Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats

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Abstract The effect of docosahexaenoic acid (DHA) on mitochondrial and peroxisomal fatty acid oxidation and on key enzymes in triglyceride metabolism was investigated in the liver of rats fed a standard diet, a cholesterol diet, and a pelleted chow diet. Unexpectedly, in all three rat models repeated administration of highly purified DHA (92% pure) at different doses and times, at a dose of 1000 mg/day per kg body weight, resulted in no significant decrease of hepatic and plasma concentration of triglycerides. The serum concentrations of cholesterol and phospholipids showed an increase in a time-dependent manner in rats fed the pelleted chow diet. The hepatic concentration of cholesterol was increased in rats fed the cholesterol diet and pelleted chow diet after administration of DHA compared to palmitic acid. In all rat models, treatment with DHA tended to increase the peroxisomal β-oxidation. This was accompanied with a significant increase (1.5-fold) of fatty acyl-CoA oxidase activity. The mitochondrial fatty acid oxidation system and carnitine palmitoyl-transferase activity, however, were almost unchanged. Moreover, palmitoyl-CoA synthetase activity was increased, whereas the palmitoyl-CoA hydrolase activity was decreased. Neither microsomal phosphatidate phosphohydrolase activity nor cytosolic phosphatidate phosphohydrolase activity was affected by DHA feeding in the three rat models. Acyl-CoA:1,2-diacylglycerol acyltransferase activity was also unaffected. In contrast to docosahexanoic acid feeding, eicosapentaenoic acid (EPA) administration possessed a hypotriglyceridemic effect and resulted in an increase of mitochondrial and peroxisomal oxidation of fatty acids. Carnitine palmitoyltransferase activity was also stimulated. Phosphatidate phosphohydrolase activity was unaffected whereas diacylglycerol acyltransferase activity was increased by EPA treatment compared with palmitic acid feeding. The results indicate that docosahexaenoic acid, in contrast to eicosapentaenoic acid, does not inhibit the synthesis and secretion of triglycerides in the liver. In addition, the results emphasize the importance that stimulation of peroxisomal β-oxidation by these n-3 fatty acids is not sufficient to decrease the serum levels of triglycerides. In addition, increased mitochondrial β-oxidation of fatty acids and thereby decreased availability of nonesterified fatty acids may be a mechanism by which EPA inhibits triglyceride, and subsequently very low density lipoprotein-triglyceride, production. Whether DHA and EPA possess different metabolic properties should be considered.—**Willumsen, N., S. Hexeberg, J. Skorve, M. Lundquist, and R. K. Berge.** Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats. *J. Lipid Res.* 1993. **34:** 13–22.

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Dietary marine fish oils, rich in n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) have triglyceride-lowering effects (1–5). Recently, we have shown that 98% pure EPA is a lipid-lowering fatty acid with both hypotriglyceridemic and hypocholesterolemic properties (6).

The mechanism by which EPA and/or DHA reduce triglyceride level is not fully elucidated. Increased fatty acid oxidation, both mitochondrial and peroxisomal, resulting in a reduced supply of fatty acids for hepatic triglyceride synthesis may be one of several mechanisms (7). Reduced hepatic lipogenesis and retarded VLDL secretion may be other operative mechanisms of dietary n-3 fatty acids (4, 8, 9).

Different results on the effect of n-3 fatty acids on hepatic concentration of triglycerides have been reported (7, 8, 10). The possibility that changes of triglycerides by EPA and DHA might be due to changes of key enzymes involved in triglyceride biosynthesis was therefore investigated.

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PALM, palmitic acid; VLDL, very low density lipoproteins.

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The aim of the present study was to investigate whether dietary pure DHA had triglyceride- and cholesterol-lowering effects and induced stimulation of mitochondrial and peroxisomal fatty acid oxidation in rats fed a standard diet, a cholesterol diet, and a pelleted chow diet. Furthermore, the dietary models were used to investigate the effect of DHA on the rate-limiting enzyme in triglyceride synthesis (phosphatidate phosphohydrolase).

Unexpectedly, we found that DHA has no triglyceride- and cholesterol-lowering effects. EPA, however, is a hypotriglyceridemic agent. The present study supports the concept that the lipid-lowering effect is dissociated from induction of peroxisomal β-oxidation and peroxisome proliferation as peroxisomal oxidation of fatty acids was stimulated with EPA and DHA as well.

Several studies have recently described the effects of fish oil on triglyceride synthesis and secretion in cultured rat liver cells (11, 12) and they suggest that triglyceride synthesis and secretion are not inhibited by docosahexanoic acid as appears to be the case with eicosapentaenoic acid. These studies support our observations that repeated administration of docosahexaenoic acid in all three animal models does not result in a decrease of serum or liver concentration of triglycerides in spite of increased peroxisomal β-oxidation.

