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Methylated Eicosapentaenoic Acid and Tetradecylthioacetic Acid: Effects on Fatty Acid Metabolism

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ABSTRACT. We introduced methyl or ethyl groups to the 2- or 3-position of the eicosapentaenoic acid (EPA) molecule to investigate whether the branching of EPA could influence its hypolipidemic effect in rats. The most effective branching involved two methyl groups in the 2-position and one methyl group in the 3-position. These EPA derivatives increased hepatic mitochondrial and peroxisomal β-oxidation and decreased plasma lipids concomitant with suppressed acetyl-coenzyme A (CoA) carboxylase (EC 6.4.1.2) and fatty acid synthase (EC 2.3.1.85) activities. This was followed by elevated activities of carnitine O-palmitoyltransferase (EC 2.3.1.21) and possibly 2,4-dienoyl-CoA reductase (EC 1.3.1.34), as well as induced mRNA levels of these enzymes and fatty acyl-CoA oxidase. The fatty acid composition in liver changed, with an increased 18:1 n-9 content, whereas the expression of Δ^9-desaturase remained unchanged. We investigated the flux of fatty acids in cultured hepatocytes, and found that oxidation of [1-^{14}C]-labeled palmitic acid increased but the secretion of palmitic acid-labeled triglycerides decreased after addition of 2-methyl-EPA. The fatty acyl-CoA oxidase (EC 1.3.3.6) activity in these cells remained unchanged. A significant negative correlation was obtained between palmitic acid oxidation and palmitic acid-labeled synthesized triglycerides. To investigate whether the hypolipidemic effect occurred independently of induced peroxisomal β-oxidation, we fed rats 2-methyl-tetradecylthioacetic acid. This compound increased the peroxisomal but not the mitochondrial β-oxidation, and the plasma lipid levels were unchanged. In conclusion, EPA methylated in the 2- or 3-position renders it more potent as a hypolipidemic agent. Furthermore, this study supports the hypothesis that the mitochondrion is the primary site for the hypolipidemic effect. BIOCHEM PHARMACOL **58**;7:1133–1143, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. methylated EPA; mitochondrial and peroxisomal β-oxidation; lipogenesis; plasma lipids; fatty acid composition

Marine fish oil rich in n-3 polyunsaturated fatty acids, particularly EPA§ and DHA, is reported to possess a number of beneficial effects on the cardiovascular system [1]. Reduction of plasma triglycerides following n-3 fatty acid treatment may change the plasma profile from atherogenic to cardioprotective. It has been assumed that EPA and DHA both possess a hypotriglyceridemic effect. Recently, animal studies [2–4] have shown that EPA, but not DHA, appears to be responsible for the triglyceride-lowering effect of fish oil, possibly through increased β-oxidation. Results from a recent clinical study seem to support this pathway as the primary site for the hypolipidemic effect of n-3 fatty acids [5]. In this paper, we fed low doses of EPA and EPA derivatives (Fig. 1) to rats. The hypolipidemic effect of EPA is dose-dependent [6]. In fact, EPA must be administered to rats at a dose of 1000 mg/day/kg body weight to be hypolipidemic [2, 7]. 2-Methyl-EPA treatment, however, was hypolipidemic at 600 mg/day/kg body weight [8]. In order to investigate whether EPA methylated in the 2- and 3-positions renders EPA more potent as a hypolipidemic agent and to differentiate between the potency of the different EPA derivatives as hypolipidemic agents, low doses of the EPA derivatives were given to rats. *In vitro* experiments were performed to further investigate the hypotriglyceridemic effect of the EPA derivatives. We also methylated a 3-thia fatty acid (Fig. 1) to further potentiate its effects on fatty acid metabolism. This new compound was administered to rats at low doses, and its effects on fatty acid oxidation and plasma lipids were investigated. By using very low doses of the methylated 3-thia fatty acid, it was possible to differentiate between the effect of this compound on mitochondrial and peroxisomal β-oxidation and on plasma lipids.

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§ *Abbreviations:* CMC, sodium carboxymethylcellulose; CPT, carnitine palmitoyltransferase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAO, fatty acyl-coenzyme A oxidase; and TTA, tetradecylthioacetic acid.

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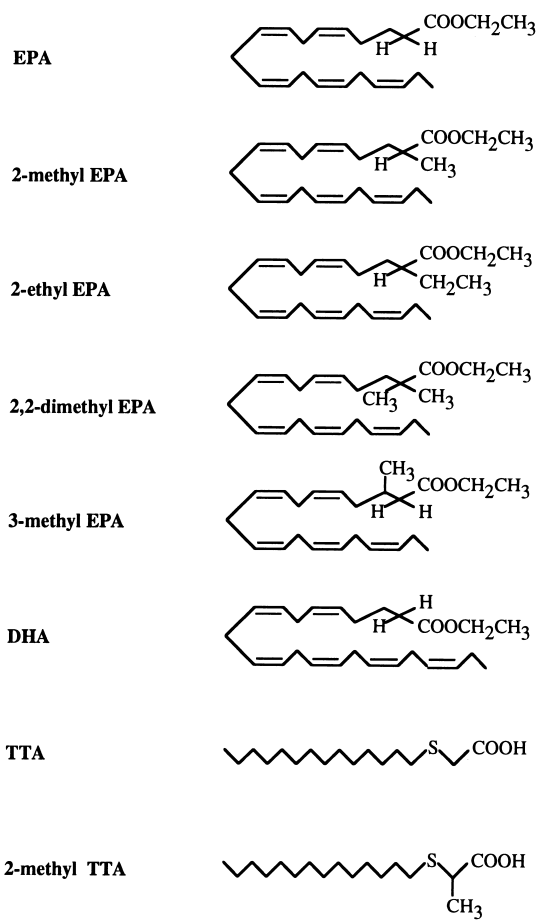

