Eicosapentaenoic Acid, But Not Docosahexaenoic Acid, Increases Mitochondrial Fatty Acid Oxidation and Upregulates 2,4-Dienoyl-CoA Reductase Gene Expression in Rats

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ABSTRACT: The aim of the present study was to investigate whether eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) was responsible for the triglyceride-lowering effect of fish oil. In rats fed a single dose of EPA as ethyl ester (EPA-EE), the plasma concentration of triglycerides was decreased at 8 h after acute administration. This was accompanied by an increased hepatic fatty acid oxidation and mitochondrial 2,4-dienoyl-CoA reductase activity. The steady-state level of 2,4-dienoyl-CoA reductase mRNA increased in parallel with the enzyme activity. An increased hepatic long-chain acyl-CoA content, but a reduced amount of hepatic malonyl-CoA, was obtained at 8 h after acute EPA-EE treatment. On EPA-EE supplementation, both EPA (20:5n-3) and docosapentaenoic acid (DPA, 22:5n-3) increased in the liver, whereas the hepatic DHA (22:6n-3) concentration was unchanged. On DHA-EE supplementation retroconversion to EPA occurred. No statistically significant differences were found, however, for mitochondrial enzyme activities, malonyl-CoA, long-chain acyl-CoA, plasma lipid levels, and the amount of cellular fatty acids between DHA-EE treated rats and their controls at any time point studied. In cultured rat hepatocytes, the oxidation of [1-^{14}C]palmitic acid was reduced by DHA, whereas it was stimulated by EPA. In the *in vivo* studies, the activities of phosphatidate phosphohydrolase and acetyl-CoA carboxylase were unaffected after acute EPA-EE and DHA-EE administration, but the fatty acyl-CoA oxidase, the rate-limiting enzyme in peroxisomal fatty acid oxidation, was increased after feeding these n-3 fatty acids. The hypocholesterolemic properties of EPA-EE may be due to decreased 3-hydroxy-3-methylglutaryl–CoA reductase activity. Furthermore, replacement of the ordinary fatty acids, i.e., the monoenes (16:1n-7, 18:1n-7, and 18:1n-9) with EPA and some conversion to DPA concomitant with increased fatty acid oxidation is probably the mechanism leading to changed fatty acid composition. In contrast, DHA does not stimulate fatty acid oxidation and, consequently, no such displacement mechanism operates. In conclusion, we have obtained evidence that EPA, and not DHA, is the fatty acid primarily responsible for the triglyceride-lowering effect of fish oil in rats.
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Administration of marine fish oil rich in n-3 polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), decreased plasma triglycerides by 25–35% in normolipidemic subjects (1,2) and in rats (3,4). Their greatest effect on plasma triglycerides is under conditions of overproduction when lipogenesis is increased (5,6). There is considerable evidence that increased dietary intakes of n-3 fatty acids reduce hepatic synthesis of very low density lipoproteins (VLDL) triglycerides in humans (5,7,8), as well as in various animal species and in HepG2 cells (9–11). In cultured rat hepatocytes, n-3 fatty acids also have been reported to impair assembly and/or secretion of VLDL (12,13). Moreover, it has been reported that changes in *de novo* fatty acid synthesis is integral to the adaptation to n-3 fatty acids. Short-term experiments with hepatocyte suspensions and primary cultures (14–16) indicate that EPA and DHA are either poor substrates for triglyceride synthesis or that these fatty acids acutely affect the triglyceride synthetic pathway. Inhibition of hepatic VLDL secretion appeared to be secondary to the effects on triglyceride synthesis in those studies. Furthermore, Otto *et al.* (13) reported a positive relationship between fasting plasma insulin and plasma triglycerides and suggested that insulin contributes to the regulation of plasma triglycerides by fish oil.

In addition to the effect on VLDL, many other pathways of hepatic fatty acid metabolism may be affected by fish oil and contribute to the triglyceride-lowering effect. Such mech-

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Abbreviations: ADGAT, acyl-CoA:1.2-diacylglycerol acyltransferase; CMC, carboxymethylcellulose; DHA, docosahexaenoic acid; DHA-EE, docosahexaenoic acid as ethyl ester; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; EPA-EE, eicosapentaenoic acid as ethyl ester; FFA, free fatty acids; GPAT, glycerol-3-phosphate acyltransferase; HMG, hydroxy-3-methylglutaryl; LCFA, long-chain fatty acids; Palm, palmitic acid; PAP, phosphatidate phosphohydrolase; VLCFA, very long-chain fatty acids; VLDL, very low density lipoprotein.

anisms include suppression of phosphatidate phosphohydrolase (PAP) (17), the enzyme catalyzing the esterification of phosphatidic acid to diglycerides, and acyl-CoA:1.2-diacylglycerolacyltransferase (ADGAT) (14), which mediates the next step. In animals fed high fish oil diets, lowered circulating triglycerides have been associated with increased peroxisomal β-oxidation (3,18) and ketone body formation (19), indicating that increased peroxisomal fatty acid oxidation may be responsible for the hypotriglyceridemic effect. Moreover, Surette *et al.* (20) have shown an association between circulating triglyceride levels and mitochondrial fatty acid oxidation after n-3 polyunsaturated fatty acid feeding. However, it is not known whether these changes in liver enzyme activities and fatty acid oxidation are related to the effect of EPA or DHA or both.

Nearly all studies that have been conducted have used a mixture of the two components of fish oil attracting the most attention, namely EPA and DHA. In our previous reports from feeding experiments in rats administered highly purified EPA and DHA as ethyl esters (EPA-EE and DHA-EE, respectively) for 10–15 d (21,22), EPA, and not DHA, appeared to be responsible for the triglyceride-lowering effect observed. Feeding rats highly purified EPA ethyl ester lowered plasma triglycerides within 1–2 d of treatment, and stimulation of mitochondrial β-oxidation was observed as a primary event (21). We also showed that mitochondrial β-oxidation was stimulated in cultured hepatocytes in the presence of EPA (21). In contrast, administration of highly purified DHA ethyl ester in three animal models produced neither hypotriglyceridemic nor hypocholesterolemic effects, and no effect on mitochondrial β-oxidation was observed, whereas peroxisomal β-oxidation was stimulated after both EPA and DHA feeding (21,22). These results confirm and extend previous findings in normo- and hypertriglyceridemic rats (23,24), perfused rat livers (25), and humans (26), but are in contrast to other reports (27,28).

It is well documented that fish oil feeding decreases plasma cholesterol in rats (29,30), whereas in human studies there is no consistent cholesterol-lowering effect after fish oil feeding (31). The mechanism behind the hypocholesterolemic effect of n-3 fatty acids in rats is not known in detail, but it has been reported that the cholesterol-lowering effect is accompanied by a reduction of the 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase activity (29).

A controversy exists concerning the efficacy of highly concentrated n-3 fatty acids in the form of alkyl esters compared to less concentrated natural triglycerides (32–34). During prolonged feeding, ethyl esters of n-3 fatty acids are highly bioavailable (35,36), whereas the markedly slower hydrolyzation by pancreatic lipase theoretically could influence the absorption of EPA-EE and DHA-EE in short-term studies. In this study we had the opportunity to address the question of whether EPA or DHA as ethyl esters executes the biological effects independent of differences in the absorption.

To precisely determine the biological role of fish oil constituents (n-3 fatty acids), it is imperative that experiments with highly purified n-3 polyunsaturated fatty acids are executed. We agree with Ikeda *et al.* (23) that determining the effects of dietary EPA and DHA on hepatic fatty acid and triglyceride synthesis and β-oxidation seems to be important to clarifying the precise mechanism(s). Therefore we studied the acute effects of a single dose of highly purified EPA-EE and DHA-EE on plasma lipids and on key enzymes in lipid metabolism, focusing on mitochondrial and peroxisomal β-oxidation, to contribute to the understanding of the triglyceride-lowering mechanism of EPA and/or DHA.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Palmitic acid (56 mCi/mmol), L-[U-^{14}C]-glycerol-3-phosphate (171 mCi/mmol), [^{14}C]acetyl-CoA (60 mCi/mmol), and $NaH^{14}CO_3$ (0.1 mCi/mmol) were purchased from the Radiochemical Center (Amersham, England). EPA-EE (purity 95%) and DHA-EE (purity 93%) were obtained from Norsk Hydro AS, Research Center (Porsgrunn, Norway). Palmitic acid (Palm) was from Sigma Chemical Co., (St. Louis, MO). All other chemicals were obtained from common commercial sources and were of reagent grade.