MATERIALS AND METHODS

Chemicals

Docosahexaenoic acid (DHA) (purity 92%) was obtained from Norsk Hydro A/S, Research Center, Porsgrunn, Norway. Palmitic acid was from Sigma Chemical Co., St. Louis, MO. All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and treatments

Male Wistar rats from Möllegaard Breeding Laboratory, Ejby, were housed in groups of two and three in bottom-grid metal-wire cages in a room maintained at 12 h light-dark cycles and at a constant temperature of 20 ± 3°C. The animals were acclimatized for at least 1 week under these conditions before the start of the experiments. During this period the rats were fed a standard pelleted diet. In the first animal model, the rats were fed a standard diet (**Table 1**). In the second animal model, the rats were fed a cholesterol diet (2% of cholesterol) that was identical to the standard diet except for addition of cholesterol which substituted for an equal amount of starch (Table 1). In the third animal model, the rats were fed a conventional pelleted chow diet, NoRM1, SDS. All animals had free access to food and water. In the first and second animal models the fatty acids were administered by gastric intubation in a volume of 0.75–1.12 ml once a day for 10 days. The animals were killed on the 11th day after a 12-h fast. The animals were separately treated from low to high dose levels with DHA and palmitic acid; the doses were 500, 1000, and 1500 mg/day per kg body weight. The control groups received only basic diet and water. In the third animal model, where a time study was run, a daily dose of 1000 mg/day per kg body weight of the fatty acids was administered by gastric intubation. Blood was collected at days 0, 3, 6, and 10 by venipuncture of the vena saphena minor. On the 11th day of administration, after a 12-h fast, the animals were killed.

Body weights were measured on the initial and final days. On the 11th day the fasted rats were lightly anesthetized and cardiac puncture was performed to obtain blood samples. The liver was removed immediately, weighed, and then chilled on ice.

The animal experiments were approved by the local ethical committee for animal experiments.

Analytical methods

Livers from individual rats were homogenized in ice-cold sucrose medium (0.25 M sucrose in 10 mM HEPES buffer, pH 7.4, 2 mM EDTA), centrifuged, and the resulting nuclear plus postnuclear fractions were used as the total homogenate.

For further analysis of postnuclear fractions from the livers of rats fed the standard and cholesterol diets, differential centrifugation samples from three animals were pooled, and a mitochondrial-enriched fraction (M), a peroxisome-enriched fraction (L), a microsomal fraction (P), and a cytosolic fraction (S) were isolated (13, 14). The M, L, P, and S fractions from the livers of pelleted chow-fed rats were isolated from five individual rats (no pooling).

TABLE 1. Composition of diets

Diet	Standard Diet	Cholesterol Diet
	g/100 g	
Cholesterol[a]	0	2
Starch	58.65	56.65
Sucrose	11	11
Soya concentrate[b]	19.35	19.35
Soya oil	5	5
Vitamin mix[c]	1	1
Mineral mix[d]	3	3
Cellulose	2	2

[a] Sigma Chemical Company, ash-free, precipitated from alcohol, 95–98%.

[b] Contains 62% protein.

[c] Milligrams/100 g: vitamin A, 80; vitamin D, 20; vitamin E (50%), 600; vitamin K (50%), 1; choline, 10,000; folic acid, 10; niacin, 200; pantothenic acid, 80; ribofiavin, 30; thiamin, 40; pyridoxin, 60; vitamin B_{12} (1%), 50; dextrin, 88,829.

[d] Grams/1000 g: $CaCO_3$, 282.87; $CaHPO_4 \cdot 2H_2O$, 208.64; KH_2PO_4, 409.57; $MgCO_3$, 0.0065; $FeSO_4 \cdot 7H_2O$, 5.69; $MnSO_4 \cdot H_2O$, 5.01; Na_2SeO_3, 0.0066; $ZnSO_4 \cdot 7H_2O$, 1.73.

Protein was assayed by Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

The enzymatic activities of palmitoyl-CoA-dependent dehydrogenase (usually termed peroxisomal β-oxidation) (15), fatty acyl-CoA oxidase (14), carnitine palmitoyltransferase (16), phosphatidate phosphohydrolase (17), acyl-CoA:1,2-diacylglycerol acyltransferase (18), palmitoyl-CoA synthetase (19), and palmitoyl-CoA hydrolase (6, 14) were determined as described.

Lipid analysis was carried out as recommended (the Monotest cholesterol enzymatic kit, Boehringer, Mannheim, Germany and Biopak triglyceride enzymatic kit, Biotrol, Paris, France).

Palmitoyl-CoA and palmitoyl-L-carnitine oxidation

Palmitoyl-CoA and palmitoyl-L-carnitine oxidation were measured as acid-soluble products. The assay medium (0.3 ml) contained 12 mM HEPES buffer (pH 7.3), 11 mM $MgCl_2$, 12 mM dithiothreitol, 5.6 mM ADP, 0.2 mM NAD^+, 0.6 mM EDTA, 60 mM KCl, and antimycin A (1 μg/ml) and 1.0–1.2 mg protein from the isolated mitochondria or postnuclear fractions. Palmitoyl-L-carnitine oxidation was measured with 80 μM [1-^{14}C]palmitoyl-L-carnitine and the palmitoyl-CoA oxidation was measured with 40 μM palmitoyl-CoA supplemented with 1 mM L-carnitine. The cyanide-sensitive fatty acid oxidation was measured in the presence of 1 mM KCN. After incubation for 120–240 sec at 30°C, oxidation was stopped by addition of 25 μl fatty acid-free bovine serum albumin (100 mg/ml) followed by 150 μl 1.5 M KOH and 500 μl 4 M $HClO_4$. After centrifugation a sample of the protein- and fatty acid-free supernatant was assayed for radioactivity.

Plasma free fatty acids were determined by an enzymatic colorimetric method (WACO Nefa C) (20).