FIG. 1. Structural formulas of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), different EPA derivatives (all as ethyl esters), tetradecylthioacetic acid (TTA), and 2-methyl-TTA.

MATERIALS AND METHODS

Chemicals

Ethyl esters of EPA (97.0% pure), DHA (91.2% pure), the EPA derivatives 2-methyl-EPA (93.8% pure), 2-ethyl-EPA (96.1% pure), 2,2-dimethyl-EPA, (95.3% pure), and 3-methyl-EPA (>90% pure), and the commercially available compound Omacor® (47.1% [w/w] EPA and 34.8% DHA) were used in the *in vivo* experiment, while in the *in vitro* experiments we used the fatty acid forms of EPA, DHA, 2-methyl-EPA, 2-ethyl-EPA, and 3-methyl-EPA (>90% pure), all obtained from Norsk Hydro AS, Research Centre. TTA was produced as described by Spydevold and Bremer [9]. 2-methyl-TTA was produced following the same method [9], except that 2-mercaptopropionic acid was used instead of mercaptoacetic acid. The purity of 2-methyl-TTA was above 95%, the molecular weight measured by MS was 302 g/mol, and the characteristic MS fragments were 229, 257, and 302. 2-*trans*, 4-*cis*-Decadienoyl-CoA was synthesized as described by Kawaguchi *et al.* [10]. [1-^{14}C] Palmitic acid (40–60 mCi/mmol) and [α-^{32}P] dCTP (3000 Ci/mmol) were from Amersham. Restriction enzymes were from Promega. Nylon membranes (Hybond N) were from Amersham. All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and Treatments

Male Wistar rats from Möllegaard Breeding Laboratory, weighing 160–180 g, were housed as pairs in metal wire cages in a room maintained at 12-hr light–dark cycles and a constant temperature of 20 ± 3°. The animals were acclimatized for at least one week under these conditions before the start of the experiments. The EPA derivatives EPA, DHA, Omacor® (all as ethyl esters), TTA, and 2-methyl-TTA were suspended in 0.1% (v/v) CMC. The different n-3 fatty acids were administered at a dose of 250 mg/day/kg body weight for 5 days, and TTA and 2-methyl-TTA were administered at a dose of 15 mg/day/kg body weight for 7 days, all by gastric intubation in a final volume of 0.5–1.0 mL once a day. The control animals received only CMC. All animals had free access to food and water (rat and mouse standard diet, from B&K Universal). The food contained the following fatty acids (mol%): 16:0 (21%), 16:1 n-7 (2%), 18:0 (4%), 18:1 n-9 (25%), 18:2 n-6 (42%), and 18:3 n-3 (6%). At the end of the feeding period, after overnight starvation, the animals were anaesthetized by Hypnorm Dormicum® (midazolam), 0.2 mL/100 g body weight. Cardiac puncture was performed to obtain blood samples, and the liver was removed. Parts of the liver were immediately frozen in liquid N_2, while the rest of the liver was chilled on ice for homogenization.

Analysis of Plasma Lipids

Plasma was prepared by centrifugation of whole blood at 1000 *g* for 10 min. Triglyceride, cholesterol, and phospholipid were measured using the Monotest triglyceride, cholesterol, and phospholipid enzymatic kit (Boehringer Mannheim).

Preparation of Subcellular Fractions

The livers were homogenized in ice-cold sucrose solution (0.25 M sucrose in 10 mM HEPES buffer pH 7.4 and 1 mM EDTA) using a Potter-Elvehjem homogenizer. The subcellular fractions were isolated as previously described [11]. Briefly, the homogenate was centrifuged at 1000 *g* for 10 min to separate postnuclear from nuclear fraction. A mitochondrial-enriched fraction was prepared from the postnuclear fraction at 10,000 *g* for 10 min. A peroxisome-enriched fraction was prepared by centrifugation of the postmitochondrial fraction at 23,500 *g* for 30 min. A microsomal-enriched fraction was isolated from the post-peroxisomal fraction at 100,000 *g* for 1 hr and 15 min. The remaining supernatant was collected as the cytosolic fraction. The procedure was performed at 0–4°, and the fractions were stored at −80°. Protein was assayed using the Bio-Rad protein assay solution.