Care of animals. One hundred fifty male Mol:WIST rats from Möllegaard Breeding Laboratory (Ejby, Denmark), weighing approximately 170 g, were housed in pairs in bottom-grid metal wire cages in a room with a controlled temperature (20 ± 3°C), humidity and air changes, and with a 12-h light/dark cycle (light 0700–1900 h). The animals were acclimatized for one week under these conditions before the start of the experiment. All animals had free access to water and food. The rats were fed a conventional pelleted chow diet, RM1 expanded diet (Special Diet Services, Whitam, United Kingdom).

EPA-EE, DHA-EE, and Palm were suspended in 0.5% sodium carboxymethylcellulose (CMC) and 0.5% α-tocopherol. A single dose of 2000 mg/kg body weight was administered by gavage in volumes of 0.8–1.0 mL. In order to provide an equal amount of energy, the control animals received Palm. There were no significant differences in food consumption or weight gain between the experimental groups.

At the time of sacrifice (2, 4, 8, 12, and 24 h after administration), rats were anesthetized with 0.6 mL Hypnorm Dormicum (fluanisone-fentanyl-midazolam) injected subcutaneously in the neck. Anesthesia depth was evaluated by means of the interdigital and palpebral reflexes. The anesthesia was sufficiently deep when there was no response to either of the reflexes (usually within 2–3 min). None of the animals responded to initial abdominal opening. Cardiac puncture was performed, blood was collected into a syringe containing 0.25 mL of 0.2 M EDTA (pH 7.4), and plasma was collected by centrifugation at 1000 × *g* for 10 min at 4°C. The animal experiments were approved by the Norwegian State Board of Biological Experiments with Living Animals.

Preparation of subcellular fractions. The livers were removed, weighed, and parts were immediately chilled on ice and the other parts freeze-clamped in liquid N_2 and stored at

–80°C. Portions of the livers from individual rats were homogenized in ice-cold sucrose-medium (0.25 M sucrose in 10 mM HEPES buffer, pH 7.4, and 1 mM EDTA). Mitochondrial, peroxisomal, microsomal, and cytoplasmic fractions were prepared as described earlier (37). The fractions were stored at –80°C.

Enzyme assays. PAP was determined by measuring the release of free phosphate in the presence of 5 mM $MgCl_2$ (38). Glycerol-3-phosphate acyltransferase (GPAT) was assayed for 3 min at 30°C, as essentially described earlier (39). The reaction was started by the addition of mitochondria or microsomes (200 μg protein), and terminated by the addition of 2.0 mL of 1-*n*-butanol. The incorporation of ^{14}C into butanol-soluble products was determined as described (39,40). ADGAT was assayed for 5 min at 37°C. The reaction was initiated by the addition of microsomes (30–40 μg protein), and terminated by the addition of 7.5 mL of 2-propanol/hexane/water (89:20:16). The extraction of triglycerides into hexane was performed as previously described (41). HMG–CoA reductase activity was measured in the isolated microsomal fraction essentially as previously described (42).

Fatty acyl-CoA oxidase activity (43) and mitochondrial β-oxidation, using radiolabeled palmitoyl-L-carnitine and L-carnitine in the presence of palmitoyl-CoA (43), were determined as described earlier. Acetyl-CoA carboxylase activity was measured in the cytosolic fraction as fixation of $^{14}CO_2$ from $NaH^{14}CO_3$ into malonyl-CoA essentially as described earlier (44). 2,4-Dienoyl-CoA reductase activity was measured in the mitochondrial fraction, and the incubation contained 50 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.01% (vol/vol) Triton X-100 (Sigma Chemical Co.), 100 μM NADPH, and 100 μg protein. The assay was started by the addition of 100 μM 2-*trans*, 4-*cis*-decadienoyl-CoA and run for 10 min at 30°C at 340 nm (45).

Determination of malonyl-CoA and long-chain acyl-CoA. Malonyl-CoA and free CoASH levels were quantitated by a modification of the high-performance liquid chromatography method originally described by Corkey *et al.* and Demoz *et al.* (46,47). Briefly, 0.1 g liver was homogenized in 1 mL 5% sulfosalicylic acid containing 50 mM dithioerythritiol. The sample was centrifuged at 600 × *g* for 10 min at 0°C. Twenty μL of the acid-soluble extracts were injected into a Spectra Physics (San Jose, CA) SP 8800 HPLC system. Absorbance measurements were made at 254 nm using a 3 mm Hypersil C18 reverse-phase column (HiChrom Ltd., Reading, Berkshire, England). The mobile phase was 0.1 M NaH_2PO_4, pH 4.9 (buffer A) and buffer B (a mixture of buffer A and methanol in a ratio of 7:3), pH 4.9. The flow rate was 2.0 mL/min. The profile of the elution was as follows: 0–10 min: 10–40% B; 10.1–17.6 min: 40–90% B. The retention times were 9.3 and 13.7 for free CoA and malonyl-CoA, respectively. Total hepatic long-chain acyl-CoA was estimated by incubating liver tissue precipitates with 1.5 M KOH at 55°C for 1 h. The liberated free CoASH found in the supernatant was determined as described previously. Individual working standards were prepared by dissolving malonyl-CoA or free CoASH, for determination of malonyl-CoA or long-chain acyl-CoA, respectively, in 5% sulfosalicylic acid containing 50 μM of dithioerythritol. They were then mixed to give a final concentration of 100 μM. Standard curves of the different CoA-esters were obtained at 25, 50, 100, and 200 mM concentrations and showed linear conditions ($r^2 > 0.999$; Ref. 47). The recovery obtained by the method was approximately 90%.

Lipid analysis. Protein was assayed by a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Lipid analyses were carried out by the Monotest cholesterol enzymatic kit (Boehringer, Mannheim, Germany), the Monotest phospholipid enzymatic kit (Boehringer), and the Biopak Triglyceride enzymatic kit (Biotrol, Paris, France). Hepatic lipids were quantified after extraction from total liver homogenates (48). Plasma free fatty acids were determined by an enzymatic colorimetric method (WACO NefaC; Waco Chemicals, Neuss, Germany) (49,50).

Total fatty acid determination. Total lipids were extracted from liver as described by Lie *et al.* (51). The lipid fractions were evaporated, saponified, 19:0 was added as internal standard, and the fatty acids esterified in 12% BF_3 in methanol. The methyl esters were separated using a Carlo Erba (Milan, Italy) 2900 gas-chromatograph ("cold on column" injection, starting at 60 and increasing at 49°C/min to 160, increasing at 1°C/min to 190, increasing at 4°C/min to 220°C), equipped with a 50 m CP-Sil 88 (Chrompack) fused silica capillary column (0.32-mm i.d.). The fatty acid composition was calculated using a Maxima 820 Chromatography Workstation software, installed in an IBM-AT, connected to the gas–liquid chromatograph and identification ascertained by standard mixtures of methyl esters (Nu-Chek-Prep, Elysian, MN). Certified reference material (CRM 162, CRM 163) from Community Bureau of Reference (BCR, Brussels, Belgium) was used.

Preparation of cultured hepatocytes. Hepatocytes were prepared as described earlier (52). In short, the rats were anesthetized as described here, and hepatocytes were isolated by collagenase perfusion, washed and plated in Dulbecco's modified Eagle's medium containing HEPES (20 mM), Ultroser G (2%), and gentamicin (50 mL) at a density of 3×10^6 cells/dish or culture flask (1×10^6 cells/mL). The culture medium was replaced after 6–7 h (2 mL/dish, medium as before). The cells were incubated at 37°C in an atmosphere of air containing 5% CO_2.

Fatty acid oxidation. Fatty acid oxidation to acid-soluble products and CO_2 (to determine the rate of β-oxidation) was measured as described earlier (53). Two mL Dulbecco's modified Eagle's medium containing HEPES (20 mM), gentamicin [1-^{14}C] Palm (0.5 μCi/mL, 200 μM), and 500 μM L-carnitine hydrochloride was added to the cultured hepatocytes, and the hepatocytes were incubated for 4 and 9 h in the presence of increasing concentrations of EPA and DHA (see Fig. 1).

The culture flasks contained a center well (Kontes, Vineland, NJ) and a folded filter paper and were closed with stopper tops (Kontes). After incubation, 300 μL of phenylethylamine/methanol (1:1, vol/vol) was added to the center

well and 300 mL of $HClO_4$ (1 M) to the cell through the stopper top by means of a syringe, and the flasks were further incubated for 1 h at room temperature to trap all $^{14}CO_2$. After incubation, the well was cut off, and radioactivity was measured by liquid scintillation counting.