Presentation of the results

The variation on the response from animal to animal was estimated separately for selected enzymes in total liver homogenates in the control group and treated animals. Three to five animals in each experimental group and eight controls were used. Data on enzyme activities in total liver homogenates are presented as means ± SD. For isolation of cellular fractions from livers of the first and second rat models, the postnuclear fractions from three animals were pooled. The cellular fractions from the third rat model were isolated separately. The tabulated values on enzymatic activities in cellular fractions of treated rats are given as means. All data were analyzed using Student's *t*-test. $P > 0.05$ was taken to be statistically insignificant.

RESULTS

Body and liver weights

Rats fed the standard diet, the cholesterol diet, and the pelleted chow supplemented with DHA, EPA, and palmitic acid at various doses gained weight at the same rate as controls (**Table 2**), indicating that appetite was not affected and that the acids were well tolerated. Rats on the different diets, whether treated with saturated or polyun-

TABLE 2. Effect of dietary EPA, DHA, and palmitic acid (PALM) on body weight and liver weight and protein content

Diet	N	Fatty Acid Addition	Dose	Body Weight		Weight Gain	Liver Weight	Relative Liver Weight[a]	Protein
				Initial	Final				
			mg/d/kg BW	*g*		*g*	*g*	*%*	*mg/g liver*
Exp. I									
S	8	none	0	197 ± 6	224 ± 17	27 ± 12	6.4 ± 0.6	2.9 ± 0.1	171.7 ± 22.9
S	3	DHA	500	191 ± 3	228 ± 11	37 ± 9	6.3 ± 0.5	2.8 ± 0.1	172.8 ± 6.2
S	3	DHA	1000	196 ± 10	208 ± 15	25 ± 8	6.9 ± 0.6	3.1 ± 0.3	164.2 ± 1.0
S	3	DHA	1500	210 ± 10	111 ± 17	25 ± 5	7.1 ± 0.4	3.0 ± 0.1	165.5 ± 14.0
S	3	PALM	1500	221 ± 6	234 ± 15	26 ± 9	7.1 ± 0.3	3.0 ± 0.1	162.5 ± 6.0
Exp. II									
C	8	none	0	203 ± 7	225 ± 11	22 ± 6	7.5 ± 0.8	3.3 ± 0.4	154.7 ± 16.0
C	3	DHA	500	216 ± 4	243 ± 3	28 ± 6	8.6 ± 0.4	3.5 ± 0.1	152.2 ± 5.0
C	3	DHA	1000	218 ± 12	254 ± 5	36 ± 7	8.2 ± 1.2	3.2 ± 0.5	153.2 ± 2.2
C	3	DHA	1500	209 ± 2	232 ± 1	23 ± 3	7.2 ± 0.2	3.1 ± 0.1	159.5 ± 9.6
C	3	PALM	1500	230 ± 10	249 ± 14	19 ± 4	8.1 ± 0.6	3.2 ± 0.1	150.5 ± 5.6
Exp. III									
P	5	PALM	1000	91 ± 5	144 ± 11	33 ± 8	4.8 ± 0.8	3.3 ± 0.3	148.3 ± 8.3
P	5	DHA	1000	94 ± 7	142 ± 8	52 ± 10	5.0 ± 0.8	3.5 ± 0.6	157.1 ± 5.0
P	5	EPA	1000	90 ± 6	149 ± 4	58 ± 3	4.6 ± 0.3	3.1 ± 0.2	160.2 ± 7.4

N, number of animals; BW, body weight; S, standard diet; C, cholesterol diet; P, pelleted, normal diet. The values are expressed as means ± SD.
[a]Relative liver weight: g liver/g body weight × 100.

saturated fatty acids, appeared healthy and looked and behaved normal. No hepatomegaly resulted after DHA, EPA, and palmitic acid feeding and no significant changes of the hepatic protein content were observed in animals fed any of the diets (Table 2).

Serum and liver lipids

The lipid-lowering effects of DHA were investigated in the three rat models. **Table 3** shows that DHA at different doses did not promote a decrease in serum triglycerides, total cholesterol, HDL-cholesterol, or phospholipids in the three rat models. Administration of DHA to pelleted chow-treated rats resulted in a time-dependent increase of the plasma cholesterol and phospholipids, whereas the concentration of triglycerides was unchanged (**Fig. 1**). A similar tendency of increased total cholesterol level in serum was also observed in rats fed a cholesterol diet after DHA administration (Table 3). In keeping with previous findings (13), serum triglycerides, cholesterol, and phospholipids were not significantly lowered after administration of palmitic acid (Table 3). In contrast, repeated administration of EPA resulted in both hypocholesterolemic and hypotriglyceridemic effects (Table 3), confirming earlier findings (6). The triglyceride-lowering effect was observed after 3 days of treatment whereas the cholesterol-lowering effect resulted after 10 days of EPA administration (Fig. 1).

Table 4 shows that the concentrations of liver triglycerides and cholesterol in the cholesterol diet group were significantly higher than those of rats fed the standard diet, whereas no significant changes of liver phospholipids between the two experimental groups were observed. Repeated administration of DHA did not result in decreased liver concentrations of triglycerides in the three rat models, whereas the hepatic levels of cholesterol tended to increase in rats fed the cholesterol diet and the pelleted chow. The hepatic phospholipid concentration was marginally affected by DHA exposure in the three dietary models (Table 4).

