this proposed mechanism is it provides no explanation for the tissue selectivity noted by us or others. For example, we noted time- and dose-dependent alterations in plasma, liver and splenocyte fatty acids associated with increasing dietary fish oil that were quite similar across all of these tissues. Yet, it is unclear why only splenocytes showed elevated α-tocopherol. Further, if there is a specific association between (n-3) PUFA and α-tocopherol within the membrane environment, then enrichment of splenocytes with α-tocopherol should have continued to increase as the percentage of fish oil in the diet was increased. This is because our data demonstrated that the concentration of these fatty acids in splenocytes continued to increase in a dose-dependent manner. Finally, in our study we examined the time-dependent nature of the changes in fatty acid composition and α-tocopherol. We found that most of the changes in fatty acid composition occurred within the first week, yet the change in splenocyte α-tocopherol was more gradual. Our observations suggest that if changes in tissue fatty acids are playing an important role in this process, it is probably through a more indirect mechanism.

The second possible explanation for the n-3 PUFA-mediated α-tocopherol enrichment of splenocytes is based on the premise that immune cells obtain their α-tocopherol via the receptor-mediated uptake of LDL. That the distribution of α-tocopherol among the lipoproteins can be significantly altered by dietary PUFA may be relevant to our findings. Carr et al.[49] fed saturated, monounsaturated and PUFA-rich diets to African green monkeys and reported that the number of molecules of α-tocopherol per LDL was significantly increased by the presence of PUFA in the diet. Interestingly, overall plasma vitamin E concentration was not altered. In our study, plasma was enriched with (n-3) PUFA, but the substantial decline in AA, meant that total long-chain PUFA concentration was not increased as the level of fish oil in the diet was increased. The study by Carr et al.[49] did not evaluate (n-3) PUFA nor did it examine whether certain PUFA may have a greater effect on the redistribution of α-tocopherol within various lipoproteins. We propose that it is possible that the inclusion of fish oil altered the fatty acid composition and α-tocopherol content of LDL particles such that uptake of these lipoproteins by immune cells was enhanced. However, it is difficult to explain why further increases in dietary fish oil did not lead to greater enrichment of immune cells. It is possible that other competing effects of (n-3) PUFA on α-tocopherol metabolism should be considered (e.g., reduced absorption, enhanced oxidative loss). Additional studies are required to determine if (n-3) PUFA affect α-tocopherol content of lipoproteins as well as uptake by immune cells.

The possibility that (n-3) PUFA directly affect the ability of immune cells to incorporate α-tocopherol is the third possible mechanism. Demoz and coworkers[50] found that supplementing the diets of mice with purified EPA led to increased levels of cytosolic antioxidant enzyme defenses. Autoimmune-prone mice fed fish oil have also been found to have increased cytosolic antioxidant enzyme levels as well as decreased indices of lipid peroxidation.[51] Such an adaptive response to increased oxidant stress may have an effect on net utilization of vitamin E in specific tissues. A 15 kDa α-tocopherol-binding protein has been identified in the cytosol of the heart and liver.[52] This protein increases the in vitro transfer of α-tocopherol from liposomes to mitochondria. Whether this protein or a similar one plays a role in the differential uptake and retention of α-tocopherol by (n-3) PUFA-enriched immune cells is uncertain at this time. Enhanced expression of a tocopherol-binding protein in response to the enrichment of immune cell membranes with (n-3) fatty acids could help protect these cells from the wide array of free radicals and oxidants (e.g., H_2O_2, superoxide

anion, lipid hydroperoxides) involved in immune and inflammatory responses.

In this study and in previous work[16,35,53] we have demonstrated that although fish oils alter immune cell fatty acid composition, they do not significantly decrease immune cell tocopherol levels. Although moderate levels of dietary fish oil may actually enhance immune cell vitamin E, this observation should not be taken as an attempt to address the issue of vitamin E requirement at the level of the (n-3) PUFA-enriched immune cell. Enrichment of cellular membranes with PUFA has been reported to increase their susceptibility to lipid peroxidation.[54] Long-chain, highly unsaturated fatty acids such as EPA and DHA in fish oil are more easily oxidized than less unsaturated fatty acids [e.g., 18:2 (n-6)].[55] When individual PUFA are compared with each other, the in vitro susceptibility to oxidation has been found to be related to the number of double bonds within the fatty acid. Therefore, rapid enrichment of immune cell membranes with easily oxidized (n-3) PUFA such as is reported in this experiment, could lead to impaired immune function because of increased lipid peroxidation. This would be particularly problematic shortly after introduction of diet, because enrichment of immune cell membranes precedes any observed increase in vitamin E content. The functional requirement of vitamin E at the level of immune cells enriched with (n-3) PUFA remains to be determined.

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