Preparation of hybridization probes. DNA fragments were labelled by random priming using the oligolabelling technique of Feinberg and Vogelstein (54), resulting in specific activities ranging from 0.8 to 5 × 10^9 cpm/mg DNA. The DNA probes were purified fragments of cloned rat genes: (i) 2,4-dienoyl-CoA reductase: 632 bp Pst I/Eco RI insert in pGem-4Z and (ii) 28 S rRNA: 1.4 kb Bam HI fragment of pA (Dr. I.L. Gonzales, personal communication).

RNA purification and analysis. Total cellular RNA was isolated by the guanidinium-thiocyanate method described by Chomczynski and Sacchi (55). The RNA concentrations were determined spectrophotometrically. Slot blotting of RNA onto nylon was carried out as described by Aasland *et al.* (56). Hybridization to immobilized RNA was performed as described by Sambrook *et al.* (57), in the presence of 50% formamide, 5 × saline–sodium–citrate, 200 μg/mL heat denatured herring-sperm DNA, 0.1% SDS, 25 mM sodium phosphate pH 6.5, 8.25% dextran sulfate at 42°C for 24 to 48 h. Filters were washed to high stringency (0.2 × saline–sodium–citrate, 0.1% NaPPi, 0.1% SDS at 65°C) and exposed on Kodak XAR-5 X-ray films in the presence of intensifying screens at −80°C. Densitometric scanning of autoradiograms was performed using the LKB Ultrogel laser-densitometer (Bromma, Sweden), or the hybridized membranes were analyzed by Instant Imager, Electronic Audioradiography (Packard, Downer's Grove, IL). When the filters were to be rehybridized, the bound probe was first stripped off in 0.1% SDS at 90–100°C for 7 min. The hybridization results were normalized to the signal of 28 S rRNA hybridization in the individual samples. Relative mRNA induction of the different genes was then calculated.

Statistical analysis. The *in vivo* results are means ± SD of duplicate or triplicate measurements on six animals of each experimental group. Results from cultured rat hepatocytes are presented as means ± SD of at least three culture dishes/flasks of hepatocytes from two or more animals.

The effects of EPA and DHA treatment compared to controls were tested by a one-way analysis of variance. Fisher's Least Square Difference was used to determine the significance of difference between the mean at 95% confidence interval using a statistical software StatView SE + Graphics on an Apple Macintosh. $P < 0.05$ was taken to be statistically significant.

RESULTS

Effect of n-3 fatty acids on oxidation of [1-^{14}C]Palm in cultured rat hepatocytes. In agreement with earlier findings (21), oxidation of [1-^{14}C]Palm was significantly higher in cells grown with L-carnitine than in cells grown without L-carnitine (data not shown). Oxidation of Palm to carbon dioxide and acid-soluble products was significantly stimulated in cultured hepatocytes in the presence of EPA, whereas it was significantly reduced in the presence of DHA (Fig. 1A). Oleic acid had no effect on Palm oxidation (data not shown). A significant difference in Palm oxidation by EPA and DHA was already observed after 4 h of incubation (Fig. 1B).

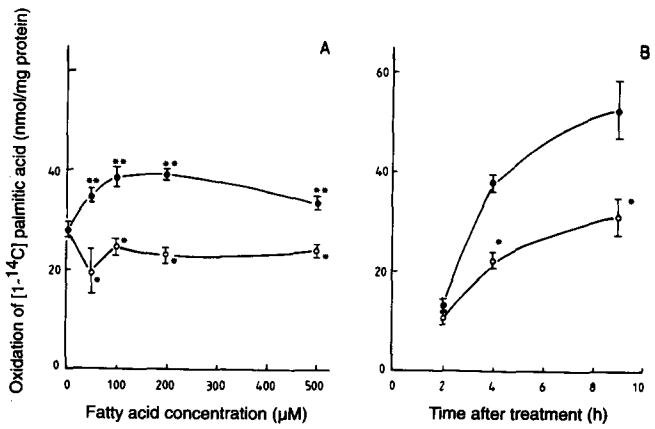

FIG. 1. Effect of eicosapentaenoic acid (EPA) (●) and docosahexaenoic acid (DHA) (○) on oxidation of [1-^{14}C]palmitic acid (0.2 mM) in cells plated and incubated with increasing concentrations (A) and for different times (B). A, cultured hepatocytes were incubated for 4 h; B, 200 μM EPA and DHA was used. In all experiments the fatty acid/bovine serum albumin ratio was 2.5:1, and 0.5 mM L-carnitine was added to the medium. Acid-soluble radioactivity was determined as described in the Materials and Methods section. Results are given as means ± SD for values obtained from three independent experiments. Asterisks correspond to the statistical comparison between values from EPA and DHA and values from DHA and controls (*) and values from EPA and controls (**).

Acute treatment. As shown in Table 1 plasma triglycerides, cholesterol and phospholipids were not changed at any of the time points studied in the different control groups. As shown in Figure 2, plasma triglycerides and cholesterol were reduced 8 h after the acute treatment with EPA-EE as compared to controls. Restoration to control levels occurred at 12 and 24 h. Plasma triglycerides and cholesterol were unaffected by DHA-EE treatment (Fig. 2). Figure 3A shows that plasma phospholipids were reduced 8 h after acute treatment with EPA-EE as compared to controls and DHA-EE administration. The plasma free fatty acid (FFA) concentration in the control and DHA-EE treated rats was significantly reduced at 4 h compared to 2 h, and this reduced level was maintained up to 12 h after feeding. The FFA concentration in the EPA-EE treated rats maintained high until 8 h time point, when it was reduced to the same level as the other two groups (Fig. 3B). No statistically significant difference was found either for hepatic triglycerides, cholesterol, phospholipids (Table 2), or for activities of PAP, acetyl-CoA carboxylase (Table 2), and GPAT (data not shown) between EPA-EE and DHA-EE treated rats and their controls at any time of the time points studied, except that PAP was reduced 8 and 12 h after acute treatment with DHA-EE as compared to controls. However, EPA-EE and DHA-EE feeding caused increased fatty acyl-CoA oxidase activity at 12 h compared to controls (Table 3).

TABLE 1
Plasma Lipids for Three Control Groups, Receiving Either Pellet Diet, Carboxymethylcellulose, or Palmitic Acid[a]

Number of animals	Time (h)	Palmitic acid			Carboxymethylcellulose			Pellet diet		
		TG	CHOL	PL	TG	CHOL	PL	TG	CHOL	PL
6	2	1.18 ± 0.57	1.45 ± 0.08	1.64 ± 0.16	1.37 ± 0.45	1.60 ± 0.31	1.66 ± 0.10	1.27 ± 0.29	1.42 ± 0.27	1.69 ± 0.12
6	4	1.16 ± 0.61	1.59 ± 0.21	1.78 ± 0.16	1.37 ± 0.60	1.45 ± 0.29	1.67 ± 0.06	1.59 ± 0.30	1.31 ± 0.28	1.59 ± 0.27
6	8	1.54 ± 0.40	1.53 ± 0.13	1.68 ± 0.15	1.10 ± 0.57	1.59 ± 0.19	1.65 ± 0.24	1.46 ± 0.04	1.54 ± 0.04	1.86 ± 0.07
6	12	1.41 ± 0.45	1.72 ± 0.28	1.85 ± 0.21	0.96 ± 0.15	1.64 ± 0.22	1.75 ± 0.11	1.37 ± 0.41	1.62 ± 0.36	1.90 ± 0.36
6	24	1.62 ± 0.25	1.61 ± 0.09	1.81 ± 0.09	1.55 ± 0.36	1.56 ± 0.21	1.68 ± 0.07	1.03 ± 0.23	1.73 ± 0.03	1.69 ± 0.17

[a]TG, triglycerides; CHOL, cholesterol; PL, phospholipids. Plasma concentrations of lipids are given in nmol/L and were analyzed as described in the Materials and Methods section. The values are expressed as means ± SD. There were no statistical differences between the groups evaluated by one-way analysis of variance and Fisher's Protected Least Square Differences used to determine differences between means at 95% confidence interval.

As shown in Table 3, the 2,4-dienoyl-CoA reductase activity was not changed at any of the time points studied in the control group, and no statistically significant difference was found for the activity between DHA-EE treated rats and their controls. As also shown in Table 3, the reductase activity was increased 4 and 12 h after acute treatment with EPA-EE as compared to controls and DHA-EE administration.