Fatty acid oxidation

Mitochondrial β-oxidation was determined by measuring acid-soluble products with palmitoyl-L-carnitine and palmitoyl-CoA as substrates. DHA exposure of the standard diet-treated rats at a dose of 1000 mg/day per kg body weight tended to increase the mitochondrial β-oxidation with both palmitoyl-L-carnitine and palmitoyl-CoA as substrates when compared to the control group (**Table 5**). However, at that dose the mitochondrial fatty acid oxidation was not induced in the cholesterol-treated group. Moreover, at a dose of 1,500 mg DHA/day per kg body weight, the mitochondrial β-oxidation was marginally affected in the standard diet-treated group, and a decreased activity was observed compared to the rats fed palmitic acid at an equal dose (Table 5). Likewise, oxidation of palmitoyl-L-carnitine in the cholesterol diet group tended to be lower compared to the standard diet group. However, this was not obtained with palmitoyl-CoA as

TABLE 3. Effect of dietary DHA, EPA, and palmitic acid (PALM) on serum lipids

Diet	N	Fatty Acid Addition	Days	Dose	Serum Lipids: Triglycerides	Total Cholesterol	HDL-Cholesterol	Phospholipids
				mg/d/kg BW	*mmol/l*			
Exp. I								
S	8	none		0	0.54 ± 0.18	1.11 ± 0.26	0.82 ± 0.33	1.01 ± 0.16
S	3	DHA		500	0.47 ± 0.19	1.05 ± 0.06	0.77 ± 0.18	0.92 ± 0.12
S	3	DHA		1000	0.63 ± 0.16	1.18 ± 0.18	0.62 ± 0.15	1.00 ± 0.20
S	3	DHA		1500	0.57 ± 0.13	1.16 ± 0.19	0.78 ± 0.09	0.93 ± 0.13
S	3	PALM		1500	0.72 ± 0.04	1.14 ± 0.18	0.80 ± 0.11	1.03 ± 0.12
Exp. II								
C	8	none		0	0.37 ± 0.14	0.93 ± 0.22	0.59 ± 0.23	0.69 ± 0.11
C	3	DHA		500	0.70 ± 0.30	1.30 ± 0.33	0.61 ± 0.12	0.89 ± 0.14
C	3	DHA		1000	0.66 ± 0.19	1.42 ± 0.30	0.52 ± 0.04	0.85 ± 0.15
C	3	DHA		1500	0.55 ± 0.11	1.68 ± 0.30[b]	0.54 ± 0.25	0.82 ± 0.24
C	3	PALM		1500	0.48 ± 0.14	1.34 ± 0.27	0.70 ± 0.17	0.92 ± 0.15
Exp. III								
P	5	PALM	10	1000	1.36 ± 0.25	3.22 ± 0.33	0.78 ± 0.07	2.63 ± 0.15
P	5	DHA	10	1000	1.31 ± 0.32	2.97 ± 0.25	0.52 ± 0.14	2.98 ± 0.28
P	5	EPA	10	1000	0.85 ± 0.10[a]	2.53 ± 0.13[a]	0.80 ± 0.67	2.30 ± 0.05[a]

N, number of animals; BW, body weight; S, standard diet; C, cholesterol diet; P, pelleted normal diet. The values are expressed as means ± SD.
[a] $P < 0.05$ between palmitic acid and EPA.
[b] $P < 0.05$ between control and DHA.