Enzyme Assays

FAO activity was determined in the peroxisomal-enriched fraction of rat livers by the coupled assay described by Small *et al.* [12]. The production of H_2O_2 was measured by monitoring the increase in dichlorofluorescein absorbance in the presence of palmitoyl-CoA. Total CPT activity in rat liver was measured in the mitochondrial-enriched fraction using palmitoyl-CoA and radiolabeled carnitine for production of butanol-soluble radiolabeled palmitoylcarnitine [13]. CPT-I activity was measured essentially as described by Bremer [14]. The assay for CPT-I contained 20 mM HEPES pH 7.5, 70 mM KCl, 5 mM KCN, 100 μM palmitoyl-CoA, 10 mg BSA/mL, and 0.6 mg tissue protein/mL. The reaction was started with 200 μM [methyl-^{14}C]L-carnitine (200 cpm/nmol). Assay conditions for CPT-II were identical, except that BSA was omitted and 0.01% (v/v) Triton X-100 was included. Tissue protein concentration was 0.1 mg/mL. 2,4-Dienoyl-CoA reductase activity was measured in the mitochondrial-enriched fraction by the method of Kunau and Dommes [15], with some minor modifications. The assay solution contained 50 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.01% (v/v) Triton X-100, 100 μM NADPH, and 6.25 μg protein. The assay was started by the addition of 100 μM 2-*trans*, 4-*cis*-decadienoyl-CoA. Fatty-acid synthase was measured in the cytosolic fraction as described by Roncari [16], with modifications according to Skorve *et al.* [17], and acetyl-CoA carboxylase was determined in the cytosolic fraction as the incorporation of $NaH^{14}CO_3$ into malonyl-CoA [18]. Mitochondrial β-oxidation was measured in the postnuclear fraction by using palmitoyl-CoA and palmitoyl-L-carnitine as substrates [19]. Peroxisomal β-oxidation was measured in the peroxisomal-enriched fraction [20].

Preparation of Hybridization Probes

DNA fragments were labeled by random priming using the oligolabeling technique of Feinberg and Vogelstein [21]. The DNA probes were purified fragments of cloned rat genes. FAO cDNA: 1400 bp *Pst*I fragment of pMJ125 [22]; CPT-I cDNA: 1600 bp *Eco*RI fragment of pBK2-CPT I [23]; CPT-II cDNA: 1600 bp *Xho*I/*Xba*I fragment of pBKS-CPT II.4 [24]; 2,4-dienoyl-CoA reductase cDNA: 632 bp *Pst*I/*Eco*RI insert in pGEM-4Z [25]. As control, we used human 28S rRNA: 1400 bp *Bam*HI fragment of pA [26] or rat PO rRNA: 1046 bp *Bam*HI/*Xho*I fragment in pBluescript II SK (z29530, provided by A. Molven, Bergen, Norway).

RNA Purification and Analysis

Total cellular RNA was isolated by the guanidinium–thiocyanate method described by Chomczynski and Sacchi [27]. The RNA concentrations were determined spectrophotometrically. Blotting of RNA onto nylon was carried out as described by Aasland *et al.* [28]. Hybridization to immobilized RNA was performed as described by Sambrook *et al.* [29] in the presence of 50% (v/v) formamide, 5× saline sodium citrate (SSC), 200 μg/mL heat-denatured herring sperm DNA, 0.1% (w/v) SDS, 25 mM sodium phosphate pH 6.5, 8.25% (w/v) dextran sulphate at 42° for 24 to 48 hr. Filters were washed to high stringency (0.2× SSC, 0.1% (w/v) NaPPi, 0.1% (w/v) SDS at 65°) and Kodak XAR-5 x-ray films were exposed in the presence of intensifying screens at −80°. Densitometric scanning of autoradiograms was performed using the LKB Ultrogel laser densitometer (Bromma). When the filters were to be rehybridized, the bound probe was first stripped off in 0.1% (w/v) SDS at 90–100° for 7 min. The hybridization results were normalized to the signal of 28S rRNA or PO rRNA hybridization in the individual samples. Relative mRNA inductions of the different genes were then calculated.

Hepatocyte Isolation and Culture Conditions

Rat liver parenchymal cells were isolated as described by Berry and Friend [30] with modifications according to Seglen [31]. The viability and number of cells in the cell suspension were determined microscopically by trypan blue exclusion. Cell viability was 90–95%. The hepatocytes were plated at a density of 2.0×10^6 in 60-mm dishes in 2 mL of Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES, 2% (w/v) Ultroser G, 0.52 mM L-carnitine, and 50 μg/mL gentamicin. Cultures were maintained in a humidified incubator at 37° in an atmosphere containing 5% CO_2. After overnight incubation, the medium was replaced with 2 mL/dish DMEM containing 20 mM HEPES and 0.52 mM L-carnitine. The cells were incubated for 4 hr in the presence of 200 μM [1-^{14}C]-labeled palmitic acid and 50, 100, or 200 μM of the different fatty acids. The fatty acids were bound to BSA (fatty acid:BSA molar ratio was 2.5:1). The medium was collected and centrifuged at 840 *g* for 5 min. The resulting cell-free medium was used for measurement of oxidation and secreted triglycerides. The cells used for measurement of synthetized triglycerides were scraped off with a rubber spatula in cold potassium phosphate buffer (50 mM, pH 7.4) with NaCl (150 mM, 0.9% [w/v]). The cell suspension was centrifuged at 840 *g* for 5 min, and the resulting cell pellet was re-suspended in a total volume of 1 mL sucrose medium (0.25 M sucrose in 10 mM HEPES buffer and 1 mM EDTA, pH 7.4) and frozen at −20°, thawed and subsequently homogenized by sonication.