Figure 4A summarizes the activities of fatty acid oxidation in mitochondrial fraction in treated rats and controls at different times after acute treatment. Oxidation of fatty acids in the mitochondrial fraction, using palmitoyl-L-carnitine as substrate, was significantly increased 8 h after acute treatment with EPA-EE as compared to controls, and this difference disappeared at 12 and 24 h (Fig. 4A). The oxidation of fatty acids in mitochondrial fraction was decreased 12 and 24 h after acute treatment with DHA-EE as compared to controls

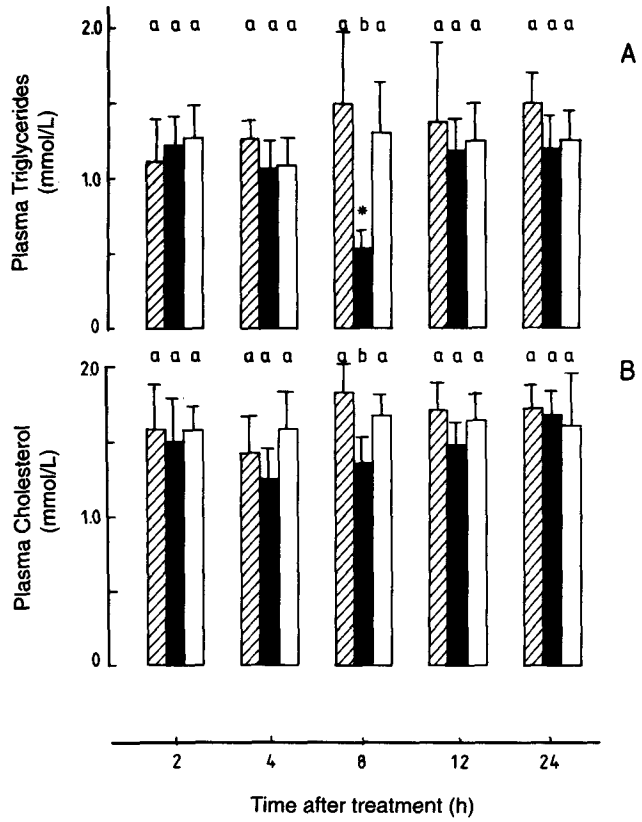

FIG. 2. Concentrations of plasma triglycerides (A) and cholesterol (B) at different times after a single oral administration of EPA as ethyl esters (EPA-EE) (black columns) and DHA as ethyl esters (DHA-EE) (open columns) in the rat and of controls (hatched columns). The results are presented as means ± SD of six rats per group. *Significantly different from each of the time points studied ($P < 0.05$). Means in a column with different superscripts are significantly different ($P < 0.05$) determined by one-way analysis of variance, Fisher's test. Abbreviations as in Figure 1.

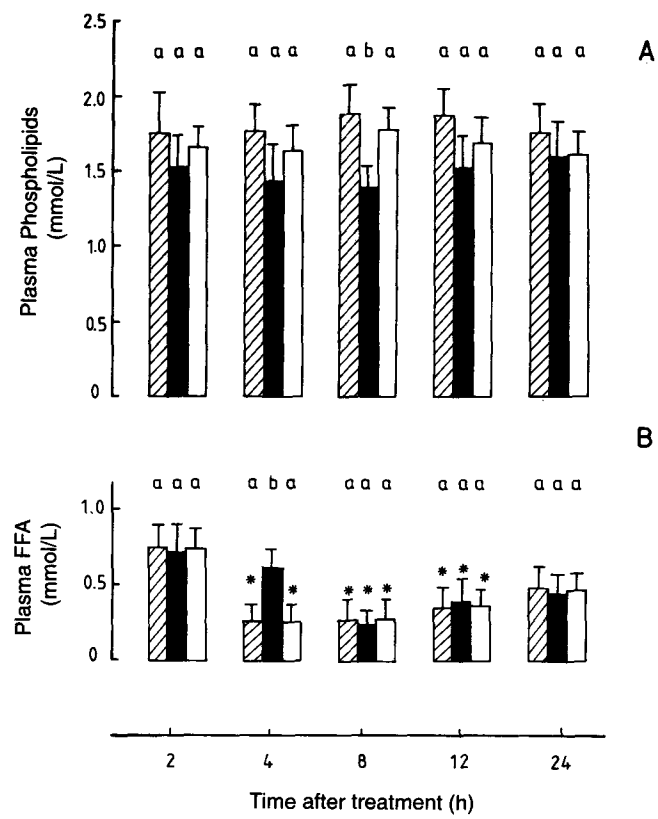

FIG. 3. Concentrations of plasma phospholipids (A) and free fatty acids (FFA) (B) at different times after a single oral administration of EPA-EE (black columns) and DHA-EE (open columns) in the rat and of controls (hatched columns). The results are presented as means ± SD of six rats per group. *Significantly different from values at two hours of individual treatment ($P < 0.05$). Means in a column with different superscripts are significantly different ($P < 0.05$) determined by one-way analysis of variance, Fisher's test. Abbreviations as in Figure 2.

TABLE 2
Liver Lipids and Activities of Phosphatidate Phosphohydrolase (PAP) and Acetyl-CoA Carboxylase at Different Times After Acute EPA-EE and DHA-EE Administration in the Rat[a]

	Hours	EPA-EE	*P*	Control	*P*	DHA-EE
Triglycerides	2	3.54 ± 0.62[a]	n.s.	3.17 ± 0.52[a]	n.s	2.89 ± 0.70[a]
(nmol/g liver)	4	3.34 ± 0.81[a]	n.s.	3.14 ± 0.63[a]	n.s.	3.06 ± 0.64[a]
	8	3.18 ± 0.56[a]	n.s.	2.90 ± 0.63[a]	n.s.	3.59 ± 0.36[a]
	12	2.86 ± 0.74[a]	n.s.	2.81 ± 0.46[a]	n.s.	3.84 ± 0.52[a]
	24	2.89 ± 0.69[a]	n.s.	3.19 ± 0.84[a]	n.s.	4.03 ± 0.93[a]
Cholesterol	2	1.40 ± 0.21[a]	n.s.	1.46 ± 0.21[a]	n.s.	1.18 ± 0.34[a]
(nmol/g liver)	4	1.22 ± 0.32[a]	n.s.	1.36 ± 0.12[a]	n.s.	1.04 ± 0.26[a]
	8	1.65 ± 0.32[a]	n.s.	1.45 ± 0.22[a]	n.s.	1.53 ± 0.42[a]
	12	1.95 ± 0.36[a]	n.s.	1.94 ± 0.21[a]	n.s.	1.78 ± 0.41[a]
	24	1.89 ± 0.21[a]	n.s.	1.92 ± 0.34[a]	n.s.	1.75 ± 0.42[a]
Phospholipids	2	12.66 ± 1.60[a]	n.s.	11.21 ± 0.57[a]	n.s.	10.99 ± 1.10[a]
(nmol/g liver)	4	10.71 ± 0.68[a]	n.s.	10.64 ± 1.80[a]	n.s.	10.26 ± 0.58[a]
	8	11.87 ± 0.93[a]	n.s.	11.48 ± 1.10[a]	n.s.	10.86 ± 1.70[a]
	12	9.54 ± 1.90[a]	n.s.	9.52 ± 1.60[a]	n.s.	11.10 ± 0.46[a]
	24	9.48 ± 2.10[a]	n.s.	9.95 ± 1.68[a]	n.s.	10.18 ± 0.63[a]
PAP	2	11.8 ± 1.2[a]	n.s.	12.8 ± 1.7[a]	n.s.	14.2 ± 1.8[a]
activity	4	12.4 ± 1.1[a]	n.s.	11.9 ± 2.0[a]	n.s.	11.5 ± 0.7[a]
(nmol/min/mg	8	11.5 ± 1.4[a]	n.s.	11.8 ± 0.6	<0.05	9.5 ± 1.2[b]
protein)	12	10.0 ± 1.3[a]	n.s.	11.3 ± 0.6[a]	<0.05	7.9 ± 0.5[b]
	24	9.2 ± 1.6[a]	n.s.	10.3 ± 1.3[a]	n.s.	9.4 ± 0.9[b]
Acetyl-CoA	2	21.4 ± 2.5[a]	n.s.	24.3 ± 2.6[a]	n.s.	28.4 ± 2.5[a]
carboxylase	4	27.3 ± 2.6[a]	n.s.	28.4 ± 1.1[a]	n.s.	23.8 ± 5.2[a]
activity	8	22.6 ± 2.1[a]	n.s.	28.7 ± 3.4[a]	n.s.	27.6 ± 2.7[a]
(nmol/min/mg	12	25.8 ± 2.3[a]	n.s.	26.9 ± 4.6[a]	n.s.	30.4 ± 2.3[a]
protein)	24	20.9 ± 1.6[a]	n.s.	26.9 ± 3.1[a]	n.s.	25.1 ± 5.1[a]

[a]PAP activity was measured in the isolated microsomal fraction, whereas the acetyl-CoA carboxylase was determined in the cytosol. Statistical comparison between eicosapentaenoic acid as ethyl ester (EPA-EE), docosahexaenoic acid as ethyl ester (DHA-EE), and controls are shown by the *P* values; n.s., not significant. [a,b]Correspond to the statistical comparison between the groups at each of the time points studied (hours), where the same letter within the same parameter means that there is no statistical difference between the corresponding groups evaluated by one-way analysis of variance and Fisher's Protected Least Square Difference used to determine differences between means at 95% confidence interval.