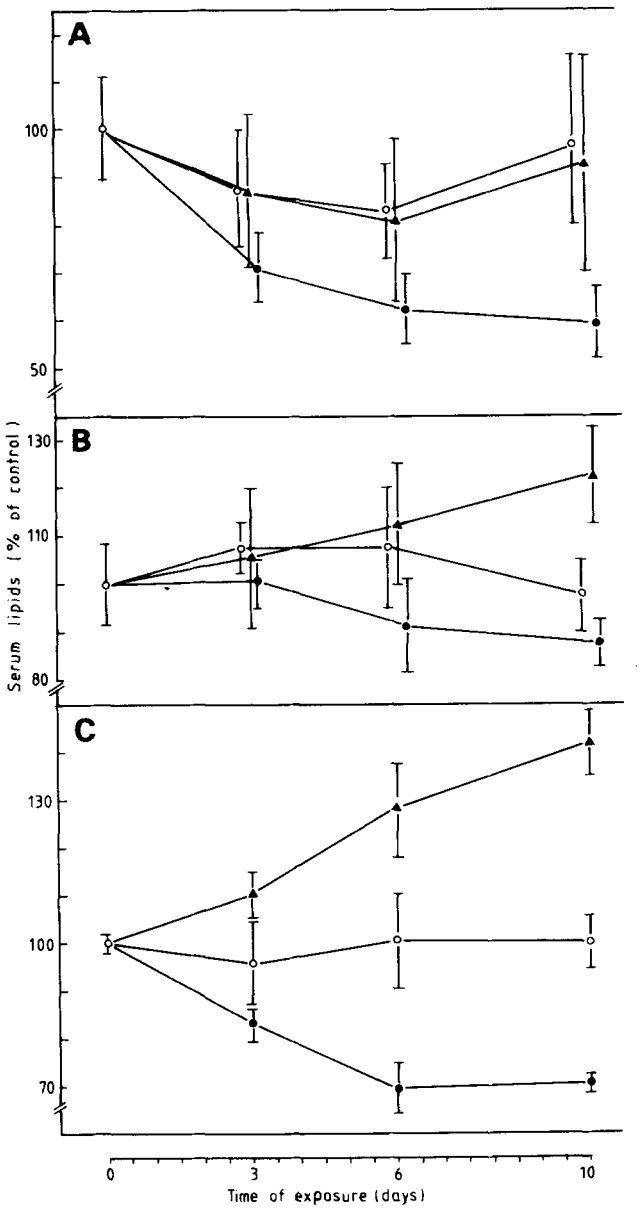

Fig. 1. Time course of changes in serum triglycerides (A), serum cholesterol (B), and serum phospholipids (C) in rats administered palmitic acid (○), docosahexaenoic acid (▲), and eicosapentaenoic acid (●). Serum lipids of the experimental groups (n = 5) are presented relative to those of control animals = 100% (n = 15). In control animals the serum triglycerides, cholesterol, and phospholipids were 1.41 ± 0.16 mmol/l, 2.70 ± 0.23 mmol/l, and 2.67 ± 0.22 mmol/l, respectively.

substrate (Table 5). In order to obtain more conclusive results regarding mitochondrial β-oxidation in DHA-treated rats, a new experiment was set up where the number of animals in each group was extended from three to five. As EPA is reported to increase the mitochondrial β-oxidation (6) and has hypotriglyceridemic effects (Table 3), administration of EPA was also included in the new experiment as a positive control. In keeping with previous findings (unpublished data) EPA significantly increased the mitochondrial β-oxidation in comparison with palmitic acid treatment (**Table 6**). With palmitoyl-CoA as substrate, a 1.5-fold increase was observed (Table 6). Furthermore, the carnitine palmitoyltransferase activity was significantly increased after repeated administration of EPA in the pelleted chow-treated rats (Table 6).

As shown in Table 6, mitochondrial fatty acid oxidation was not enhanced with DHA exposure. Likewise, there was not a significant increase in the activity of carnitine palmitoyltransferase after DHA administration.

In the presence of KCN, the palmitoyl-CoA oxidation tended to increase after administration of DHA (Table 5), reflecting an increased peroxisomal β-oxidation. The dose-response data show that DHA tended to increase the peroxisomal β-oxidation and fatty acyl-CoA oxidase activity in the two first animal models (S and C) (**Table 7**). Repeated administration of DHA and EPA significantly increased the peroxisomal β-oxidation and fatty acyl-CoA oxidase activity in the peroxisome-enriched fractions from pelleted chow-treated rats. Addition of cholesterol does not seem to inhibit the peroxisomal β-oxidation as the peroxisomal enzyme activities for the group fed the cholesterol diet were not significantly different from the group fed the standard diet.

Key enzymes involved in synthesis of triglycerides

As the availability of substrates along with an inhibition of key enzymes in triglyceride biosynthesis may contribute to changes of hepatic lipid formation, we examined whether phosphatidate phosphohydrolase activities and acyl-CoA:1,2-diacylglycerol acyltransferase were changed after feeding DHA and EPA. **Table 8** shows that no effect on phosphatidate phosphohydrolase activity was observed in pelleted chow-treated animals. A similar tendency was observed in the two other rat models. EPA led to 80% greater ($P < 0.05$) diacylglycerol acyltransferase activity whereas the enzyme activity was unaffected in DHA-treated rats compared to those fed palmitic acid (Table 6). Repeated administration of the hypolipidemic 3-thia fatty acid analogue revealed a strong correlation between the rate of phosphatidate hydrolysis and the plasma triglyceride concentration (21). However, no translocation of the enzyme activity was observed (21).

Formation of esterified fatty acids is catalyzed by long-chain acyl-CoA synthetase (palmitoyl-CoA synthetase). Table 8 shows that DHA stimulated the palmitoyl-CoA synthetase activity in pelleted chow-treated rats. Repeated administration of DHA decreased the palmitoyl-CoA hydrolase activity in the standard diet group and the pelleted chow-treated animals (Table 8).

TABLE 4. Effects of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and palmitic acid (PALM) on hepatic lipids

Diet	N	Fatty Acid Addition	Dose	Hepatic Lipids: Triglycerides	Hepatic Lipids: Cholesterol	Hepatic Lipids: Phospholipids
			mg/d/kg BW		*μmol/g liver*	
Exp. I						
S	8	none	0	25.2 ± 7.0	5.8 ± 1.7	19.6 ± 1.9
S	3	DHA	500	23.2 ± 12.8	6.1 ± 1.6	21.4 ± 1.0
S	3	DHA	1000	35.3 ± 4.3	9.7 ± 1.9	22.1 ± 1.4
S	3	DHA	1500	24.0 ± 9.5	5.9 ± 1.6	20.4 ± 2.2
S	3	PALM	1500	43.3 ± 17.6	6.6 ± 1.9	19.6 ± 2.3
Exp. II						
C	8	none	0	54.5 ± 12.1[b]	22.1 ± 3.3[b]	20.3 ± 5.1
C	3	DHA	500	73.9 ± 3.2	31.9 ± 4.1[a]	20.6 ± 4.0
C	3	DHA	1000	62.6 ± 20.2	38.2 ± 4.0[a]	22.7 ± 1.5
C	3	DHA	1500	50.8 ± 7.7	41.2 ± 2.6[a]	24.7 ± 3.8
C	3	PALM	1500	85.4 ± 10.6	36.1 ± 4.8	22.0 ± 4.9
Exp. III						
P	5	PALM	1000	5.33 ± 1.20	1.92 ± 0.53	17.40 ± 0.80
P	5	DHA	1000	6.00 ± 1.20	3.54 ± 0.43[a]	19.08 ± 0.80
P	5	EPA	1000	6.57 ± 0.96	3.85 ± 1.08[a]	18.18 ± 1.20

N, number of animals; BW, body weight: S, standard diet; C, cholesterol diet; P, pelleted normal diet. Values represent means ± SD.