Determination of Fatty Acid Oxidation in Cultured Hepatocytes

The rate of β-oxidation was measured as oxidation of [1-^{14}C] palmitic acid to acid-soluble products. One hundred microliters of 6% (w/v) BSA was added to 250 μL cell-free medium and precipitated with 1.0 mL 1 M ice-cold $HClO_4$ and 150 μL 0.1 M KOH. The mixture was shaken vigorously and centrifuged at 2500 *g* for 10 min and 500 μL of

TABLE 1. Effect of EPA derivatives on mitochondrial and peroxisomal fatty acid oxidation, and gene expression of fatty acyl-CoA oxidase

Treatment	Plasma lipids (mmol/L)	Mitochondrial β-oxidation		Peroxisomal β-oxidation (nmol/mg/min)	Fatty acyl-CoA oxidase mRNA (fold increase)
		Palmitoyl-L-carnitine (nmol/mg/min)	Palmitoyl-CoA (nmol/mg/min)		
2-Methyl-EPA	3.72 ± 0.61	0.064 ± 0.009	0.028 ± 0.013	1.17 ± 0.62	1.04 ± 0.11
2-Ethyl-EPA	2.94 ± 0.32	0.086 ± 0.014	0.042 ± 0.012	2.01 ± 0.85	1.58 ± 0.19
2,2-Dimethyl-EPA	2.73 ± 0.46*†	0.123 ± 0.056*†	0.061 ± 0.028*†	4.37 ± 0.38*†	2.75 ± 0.81*†
3-Methyl-EPA	2.54 ± 0.39*†	0.102 ± 0.040*	0.042 ± 0.019	2.01 ± 0.27	1.72 ± 0.52
EPA	3.49 ± 0.53	0.054 ± 0.031	0.027 ± 0.018	1.49 ± 0.25	1.09 ± 0.17
Omacor	3.26 ± 0.28	0.042 ± 0.015	0.027 ± 0.012	1.12 ± 0.34	0.93 ± 0.24
DHA	3.45 ± 0.24	0.050 ± 0.016	0.022 ± 0.007	1.17 ± 0.36	0.87 ± 0.12
CMC	3.55 ± 0.50	0.038 ± 0.024	0.022 ± 0.009	1.05 ± 0.59	1.00 ± 0.24

The values are given as means ± SD for 4–8 animals. The values of plasma lipids are given as the summation of plasma triglycerides, plasma cholesterol, and plasma phospholipids. The rats were treated for 5 days with the different fatty acids at a dose of 250 mg/day/kg body weight.

*Significantly different ($P < 0.05$) from CMC.

†Significantly different from EPA.

the supernatants was counted in an LKB Wallac 1219 Rackbeta liquid scintillation counter. One set of samples containing the different media was precipitated immediately as described above and used as background activities.

Extraction and Separation of Radiolabeled Lipids from the Cells and the Medium

Medium and cellular lipids were extracted with chloroform: methanol according to Folch *et al.* [32]. Briefly, 4 mL of chloroform:methanol (2:1 v/v), 20 μL foetal bovine serum, and 15 μL of 5 M HCI was added to 1 mL cell-free medium serum and shaken vigorously before centrifugation at 840 *g* for 5 min. The cells were scraped off the plates and harvested, and 500 μL H_2O was added, before it was shaken and frozen. The cells were then sonicated. Four hundred μL of the sonicated cell suspension was shaken with 8 mL chloroform:methanol (2:1, v/v), and after 30 min 2 mL of a 0.9% (w/v) NaCl solution (acidified to pH 2) was added and the sample was shaken once more and centrifuged at 840 *g* for 5 min. The methanol–water phase was re-extracted with chloroform:methanol (2:1, v/v). The combined organic phases were subsequently evaporated under a stream of N_2. Finally, the residual lipids were re-dissolved in 200 μL chloroform:methanol (2:1 v/v) acidified by adding 1 drop of concentrated acetic acid per 5 mL solution, and kept on ice. The lipids were separated by TLC on silica gel plates using *n*-hexane:diethylether:acetic acid (65:35:1, v/v/v) as the non-polar mobile phase. Lipids were visualized using iodine vapor, and the TLC foils were cut into sections and counted by liquid scintillation.

Determination of Fatty Acid Composition

Total lipids were extracted from liver as described by Lie *et al.* [33]. The lipid fractions were evaporated, saponified, 19:0 added as internal standard, and the fatty acids esterified in 12% (v/v) BF_3 in methanol. The methyl esters were separated using a Carlo Erba 2900 gas chromatograph ("cold on column" injection, $60^{49°/min}$ $160^{1°/min}$ $190^{4°/min}$ 220°), equipped with a 50-m CP-sil 88 (Chrompack) fused silica capillary column (i.d. 0.32 mm). The fatty acid composition was calculated using a Maxima 820 Chromatography Workstation software, installed in an IBM-AT and connected to the GLC, and identification ascertained by standard mixtures of methyl esters (Nu-Chek).

Presentation of Results

The *in vivo* results are reported as means ± SD from 3 to 8 animals. The *in vitro* results are reported as means ± SD from 4–6 observations. Statistical analysis was by one-way Anova.