(Fig. 4A). Thus, statistically significant difference was found for mitochondrial β-oxidation between the EPA-EE and DHA-EE treated rats from 8 to 24 h after receiving the diets, using palmitoyl-L-carnitine (Fig. 4A) and palmitoyl-CoA as substrates (data not shown). When rats were treated with EPA-EE, the 2,4-dienoyl-CoA reductase mRNA concentration in-

TABLE 3
Fatty Acyl-CoA Oxidase Activity and 2,4-Dienoyl-CoA Reductase Activity at Different Times After Acute EPA-EE and DHA-EE Administration in the Rat[a]

	Hours	EPA-EE	*P*	Controls	*P*	DHA-EE
Fatty acyl-CoA	2	13.3 ± 2.4[a]	n.s.	15.0 ± 3.6[a]	n.s.	19.0 ± 1.4[a]
oxidase activity	4	17.9 ± 1.6[a]	n.s.	14.6 ± 3.0[a]	n.s.	17.7 ± 2.9[a]
(nmol/min/mg	8	17.7 ± 2.3[a]	n.s.	13.6 ± 2.0[a]	n.s.	16.6 ± 2.4[a]
protein)	12	19.4 ± 2.1[b]	<0.05	14.7 ± 2.3[a]	<0.05	19.1 ± 1.3[a]
	24	16.8 ± 1.8[a]	n.s.	13.1 ± 2.6[a]	n.s.	15.4 ± 2.7[a]
2,4-Dienoyl-CoA	2	1.28 ± 0.18[a]	n.s.	1.30 ± 0.16[a]	n.s.	1.65 ± 0.24[a]
reductase activity	4	1.91 ± 0.31[a]	<0.05	1.23 ± 0.28[a]	n.s.	1.26 ± 0.36[a,b]
(nmol/min/mg	8	2.02 ± 0.26[b]	<0.05	1.07 ± 0.33[a]	n.s.	0.88 ± 0.14[b]
protein)	12	1.65 ± 0.10[b]	<0.05	0.94 ± 0.17[a]	<0.05	0.94 ± 0.18[b]
	24	1.18 ± 0.07[a]	n.s.	1.05 ± 0.14[a]	n.s.	1.17 ± 0.15[b]

[a]The acyl-CoA oxidase activity was determined in the isolated peroxisome-enriched fraction, whereas the 2.4-dienoyl-CoA reductase activity was measured in the isolated mitochondrial fraction. The values are expressed as means ± SD of six rats per group. Statistical comparison between EPA-EE, DHA-EE, and controls are shown by the *P* values. [a,b]Correspond to the statistical comparison between the groups at each of the time points studied (hours), where the same letter within one enzyme activity means that there is no statistical differences between the corresponding groups ($P < 0.05$). Abbreviations as in Table 2.

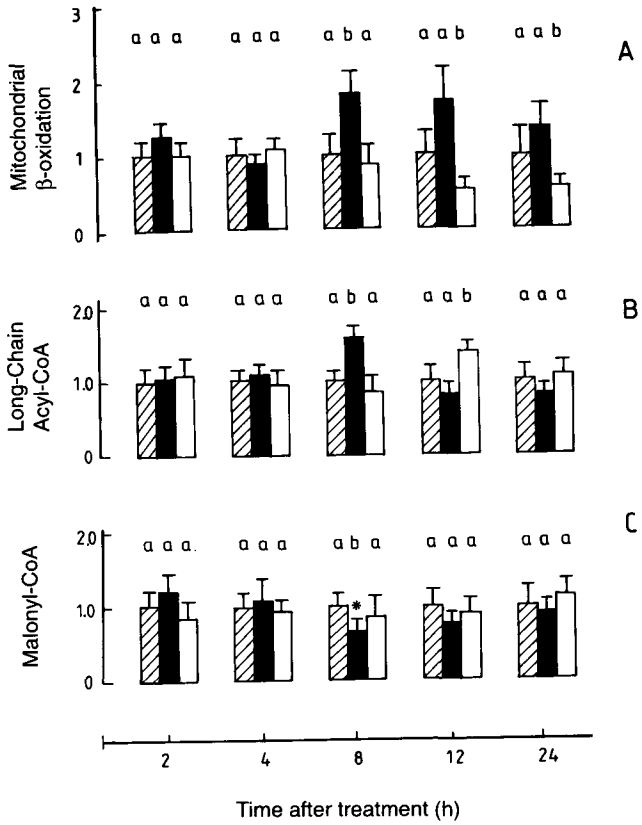

FIG. 4. Relative specific activities of mitochondrial β-oxidation, with palmitoyl-L-carnitine as substrate (A), relative concentration of hepatic long-chain acyl-CoA (B), and relative concentration of liver malonyl-CoA (C) at different times after a single oral administration of EPA-EE (black columns) and DHA-EE (open columns) in the rat. The relative values are calculated to those of palmitic acid-fed controls (hatched columns) = 1.0 at each of the time points studied. The results are presented as means of six rats per group. Means in the column with different superscripts are significantly different ($P < 0.05$) determined by one-way analysis of variance, Fisher's test. *Significantly different from values at two hours of individual treatment ($P < 0.05$). Abbreviations as in Figure 2.

creased significantly 4 h after treatment (Fig. 5), and continued to rise more than 1.5-fold above control values at 12 h after exposure. No evidence was found for induction of 2,4-dienoyl-CoA reductase after acute administration of DHA-EE during this experiment compared to the 28 S rRNA (Fig. 5).

As shown in Figure 4B, hepatic long-chain acyl-CoA content was increased 8 h after acute treatment with EPA-EE as compared to controls and DHA-EE treated rats. At 12 h the long-chain acyl-CoA level in EPA-EE treated rats was normalized. In contrast to the changes of long-chain acyl-CoA level observed after EPA-EE feeding, the malonyl-CoA level was reduced from the second to the eighth hour (Fig. 4C), where the mitochondrial β-oxidation was increased (Fig. 4A), and at 8 h the malonyl-CoA values became significantly lower in EPA-EE treated rats than in controls (Fig. 4C).

As shown in Table 4, the total amount of the different fatty acids in the liver was not changed at any of the time points studied in the Palm treated group (controls). Treating the rats with Palm did not change the fatty acid composition, including the amount of Palm (16:0) compared to rats only treated with CMC (data not shown). The total amounts of monoenes and polyenes were decreased and increased, respectively, 4 h after acute treatment with EPA-EE as compared to Palm (controls) and DHA-EE administration (Table 4). Palmitoleic acid (16:1n-7) and vaccenic acid (18:1n-7) were significantly reduced after EPA-EE treatment compared to DHA-EE feeding, whereas the amount of oleic acid (18:1n-9) was reduced compared to controls. No statistically significant difference was found for either stearic acid (18:0), linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), and arachidonic acid (20:4n-6) between EPA-EE, DHA-EE, and Palm treated rats at any of the time points studied. The total amount of cellular EPA (20:5n-3) and docosapentaenoic acid (DPA) (22:6n-3) increased from the second to the fourth hour after EPA-EE administration, and these fatty acids were increased at all time points studied with EPA-EE compared to controls and DHA-EE feeding. Table 4 further shows that the amount of cellular DHA (22:6n-3) increased at all time points studied in DHA-EE treated rats compared to controls except at 8 h, but 22:6n-3 did not reach a peak at 4 h as 20:5n-3 after EPA-EE administration. Moreover, at all time points studied, the fold increase of 22:6n-3 after DHA-EE treatment was much lower than the increase of 20:5n-3 after EPA-EE feeding. No statistically significant difference was found for 22:5n-3 between controls and DHA-EE administration at any of the time points studied (Table 4). Noteworthy, however, was the fact that DHA-EE supplementation elevated the amount of 20:5n-3 the first 8 h after administration.