[a] $P < 0.05$ between control and treated rats.

[b] $P < 0.05$ between standard diet and cholesterol diet.

TABLE 5. Effect on dietary DHA and palmitic acid on mitochondrial β-oxidation

Diet	N	Fatty Acid	Dose	Mitochondrial β-oxidation in E-Fraction[a]: Palmitoyl-Carnitine	Mitochondrial β-oxidation in E-Fraction[a]: Palmitoyl-CoA	Mitochondrial β-oxidation in E-Fraction[a]: Palmitoyl-CoA + CN	Mitochondrial β-Oxidation in M-Fraction[b]: Palmitoyl-Carnitine	Mitochondrial β-Oxidation in M-Fraction[b]: Palmitoyl-CoA
			mg/d/kg BW					
S	8	none		230.8 ± 17.2 (1.57 ± 0.03)	112.6 ± 16.8 (0.77 ± 0.07)	8.4 ± 0.4	129.3	42.2
S	3	DHA	500	346.1 ± 12.9 (1.42 ± 0.10)	142.3 ± 17.8 (0.82 ± 0.14)	9.6 ± 0.8	139.3	46.4
S	3	DHA	1000	296.5 ± 14.5 (1.81 ± 0.10)	189.3 ± 16.3 (1.15 ± 0.06)	11.3 ± 1.1	154.5	47.9
S	3	DHA	1500	182.3 ± 37.3 (1.19 ± 0.20)	144.1 ± 13.4 (0.87 ± 0.10)	12.4 ± 0.6	99.6	41.1
S	3	PALM	1500	225.7 ± 21.3 (1.39 ± 0.14)	145.3 ± 19.2 (0.83 ± 0.13)	8.1 ± 0.3	126.4	40.3
C	8	none	0	123.1 ± 27.8 (0.89 ± 0.21)	120.1 ± 17.8 (0.89 ± 0.16)	8.2 ± 0.3	95.2	34.5
C	3	DHA	500	183.4 ± 10.3 (1.20 ± 0.05)	133.0 ± 14.8 (0.82 ± 0.13)	9.4 ± 0.1	103.2	21.4
C	3	DHA	1000	155.9 ± 14.3 (0.98 ± 0.10)	147.6 ± 21.5 (0.96 ± 0.16)	11.7 ± 0.6	105.4	24.0
C	3	DHA	1500	221.8 ± 23.6 (1.40 ± 0.27)	162.3 ± 9.5 (1.02 ± 0.04)	12.6 ± 1.2	102.9	32.7
C	3	PALM	1500	125.8 ± 30.4 (0.84 ± 0.26)	129.4 ± 15.4 (0.57 ± 0.20)	8.0 ± 0.3	85.2	25.8

N, number of animals; BW, body weight; S, standard diet; C, cholesterol diet; E-fraction, post-nuclear fraction; M-fraction, isolated mitochondrial fraction from pooled samples of three rats.

[a] The tabulated values represent means ± SD. The activities of mitochondrial β-oxidation are expressed as nmol/min per g liver and the specific activities (nmol/min per mg protein) are given in parentheses.

[b] The tabulated values represent the means in isolated mitochondrial fraction obtained from pooled post-nuclear fraction of three rats. The activity of mitochondrial β-oxidation is expressed as nmol/min per mitochondrial protein per g liver.

TABLE 6. Effect of dietary DHA, EPA, and palmitic acid on mitochondrial β-oxidation, carnitine palmitoyltransferase activity, and diacylglycerol acyltransferase activity

Diet	N	Fatty Acid	Dose	Mitochondrial β-Oxidation[a]		Carnitine Palmitoyl Transferase Activity[b]	Diacylglycerol Acyltransferase[c]
				Palmitoyl-Carnitine	Palmitoyl-CoA		
			mg/d/kg BW				
P	5	PALM	1000	250.2 ± 16.4 (116.1 ± 4.9)	141.6 ± 12.4 (49.1 ± 2.2)	617 ± 64	0.27 ± 0.04
P	5	DHA	1000	258.0 ± 18.3 (137.1 ± 18.9)	152.1 ± 6.7 (50.0 ± 1.6)	755 ± 84	0.36 ± 0.09
P	5	EPA	1000	295.4 ± 10.1[d] (137.8 ± 3.3)[d]	217.5 ± 13.2[d] (56.7 ± 4.6)[d]	933 ± 138[d]	0.49 ± 0.08[d]

N, number of animals; BW, body weight; P, pelleted normal diet. Values represent means ± SD.
[a]The activity of mitochondrial β-oxidation was measured in total homogenate (E-fraction) and expressed as nmol/min per g liver. The values in parentheses give the mitochondrial fatty acid oxidation in isolated mitochondrial fraction and are expressed as nmol/min mitochondrial protein per g liver.
[b]Carnitine palmitoyltransferase activity was measured in the mitochondrial fraction (M) and expressed as nmol/min per mitochondrial protein per g liver.
[c]Diacylglycerol acyltransferase activity was measured in the microsomal fraction and expressed as nmol/min per mg protein.
[d]$P < 0.05$ between control and treated rats.