RESULTS

Activities and mRNA Levels of Mitochondrial and Peroxisomal β-Oxidation Enzymes

Mitochondrial β-oxidation in liver, measured as acid-soluble products with palmitoyl-carnitine and palmitoyl-CoA as substrates (Table 1), was unchanged compared to control animals (CMC). This was expected, since EPA needs to be administered at high doses (≥1000 mg/day/kg body weight) to increase mitochondrial β-oxidation [2, 7]. The rate of mitochondrial β-oxidation, however, increased in liver after 3-methyl- and especially 2,2-dimethyl-EPA feeding (Table 1). As shown in Fig. 2A, mitochondrial CPT activity was significantly elevated in rats fed 2,2-dimethyl-EPA, giving a 35% increase compared to control animals. The increased CPT activity was accompanied by a 1.8-fold increase in the mRNA content of CPT-II (Fig. 2B), whereas the expression of CPT-I remained unchanged (Fig. 2C). Mitochondrial 2,4-dienoyl-CoA reductase enzyme activity tended to increase in rats fed 2-ethyl-EPA and 2,2-dimethyl-EPA, although not significantly (Fig. 3A). The gene expression of 2,4-dienoyl-CoA reductase significantly increased 1.7-fold in rats fed 2,2-dimethyl-EPA (Fig. 3, B and C). Peroxisomal β-oxidation tended to

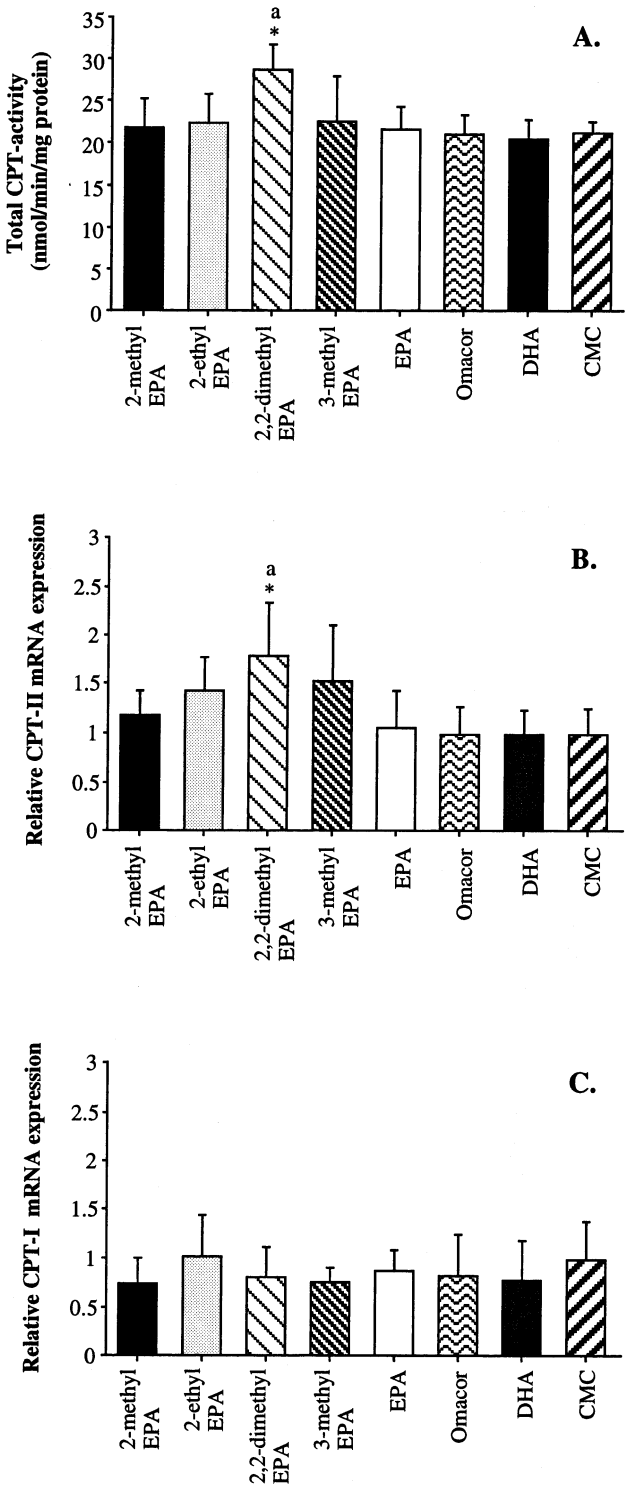

FIG. 2. Effects of EPA derivatives on (A) enzyme activity of total CPT, (B) CPT-II gene expression, and (C) CPT-I gene expression in rat liver. The rats were treated for 5 days with the different fatty acids at a dose of 250 mg/day/kg body weight. The measurements of enzyme activity and relative mRNA levels were as described in Materials and Methods. *Significantly different from control (CMC) and [a]significantly different from EPA ($P < 0.05$). Data are given as means ± SD of 6–7 animals.