As shown in Table 5, the HMG–CoA reductase activity in the control group was increased at 8 h and this increase was maintained to 12 h after feeding. As also shown in Table 5, the HMG–CoA reductase activity was reduced 8 h after acute treatment with EPA-EE as compared to controls and DHA-EE administration.

DISCUSSION

It has been assumed that EPA or DHA, the major polyunsaturated fatty acids of fish oil, or both are responsible for the triglyceride-lowering effect. Most studies have used fish oil with various contents of EPA and DHA, and only a few studies have examined the effect of EPA and DHA separately to determine the effects of each fatty acid. The present study reports that EPA, and not DHA, is the fatty acid primarily responsible for the triglyceride-lowering effect of fish oil in rats. These acute effects are consistent with observations in normolipidemic and hyperlipidemic rats (21–24). Studies in perfused livers and cultured cells have shown that EPA reduces triglyceride synthesis/secretion in perfused rat liver (19,58), in rat (12,59), rabbit hepatocytes (12,59,60), and in HepG2 cells (9,61). Furthermore, Zhang *et al.* (58) observed in a liver perfusion study that only EPA, and not DHA, decreased triglyceride synthesis and VLDL secretion. In contrast, Williams *et al.* (62) observed that feeding pure DHA re-

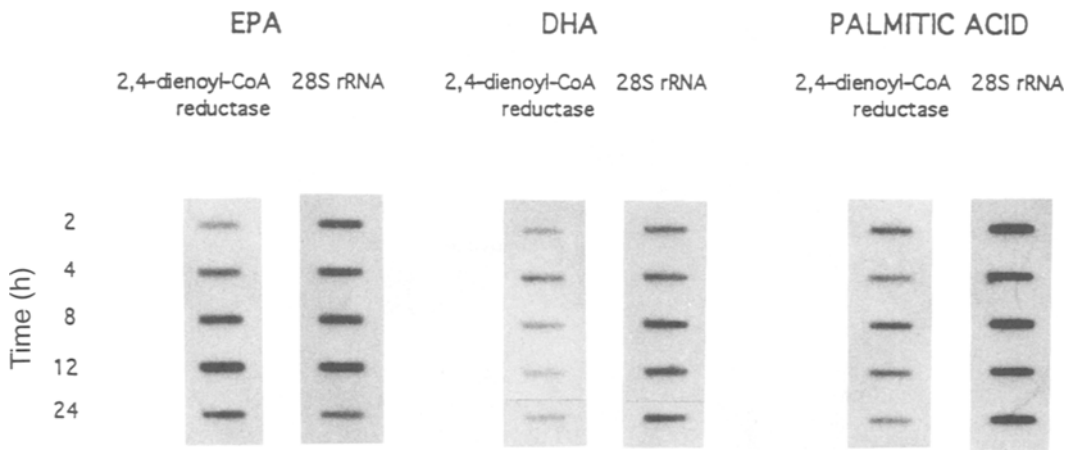

FIG. 5. Kinetics of 2,4-dienoyl-CoA reductase and 28S rRNA after acute treatment with DHA-EE and palmitic acid. One representative slot blot of a total of three from six rats in each group is shown. Mouse 28S rRNA was used as a probe to evaluate the amount of RNA applied on the filter, which was 5, 2, and 1 μg. Abbreviations as in Figure 1.

duced hepatic triglyceride secretion *in vivo*. Similarly, Martin *et al.* (63) reported a reduction of triglyceride secretion in rat hepatocytes following incubation with DHA, but concluded that inhibition of hepatocyte triglyceride synthesis is not obligatory for the 22:6, n-3-induced diminution of triglyceride secretion.

In humans, supplementation with purified EPA has lowered triglycerides in plasma (64–67), whereas the few studies reported on purified DHA on human serum lipoprotein levels are conflicting (26–28,68). Worne and Smith (68) reported a marked reduction in total serum lipids by EPA, by DHA, and by arachidonic acid administration (triglyceride levels were not measured), and Hirai *et al.* (27) showed a significant reduction of serum triglycerides and cholesterol in hypertriglyceridemic patients of Type IIa and Type IIb hyperlipidemia following both highly purified EPA and DHA ingestion. In contrast, a report by Harris (26) at the recent ISSFAL meeting found that EPA, but not DHA, appeared to be responsible for the triglyceride-lowering effect of fish oil in humans. This is in contrast to the Tromsø study, reported at the same meeting, where it was concluded that DHA was more efficient than EPA in decreasing plasma triglyceride concentrations (28). The cause of the differences between the results in the human studies is not clear, but one explanation might be that the Japanese and Norwegian people consume more fish and thereby have higher basal values of EPA and DHA compared to the healthy subjects in Kansas City, Missouri, where fish consumption is lower.

A large number of reports have been published demonstrating that ethyl esters of n-3 fatty acids execute the same biological effects as other chemical forms of these fatty acids (67,69). However, it is clear that the *in vitro* hydrolysis of ethyl esters of very long-chain n-3 fatty acids is markedly reduced as compared to the free forms and triglycerides of these fatty acids (34,70). During prolonged feeding, ethyl esters of n-3 fatty acids are highly bioavailable, whereas the markedly slower hydrolyzation by pancreatic lipase theoretically could influence the absorption of n-3 fatty acids in short-term studies. Our results from fatty acid composition in liver of rats treated with a single dose of EPA-EE and DHA-EE show that EPA-EE feeding increases (20:5n-3) after two hours and that feeding EPA-EE also increases DPA (22:5n-3), whereas DHA (22:6n-3) remains unchanged (Table 4). Furthermore, we observed triglyceride-lowering effect by EPA-EE after eight hours (Fig. 2). Concerning DHA-EE feeding, an increase in fatty acid composition in liver was observed after two hours. Our results show that EPA-EE and DHA-EE are rapidly absorbed and exert their effects on plasma lipids and key enzyme activities of lipid metabolism within hours. Thus, the discrepancy regarding the separate effects of EPA and DHA is not due to the bioavailability of ethyl esters of n-3 fatty acids compared to n-3 fatty acids esterified to triacylglycerol or phosphoacylglycerol.

We had the opportunity in this study to address the question of the mechanism behind this lipid-lowering effect. EPA-EE treatment for either hours (Figs. 2 and 3) or given over a 15-d period (21) to normolipidemic rats decreased plasma triglycerides, cholesterol, and phospholipids. Prolonged treatment with EPA-EE is reported to increase the activity of acyl-CoA:diacylglycerol acyltransferase and to decrease the acetyl-CoA carboxylase activity (21). No change of the last enzyme activity was observed after acute treatment (Table 2). EPA-EE increased mitochondrial fatty acid oxidation (Fig. 4A) and, most plausible, also peroxisomal β-oxidation, as the fatty acyl-CoA oxidase activity was increased (Table 3). EPA-EE treatment either for prolonged feeding (21) or given acutely to rats had no effect on the PAP activity (Table 2). It is conceivable that, whereas the hypolipidemic effect of EPA-EE after prolonged treatment (18,21) may be influenced by alterations of the triglyceride biosynthesis and lipogenesis, its effect after acute treatment appears to be independent of changes of these pathways. The triglyceride-lowering effect after acute EPA-EE treatment may be a consequence of its effect on hepatic fatty acid oxidation.