DISCUSSION

Experimental data from dietary sulfur-substituted fatty acid analogues have strongly suggested that the minimal structural requirement for peroxisome proliferation may be a carboxylic acid group linked to a hydrophobic backbone with poor susceptibility to β-oxidation (22). The stimulation of peroxisomal β-oxidation with EPA and DHA, but not with palmitic acid, conforms to the previously defined requirement for initiation of peroxisome proliferation. Production of liver peroxisomes accompanied by stimulated peroxisomal β-oxidation and fatty acyl-CoA oxidase activity is observed after feeding certain high fat diets, especially diets rich in C_{20}–C_{22} fatty acids which are relatively poorly oxidized by the mitochondrial β-oxidation (23). The present study shows for the first time that administration of highly purified DHA stimulates peroxisomal β-oxidation. It is possible that induction of peroxisomal β-oxidation by a high fat diet (14, 19, 23) is due to its content of DHA and/or EPA, i.e., fatty acids that are poorly oxidized by mitochondria and that seem to be dependent on peroxisomes for efficient chain shortening (23).

It has been suggested that the hypoglyceridemic effect of pure EPA results from reduced supply of fatty acids for hepatic triglyceride synthesis because of increased fatty

TABLE 7. Effect of dietary DHA and palmitic acid on peroxisomal β-oxidation and acyl-CoA oxidase

Diet	N	Fatty Acid	Dose	Peroxisomal β-Oxidation		Fatty Acid CoA-Oxidase	
				E-Fraction	L-Fraction	E-Fraction	L-Fraction
			mg/kg/d	*nmol/min/mg protein*			
S	8	none	0	3.5 ± 0.6	8.3	9.6 ± 1.0	15.6
S	3	DHA	500	4.3 ± 0.6	10.8	13.4 ± 1.8[a]	27.4
S	3	DHA	1000	5.0 ± 0.2[a]	14.1	15.9 ± 1.7[a]	34.8
S	3	DHA	1500	4.9 ± 0.5[a]	12.7	15.2 ± 0.1[a]	29.7
S	3	PALM	1500	3.6 ± 0.7	8.1	11.1 ± 1.6	21.8
C	8	none	0	3.1 ± 0.3	9.4	9.7 ± 1.6	21.0
C	3	DHA	500	3.9 ± 0.5	8.7	12.1 ± 1.5	18.5
C	3	DHA	1000	4.5 ± 0.3[a]	11.2	13.6 ± 0.5[a]	24.5
C	3	DHA	1500	5.0 ± 1.0[a]	11.0	15.6 ± 3.9[a]	25.7
C	3	PALM	1500	3.2 ± 0.2	6.2	10.9 ± 1.7	17.8
P	5	PALM	1000	ND	4.8 ± 0.7	8.4 ± 1.2	15.1 ± 3.4
P	5	DHA	1000	ND	8.9 ± 0.4[a]	13.2 ± 4.0[a]	24.0 ± 1.2[a]
P	5	EPA	1000	ND	9.3 ± 1.8[a]	13.6 ± 4.0[a]	28.8 ± 5.4[a]

N, number of animals; S, standard diet; C, cholesterol diet; P, pelleted diet; ND, not determined. Values represent means or means ± SD. Activity of peroxisomal β-oxidation and fatty acyl-CoA oxidase was measured in the post-nuclear fraction (E) and in the peroxisome-enriched fraction (L).
[a]$P < 0.05$ between control and treated rats.

TABLE 8. Effect of dietary DHA and palmitic acid on hepatic enzyme activity

Diet	N	Fatty Acid		Phosphatidate Phosphohydrolase P-Fraction	Phosphatidate Phosphohydrolase S-Fraction	Palmitoyl-CoA Hydrolase E-Fraction	Palmitoyl-CoA Synthetase E-Fraction
				nmol/min/mg protein		*nmol/min/mg protein*	
S	8	none	0	9.61	2.30	51.2 ± 0.5	44.3 ± 6.7
S	3	DHA	500	9.60	1.55	47.7 ± 4.1	38.6 ± 1.8
S	3	DHA	1000	9.95	1.56	45.2 ± 6.0	43.6 ± 2.1
S	3	DHA	1500	9.90	1.62	43.7 ± 2.8[a]	44.7 ± 7.0
S	3	PALM	1500	9.75	1.63	47.8 ± 3.7	38.8 ± 3.6
C	8	none	0	9.21	1.33	47.2 ± 3.5	43.6 ± 6.7
C	3	DHA	500	8.62	1.40	48.8 ± 2.9	40.9 ± 6.1
C	3	DHA	1000	7.60	1.36	48.0 ± 8.1	47.2 ± 8.4
C	3	DHA	1500	9.21	1.55	47.4 ± 4.0	41.6 ± 5.0
C	3	PALM	1500	8.01	1.59	46.3 ± 3.4	46.1 ± 5.9
P	5	PALM		10.23 ± 0.76	5.41 ± 0.70	40.5 ± 2.5	42.9 ± 2.3
P	5	DHA		10.86 ± 0.61	5.33 ± 0.49	36.6 ± 1.4[a]	53.4 ± 6.0[a]
P	5	EPA		11.58 ± 0.60	6.02 ± 0.38	32.6 ± 1.6[a]	46.1 ± 1.4

N, number of animals; S, standard diet; C, cholesterol diet; P, pelleted diet. Values represent means or means ± SD. The activity of phosphatidate phosphohydrolase was measured in the microsomal-enriched fraction (P) and in the cytosolic-enriched fraction (S). Palmitoyl-CoA hydrolase activity and palmitoyl-CoA synthetase activity were measured in the post-nuclear fraction (E).