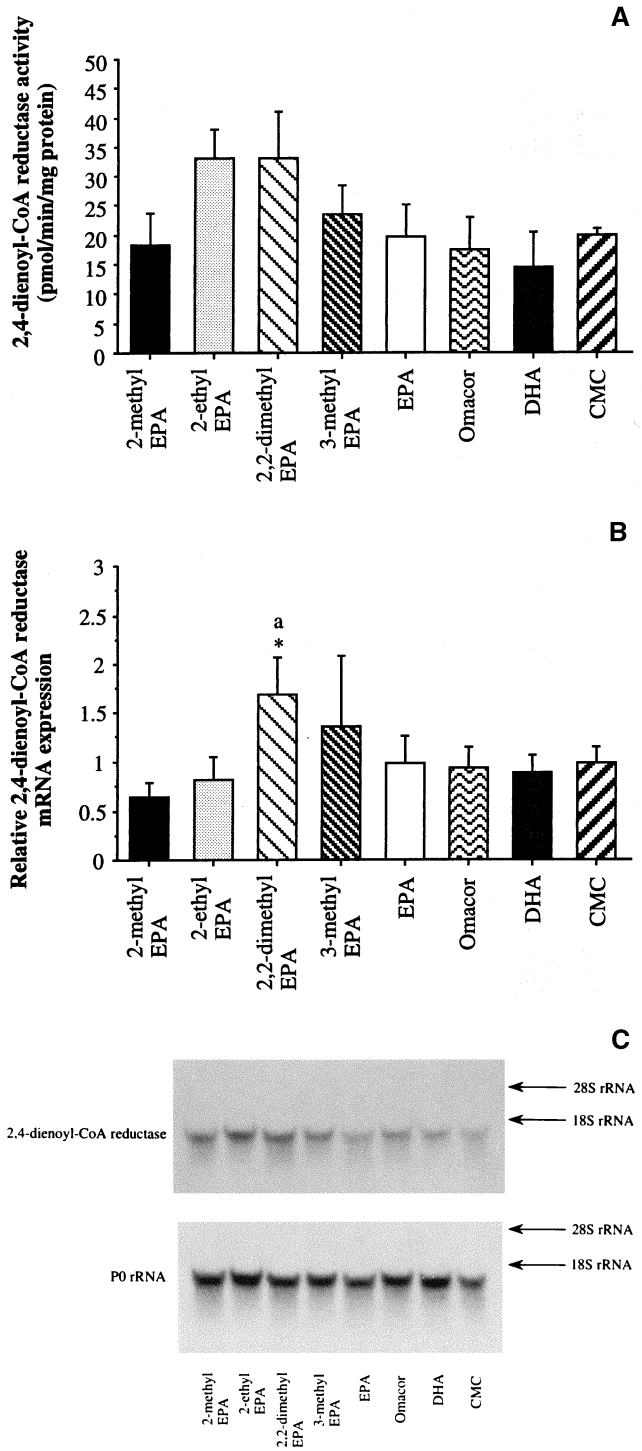

FIG. 3. Effects of EPA derivatives on (A) enzyme activity of 2,4-dienoyl-CoA reductase, (B) 2,4-dienoyl-CoA reductase gene expression, and (C) a representative Northern blot of 2,4-dienoyl-CoA reductase. The rats were treated for 5 days with the different fatty acids at a dose of 250 mg/day/kg body weight. The measurements of enzyme activity and relative mRNA levels were as described in Materials and Methods. *Significantly different from CMC, and [a]significantly different from EPA ($P < 0.05$). Data are given as means ± SD of 3–7 animals.

increase 2-fold in rats fed 2-ethyl-EPA and 3-methyl-EPA, and significantly increased 4-fold in rats fed 2,2-dimethyl-EPA (Table 1). The gene expression of FAO increased in the same manner. EPA, DHA, and Omacor® at low doses did not change the peroxisomal β-oxidation or gene expression of FAO.

Plasma Lipids

As shown in Table 1, administration of low doses (250 mg/day/kg body weight) of 2,2-dimethyl- and 3-methyl-EPA to rats resulted in a 22 and 28% reduction, respectively, in total plasma lipids (triglycerides, cholesterol, and phospholipids) as compared to CMC (control). EPA feeding did not have any effect on plasma lipids at this low dose. The level of plasma lipids was also unchanged in rats fed DHA or Omacor®.

Cultured Hepatocytes

In order to study the flux of fatty acids in whole cells, experiments in cultured hepatocytes were performed. The results showed that the oxidation of [1-^{14}C] palmitic acid to acid-soluble products increased 21% after addition of 100 μM 2-methyl-EPA compared to oleic acid, while the oxidation did not change significantly after addition of EPA, 2-ethyl-EPA, and 3-methyl-EPA (Fig. 4A). The secretion of palmitic acid-labeled triglycerides decreased by 46% in isolated hepatocytes after addition of 2-methyl-EPA, compared to oleic acid (Fig. 4B), while EPA, 2-ethyl-EPA and 3-methyl-EPA tended to decrease this secretion, although not significantly. The cell-associated palmitic acid-labeled triglycerides were unchanged (results not shown). We also measured the enzyme activity of FAO in these cells incubated with different EPA derivatives for 4 hr, and found no difference as compared to oleic acid (results not shown). As 2-methyl-EPA was the most potent of the EPA derivatives *in vitro*, we used this fatty acid in a correlation study. 2-methyl-EPA, EPA, and DHA were added to a final concentration of 50, 100, or 200 μM, and a correlation of palmitic acid-labeled oxidation versus palmitic acid-labeled synthesis of triglycerides (secreted and cell-associated triglycerides) was plotted (Fig. 5). The negative correlation between fatty acid oxidation and triglyceride synthesis is evident.