TABLE 4
Changes in Fatty Acid Composition in Liver of Rats Treated with EPA-EE, DHA-EE, and Palmitic Acid (Palm) (controls)

Fatty acid	Treatment/hours	Fatty acid composition (mol%) 2	4	8	24
16:0	Palm	20.6 ± 1.2[a]	22.2 ± 1.3[a]	20.5 ± 1.1[a]	23.5 ± 2.0[a]
	EPA-EE	19.8 ± 0.7[a]	18.9 ± 1.1[a]	19.6 ± 1.5[a,c]	23.4 ± 0.9[a,c]
	DHA-EE	21.3 ± 0.3[a]	21.3 ± 1.4[a]	23.3 ± 0.9[a]	20.3 ± 1.6[a]
16:1n-7	Palm	2.4 ± 0.5[a]	2.7 ± 0.8[a]	1.9 ± 0.4[a]	3.1 ± 1.6[a]
	EPA-EE	2.2 ± 0.1[a]	2.0 ± 0.2[a,c]	1.6 ± 0.3[b,c]	1.7 ± 0.1[b]
	DHA-EE	2.5 ± 0.1[a]	2.7 ± 0.4[a]	2.8 ± 0.4[a]	2.0 ± 0.4[a]
18:0	Palm	16.8 ± 1.3[a]	17.5 ± 1.6[a]	17.6 ± 1.5[a]	15.1 ± 3.0[a]
	EPA-EE	16.5 ± 0.9[a]	17.1 ± 0.5[a]	16.9 ± 1.0[a]	16.8 ± 0.4[a]
	DHA-EE	16.0 ± 0.5[a]	15.2 ± 1.2[a]	16.5 ± 0.4[a]	16.3 ± 0.6[a]
18:1n-7	Palm	3.3 ± 0.2[a]	3.7 ± 0.6[a]	4.0 ± 1.0[a]	4.1 ± 1.1[a]
	EPA-EE	3.0 ± 0.3[a,c]	3.2 ± 0.3[a,c]	3.7 ± 0.4[a]	3.3 ± 0.3[a]
	DHA-EE	4.0 ± 0.3[a]	4.7 ± 0.5[a]	4.0 ± 0.3[a]	4.2 ± 0.6[a]
18:1n-9	Palm	7.0 ± 1.4[a]	7.8 ± 0.6[a]	5.8 ± 0.2[a]	7.8 ± 2.4[a]
	EPA-EE	6.5 ± 1.1[a]	5.6 ± 0.4[a,d]	5.3 ± 0.4[a]	6.5 ± 0.8[a]
	DHA-EE	5.7 ± 0.5[a]	6.5 ± 0.7[a]	6.2 ± 0.7[a]	5.1 ± 0.4[a]
18:2n-6	Palm	14.3 ± 1.1[a]	13.2 ± 1.3[a]	14.4 ± 1.0[a]	14.8 ± 1.1[a]
	EPA-EE	13.8 ± 0.5[a]	12.5 ± 6.6[a]	13.2 ± 1.0[a]	14.0 ± 1.2[a]
	DHA-EE	13.8 ± 0.4[a]	13.4 ± 1.0[a]	13.3 ± 0.6[a]	14.9 ± 1.2[a]
18:3n-3	Palm	0.2 ± 0.1[a]	0.1 ± 0.0[a]	0.2 ± 0.0[a]	0.2 ± 0.0[a]
	EPA-EE	0.2 ± 0.1[a]	0.2 ± 0.1[a]	0.2 ± 0.0[a]	0.2 ± 0.1[a]
	DHA-EE	0.2 ± 0.0[a]	0.2 ± 0.1[a]	0.2 ± 0.0[a]	0.3 ± 0.0[a]
20:4n-6	Palm	18.0 ± 2.9[a]	16.8 ± 0.8[a]	19.2 ± 1.4[a]	15.7 ± 2.3[a]
	EPA-EE	16.4 ± 1.3[a]	16.7 ± 1.5[a]	18.3 ± 2.2[a]	16.1 ± 0.8[a]
	DHA-EE	16.8 ± 0.5[a]	15.0 ± 1.2[a]	15.8 ± 1.6[a]	18.0 ± 1.8[a]
20:5n-3	Palm	1.1 ± 0.4[a]	0.9 ± 0.2[a]	0.9 ± 0.1[a]	1.0 ± 0.1[a]
	EPA-EE	4.2 ± 1.3[a,c,d]	7.5 ± 0.7[b,c,d]	5.4 ± 1.2[b,c,d]	4.4 ± 0.4[a,c,d]
	DHA-EE	1.9 ± 0.3[a,e]	1.5 ± 0.7[a,e]	1.6 ± 0.7[a,e]	1.2 ± 0.1[a]
22:5n-3	Palm	1.0 ± 0.2[a]	1.1 ± 0.1[a]	1.2 ± 0.2[a]	1.2 ± 0.1[a]
	EPA-EE	1.8 ± 0.2[a,d]	2.8 ± 0.1[b,c,d]	2.8 ± 0.7[b,c,d]	3.0 ± 0.6[b,c,d]
	DHA-EE	1.4 ± 0.3[a]	1.4 ± 0.1[a]	1.2 ± 0.1[a]	1.3 ± 0.1[a]
22:6n-3	Palm	7.8 ± 0.6[a]	7.2 ± 0.8[a]	7.9 ± 1.1[a]	7.0 ± 1.4[a]
	EPA-EE	7.2 ± 0.5[a,c]	7.2 ± 0.5[a,c]	6.9 ± 0.8[a,c]	7.0 ± 0.5[a,c]
	DHA-EE	9.7 ± 1.1[a,e]	9.7 ± 1.5[a,e]	8.8 ± 0.6[a]	10.1 ± 0.3[a,e]
Σ monoenes	Palm	13.5 ± 1.9[a]	14.7 ± 1.8[a]	12.4 ± 1.5[a]	15.6 ± 5.2[a]
	EPA-EE	12.5 ± 0.8[a]	11.3 ± 0.5[a,c,d]	11.4 ± 1.2[a]	12.0 ± 1.0[a]
	DHA-EE	12.9 ± 0.8[a]	14.8 ± 1.4[a]	13.6 ± 1.3[a]	11.9 ± 0.6[a]
Σ polyenes	Palm	44.4 ± 3.2[a]	41.6 ± 1.8[a]	45.7 ± 0.8[a]	41.8 ± 4.4[a]
	EPA-EE	45.5 ± 2.5[a]	49.1 ± 1.2[a,c,d]	48.8 ± 1.7[a,c,d]	44.3 ± 0.8[a]
	DHA-EE	46.0 ± 1.6[a]	43.8 ± 1.4[a]	42.6 ± 2.4[a]	47.7 ± 2.5[a]
n-3/n-6	Palm	0.30 ± 0.03[a]	0.30 ± 0.03[a]	0.29 ± 0.05[a]	0.30 ± 0.03[a]
	EPA-EE	0.42 ± 0.03[a,c,d]	0.58 ± 0.07[b,c,d]	0.47 ± 0.08[a-d]	0.40 ± 0.07[a]
	DHA-EE	0.41 ± 0.05[a,e]	0.42 ± 0.08[a,e]	0.38 ± 0.04[a]	0.37 ± 0.04[a]

[a,b]Values in a row with different superscripts are significantly different ($P < 0.05$). Abbreviations as in Table 2.
[c]$P < 0.05$ for difference between EPA-EE and DHA-EE treated rats.
[d]$P < 0.05$ for difference between EPA-EE and controls.
[e]$P < 0.05$ for difference between DHA-EE and controls.

The results from this study demonstrated that both EPA-EE and DHA-EE administration resulted in the induction of fatty acyl-CoA oxidase activity (Table 2). As the fatty acyl-CoA oxidase mRNA levels were increased (Vaagenes, H., Asiedu, D., Demoz, A., and Berge, R.K., unpublished results), the induction of fatty acyl-CoA oxidase by these peroxisome proliferators is partly, if not entirely, due to an increase in the transcription rate of the gene. EPA-EE lowered plasma triglycerides, but DHA-EE supplementation had no hypotriglyceridemic effect. Thus, increased triglyceride clearance by fish oil is not due to enhanced activity of peroxisomal β-oxidation, which confirms and extends previous reports by hypolipidemic peroxisome proliferating fatty acid analogues (71).

To our knowledge, the results from this study demonstrate for the first time that EPA-EE (but not DHA-EE) treatment results in the induction of rat liver 2,4-dienoyl-CoA reductase gene expression, an enzyme which is necessary for oxidation

TABLE 5
HMG–CoA Reductase Activity at Different Times After Acute EPA-EE and DHA-EE Administration in the Rat[a]

	Hours	EPA-EE	*P*	Control	*P*	DHA-EE
HMG–CoA	2	184 ± 98[a]	n.s.	91 ± 16[a]	<0.05	240 ± 22[a]
reductase activity	4	241 ± 34[a]	n.s.	150 ± 47[a]	n.s.	228 ± 22[a]
(pmol/min/mg protein)	8	152 ± 54[a]	<0.05	237 ± 40[b]	<0.05	335 ± 17[b]
	12	186 ± 129[a]	n.s.	269 ± 79[b]	n.s.	266 ± 79[a,b]
	24	344 ± 59[a]	n.s.	233 ± 97[a,b]	n.s.	270 ± 22[a]

[a]The 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase activity was measured in the isolated microsomal fraction. Statistical comparison between EPA-EE, DHA-EE, and controls is shown by the *P* values.
[a,b]Correspond to the statistical comparison between the groups at each of the time points studied (hours), where the same letter within one column means that there is no statistical differences between the corresponding groups. Abbreviations as in Table 2.

of unsaturated fatty acids. Furthermore, the induced activity of 2,4-dienoyl-CoA reductase on the transcriptional induction of the enzyme gene expression by EPA-EE supports previous reports that modification of the mitochondrial fatty acid oxidation system is related to the mitochondrial induction produced by non-β-oxidizable fatty acid analogues, i.e., 3-thia fatty acids (71) and by fish oil (72). Increased fatty acid oxidation of EPA, but not DHA (free forms of the n-3 fatty acids), seen in cultured rat hepatocytes (Fig. 1) is in keeping with the animal data. Thus, the triglyceride-lowering effect of EPA may be due to the increased mitochondrial fatty acid oxidation.