[a] $P < 0.05$ between control and treated rats.

acid oxidation (6). Thus, it is important to determine whether increases in fatty acid oxidation are a result of an increase in mitochondrial and/or peroxisomal oxidation. This study demonstrates that at a hypotriglyceridemic dose, EPA is shown to increase the mitochondrial fatty acid oxidation system along with stimulation of carnitine palmitoyltransferase (Table 6). The findings that increased peroxisomal β-oxidation and fatty acyl-CoA oxidase activity was observed with both EPA and DHA (Table 7) and that DHA has no lipid-lowering effect (Fig. 1) indicate that EPA as a peroxisome proliferator might not be crucial for the hypolipidemic effect observed. It should also be emphasized that mitochondria are the quantitatively dominating organelles in liver cells compared with peroxisomes, implying that a 1.5-fold increase in mitochondria β-oxidation might have greater impact in the total β-oxidation of fatty acids than a 1.5-fold increase in peroxisomal β-oxidation.

Thus, increased mitochondrial fatty acid oxidation, *and not peroxisomal β-oxidation, emphasizes the importance* of substrate availability as a determinant of rate of triglyceride biosynthesis and VLDL output. As stimulation of peroxisomal fatty acid oxidation by DHA was not sufficient to decrease plasma levels of triglycerides, the data indicate that hypotriglyceridemia could be dissociated from induction of peroxisomal β-oxidation, which confirms previous findings (13).

In the present study no significant effect on the peroxisomal β-oxidation and fatty acyl-CoA oxidase activity was found by addition of cholesterol in the diet. This is in contrast to the findings of Hayashi, Hashimoto, and Nakata (24) who showed that addition of cholesterol to the diet increased peroxisomal β-oxidation 1.4-fold.

Different results of the effect of n-3 fatty acids on hepatic concentration of triglycerides have been reported (7, 8, 10). In some studies treatment with marine oils containing n-3 fatty acids reduces the hepatic triglyceride content (8). In others, however, such a reduction cannot be demonstrated (7, 10). In HepG2 cells, Wong, Fisher, and Marsh (25) observed that EPA was inhibitory to triglyceride synthesis, but that DHA was not. In another study, Zhang et al. (11) presented results suggesting that triglyceride synthesis and secretion were not inhibited by DHA, as appears to be the case with EPA. In the present study dietary highly purified DHA did not decrease the hepatic concentration of triglycerides in the three rat models (Table 4). These contradictory results are probably due to dissimilarity in the experimental models used and to possible metabolic differences between EPA and DHA.

Phosphatidate phosphohydrolase and acyl-CoA:1,2-diacylglycerol acyltransferase may have considerable potential for regulation of triglycerides as they catalyze the key intermediate and last esterification step of glycerolipid synthesis. The lack of effect of dietary DHA on diacylglycerol acyltransferase activity and the stimulatory effect observed with EPA (Table 6) suggest that this is not the site at which n-3 fatty acids modulate triglyceride synthesis. This finding is in agreement with data obtained after feeding fish oil (26) and saturated 3-thia fatty acids (27). Rustan et al. (18), however, have observed that EPA inhibited this enzyme activity in cultured hepatocytes.

Phosphatidate phosphohydrolase is a key enzyme in regulation of triglyceride synthesis (28). The activity occurs in the cytosol and microsomes and it has been sug-

gested that stimulation of the enzyme activity may be attributed to a translocation of the cytosol enzyme to microsomes (28). Repeated administration of the hypolipidemic 3-thia fatty acid analogue revealed a strong correlation between the rate of phosphatidate hydrolysis and the plasma triglyceride concentration (21). However, no translocation of the enzyme activity was observed (21). In the present study, the microsomal as well as the cytosolic phosphatidate phosphohydrolase activities were not found to be affected by DHA and EPA, suggesting that this is not the site at which n-3 fatty acids modulate triglyceride synthesis (Table 8). By way of contrast, previous studies by Wong and Marsh (12) and Marsh, Topping, and Nestel (26) have shown an inhibition of phosphatidate phosphohydrolase after administration of high fat diets which, they suggest, could explain the decreased triglyceride synthesis.

In summary, administration of highly purified DHA in three animal models did not produce hypotriglyceridemic or hypocholesterolemic effects. In addition, a significant induction of mitochondrial and peroxisomal fatty acid oxidation was shown, both in EPA- and DHA-treated animals. The results emphasize the importance of the fact that stimulation of peroxisomal fatty acid oxidation by DHA and EPA is not sufficient to decrease the serum levels of triglycerides. EPA, however, has a triglyceride-lowering effect. The mitochondrial fatty acid oxidation was stimulated by EPA treatment whereas DHA exposure was without effect. These results suggest that increased mitochondrial oxidation of fatty acids and thereby decreased availability of nonesterified fatty acids may be a mechanism by which EPA inhibits triglyceride, and subsequently VLDL-triglyceride, production. Moreover, the present study strongly supports the assumption that EPA and DHA possess different metabolic properties. Both in vivo and in vitro studies to further explore the different mechanisms of highly purified DHA and EPA on lipids and key enzymes in the lipid metabolism are needed.

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