Effect on Lipogenesis

Figure 6 shows the activities of the two enzymes necessary for fatty acid synthesis, acetyl-CoA carboxylase and fatty-acid synthase. Acetyl-CoA carboxylase, the rate-limiting enzyme of lipogenesis, was reduced by 37% in rats fed 3-methyl-EPA, while the activity of fatty-acid synthase decreased about 30% after administration of 2,2-dimethyl- and 3-methyl-EPA compared to CMC.

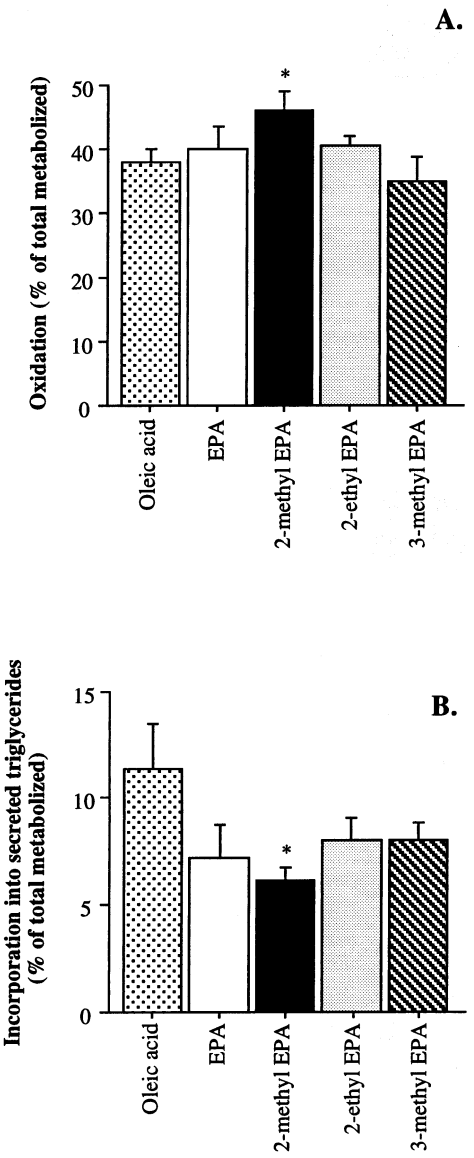

FIG. 4. Effects of oleic acid, EPA, 2-methyl-EPA, 2-ethyl-EPA, and 3-methyl-EPA on (A) the oxidation of [1-^{14}C]-labeled palmitic acid and (B) the incorporation of [1-^{14}C]-labeled palmitic acid into secreted triglycerides in hepatocytes. The cells were incubated for 4 hr in the presence of 200 μM [1-^{14}C]-labeled palmitic acid and 100 μM of the different fatty acids. The results are given as percentage of total metabolized palmitic acid and are the means ± SD of six independent observations. *Significantly different from oleic acid ($P < 0.05$).

2-Methyl Group in a 3-Sulphur-Substituted Fatty Acid Analogue

TTA is a saturated fatty acid analogue and peroxisome proliferator [34,35]. TTA was methylated in the 2-position, and administered to rats at a low dose (15 mg/day/kg body weight) for 7 days. At this low dose, we did not expect TTA to affect fatty acid metabolism, since the effects of TTA were shown earlier to be dose-related [36, 37]. 2-Methyl TTA or TTA did not change the hepatic mitochondrial β-oxidation as compared to control (CMC) (Table 2). The

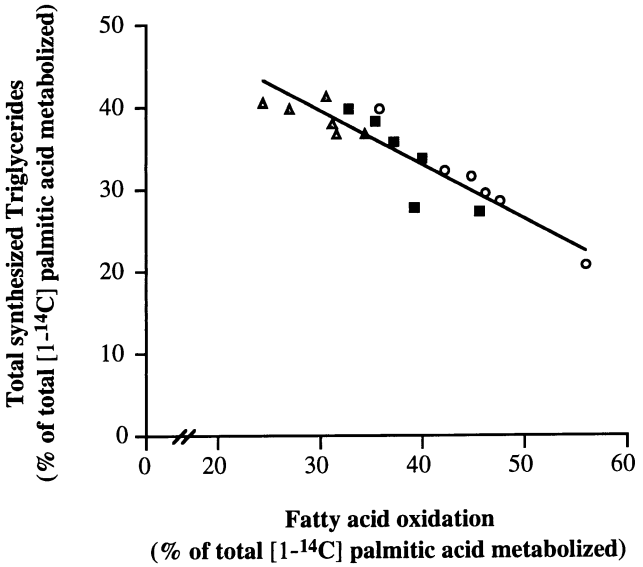

FIG. 5. Correlation between the oxidation of radiolabeled palmitic acid and synthesis of radiolabeled triglycerides (secreted and cell-associated). The hepatocytes were incubated for 4 hr in the presence of 200 μM [1-^{14}C]-labeled palmitic acid and 50, 100, or 200 μM of 2-methyl-EPA (○), EPA (■), or DHA (▲). Results in % of total radiolabeled palmitic acid metabolized are given from two separate experiments as the means of three different observations.

plasma lipids (triglycerides, cholesterol, and phospholipid) were not significantly changed in rats fed 2-methyl-TTA or TTA. In addition, the enzyme activities of CPT-I and CPT-II were unchanged, as was the mRNA level of CPT-II. The mRNA level of CPT-I, however, decreased in rats fed 2-methyl-TTA. The enzyme activity of FAO in liver increased almost 2-fold in rats fed 2-methyl-TTA compared to control, while TTA did not significantly change the FAO activity (Table 2).