McGarry and Foster (73) have shown that fatty acid oxidation is inhibited by malonyl-CoA, *via* inhibition of carnitine palmitoyltransferase I. Depression of the hepatic malonyl-CoA content (Fig. 4C) therefore appears to be the mechanism by which EPA stimulates mitochondrial fatty acid oxidation. It is tempting to suggest that EPA, by decreasing malonyl-CoA, may promote acyl carnitine formation and, consequently, acylcarnitine transport across the inner mitochondrial membrane followed by β-oxidation of long-chain acyl-groups to acetyl-CoA.

A number of animal studies have demonstrated increased hepatic β-oxidation capacity and ketone body production after fish oil feeding (17,19,25,74,75). Surette *et al.* (20) observed an increased activity of hepatic carnitine palmitoyl transferase I in fish oil-fed Syrian hamsters, which was highly correlated with the diet-induced change in serum triacylglycerol concentrations. These findings are in agreement with the results in this study and previous findings (21,22), and one reason for the different results with EPA and DHA might be that the bioavailability of EPA and DHA for the mitochondrial and peroxisomal β-oxidation is different. Very long chain fatty acids (VLCFA) (>C_{20}) are oxidized predominantly, if not exclusively, by peroxisomes (76,77) and very long chain fatty acyl-CoA synthetase (necessary for the activation of the fatty acids to their CoA derivatives, a prerequisite for β-oxidation and esterification) is present in endoplasmic reticulum and peroxisomes, but not in mitochondria (77,78). Apparently, the site of activation determines the subsequent fate of VLCFA. VLCFA are not oxidized by isolated mitochondria, indicating that the absence of very long chain acyl-CoA synthetase from mitochondria is the reason why these organelles do not oxidize VLCFA (79). Because DHA is a good substrate for peroxisomes and increases peroxisomal β-oxidation, but not the mitochondrial β-oxidation (Table 3 and Fig. 4), we suggest that DHA is mainly metabolized (chain-shortened/retroconverted) by peroxisomes. This is in agreement with studies in isolated liver cells (80). In contrast, EPA and other long-chain fatty acids (LCFA; C_{16}–C_{20}) are mainly directed to mitochondrial β-oxidation, as long-chain acyl-CoA synthetase (C_{16}–C_{20}) is located in mitochondria and they have a lower apparent K_m for LCFA (81,82). The different results of EPA and DHA on triglyceride-lowering effect and hepatic fatty acid oxidation may partly reflect the diversion of EPA (as a LCFA) to mitochondrial β-oxidation and ketogenesis, whereas DHA is diverted to the peroxisomes for further metabolism.

Circulating FFA and glycerol are mainly taken up by the liver where they are partially converted into triglycerides for later export to the circulation in the form of VLDL-triglycerides. Several studies have reported a marked reduction in FFA concentration after fish oil supplementation, indicating that decreased lipolysis from adipose tissue could be the reason for decreased VLDL synthesis and/or secretion (13,75, 83). However, the activities of lipoprotein lipase and hepatic lipase have uniformly shown no change after fish oil feeding both in humans (84,85) and in animals (86,87). Otto *et al.* (88) observed a dose-dependent reduction in plasma FFA by n-3 fatty acids in rats fed *ad libitum*, but not in rats fasted overnight (88). In the present study, administration of EPA-EE reduced the plasma FFA at eight hours, whereas plasma FFA in DHA-EE treated animals showed a marked decrease in plasma FFA after only two hours. At that time the plasma FFA in EPA-EE treated rats were significantly higher than in DHA-EE treated rats and controls (Fig. 3B). A possible explanation for this might be the recently published observations by Raclot and Groscolas (89) that individual fish oil n-3 polyunsaturated fatty acids are selectively mobilized from and stored in adipose tissue. The preferential mobilization of EPA could contribute to its maintenance in the circulation at four hours compared to DHA, and that this effect was overcome at later time points.

In general, hepatic fatty acid oxidation is proportional to plasma FFA concentration (90) but, at equal FFA concentrations, perfused livers from fish oil-adapted rats have shown

significantly higher rates of ketogenesis than livers from animals fed chow or safflower oil (19). This is in agreement with our results where all three fatty acid supplements resulted in decreased concentrations of plasma FFA at eight hours, but at the same time point mitochondrial β-oxidation was significantly increased in the EPA-EE treated rats (Fig. 4). This supports our data that decreased plasma triglycerides following EPA-EE supplementation is due to increased mitochondrial β-oxidation, resulting in decreased amount of substrate for triglyceride synthesis, and is not due to increased lipolytic activity.

There is no consistent change in plasma cholesterol after fish oil feeding in humans, whereas the cholesterol-lowering effect of fish oil in rats is well documented (29,31). One explanation might be that rats are deficient in lipid transfer protein (91) and in this species fish oils lower plasma cholesterol concentrations, probably as a consequence of this lack. In humans and marmosets who have lipid transfer protein, there may be even a small increase in plasma cholesterol after ingestion of fish oil and EPA (as ethyl ester), respectively (92,93). Human studies also have demonstrated that fish oil reduces the expected increase in plasma cholesterol levels with an atherogenic diet (94). As with the hypotriglyceridemic effect, it remains unknown whether EPA or DHA or both are responsible for the cholesterol-lowering effect in rats. In this and a previous study, we have observed a hypocholesterolemic effect of EPA-EE, and not DHA-EE, associated with a significant inhibition of HMG–CoA reductase activity (Table 5) (22). This suggests that EPA-EE, in contrast to DHA-EE treatment, might decrease cholesterol biosynthesis, thereby contributing to the decrease in VLDL secretion.

Another important observation made in this study was that EPA-EE, but not DHA-EE, given acutely affected the cellular fatty acid composition by decreasing the amounts of monoenes (16:1n-7, 18:1n-7, and 18:1n-9) and increasing the amount of polyenes (20:5n-3 and 22:6n-3). Replacement of the ordinary fatty acids, i.e., the monoenes with EPA and some conversion to DPA concomitant with increased fatty acid oxidation, is probably the mechanism leading to changed fatty acid composition. The paradox that administration of pure EPA-EE did not increase DHA concentration in liver (Table 4) may be explained by reduced Δ^6 desaturase activity as 22:5n-3 is metabolized to 22:6n-3 through three steps: namely elongation to 24:5n-3, Δ^6-desaturation to 24:6n-3, and finally β-oxidation to DHA (95). Indeed, dietary supplementation with n-3 fatty acids depresses rat liver Δ^6-desaturase activity (96), which is necessary for the second step of the conversion of 22:5n-3 to 22:6n-3.

The results from this study argue against a direct conversion of EPA to DHA, but rather in favor of retroconversion of EPA to DHA (Table 4). Retroconversion of DHA to EPA also has been reported by others (23,27). This implies that it will be difficult to study the unique effects of DHA as some of it will always be converted to EPA. Whether the effects observed in the DHA-EE treated group were due to the EPA generated from DHA, and not from DHA itself, must then be considered.

In conclusion, we have shown that EPA is the hypotriglyceridemic fatty acid in fish oils, and increased mitochondrial fatty acid oxidation may be responsible. DHA has little impact on serum lipid levels, but can be retroconverted to EPA. In summary, the present observations provide evidence for the following events in the liver following exposure to EPA: (i) inhibition of malonyl-CoA formation possibly due to increased long-chain acyl-CoA levels (Fig. 4) (most plausible attributed to EPA-CoA); (ii) increased mitochondrial fatty acid oxidation; (iii) incorporation into triglycerides and phospholipids and displacement of easily oxidizable fatty acids into β-oxidation in mitochondria; and (iv) decreased production of VLDL-triglycerides.

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