Fatty Acid Composition

Detectable amounts of 3-methyl-EPA, but only trace amounts of the other EPA derivatives, were found in liver (Table 3). 3-Methyl-EPA, but none of the other EPA derivatives, increased the hepatic amount of 18:1 n-9 compared to EPA-treated rats. The gene expression of Δ^9-desaturase, however, was not significantly changed (data not shown). 3-Methyl-EPA also decreased the hepatic amount of 20:5 n-3 compared to EPA feeding. 3-Methyl-EPA as well as EPA increased the hepatic amount of 22:5 n-3 compared to control. DHA, however, did not significantly increase the 22:5 n-3 content. No change in the hepatic concentration of 22:6 n-3 was found after the different dietary treatments.

DISCUSSION

In attempt to differentiate between the potency of EPA and the different EPA derivatives on fatty acid metabolism and hypolipidemic properties, low doses of 2-methyl-, 2-ethyl-, 2,2-dimethyl-, and 3-methyl-EPA as well as EPA, DHA, and Omacor® (combination of EPA and DHA) were administered to rats. This study shows that, at low doses (250 mg/day/kg body weight), methylated EPA potentiated the hypolipidemic effect of EPA, as the order of the lipid-lowering effects of the EPA derivatives was 3-methyl-EPA ≥ 2,2-dimethyl-EPA > 2-ethyl-EPA > 2-methyl-EPA = EPA = CMC. Obviously, the position and length of the alkyl group attached to the EPA molecule was of vital importance for the hypolipidemic effect of the EPA derivatives. The data indicated that a single methyl or ethyl substituent at carbon atom number two was not sufficient to impart hypolipidemic properties to EPA. Two methylations in the 2-position, or one methylation in the 3-position, however, more profoundly potentiated the hypolipidemic effect of EPA. The data suggest that the efficiency of the different EPA derivatives as lipid-lowering agents

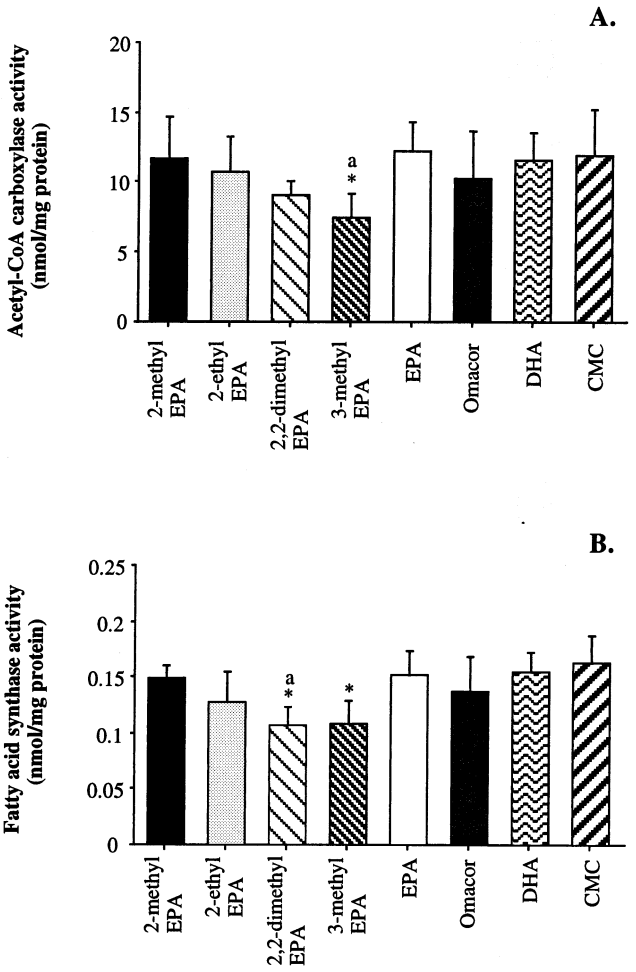

FIG. 6. Activity of the lipogenic enzymes (A) acetyl-CoA carboxylase and (B) fatty-acid synthase. The rats were treated for 5 days with the different fatty acids at a dose of 250 mg/day/kg body weight. The enzyme activities were measured as described in Materials and Methods. Data are given as means ± SD and N = 6. *Significantly different ($P < 0.05$) from CMC, [a]significantly different from EPA.

TABLE 2. The effects of tetradecylthioacetic acid (TTA) and 2-methyl-TTA on liver and plasma parameters

		CMC	TTA	2-Methyl-TTA
CPT-I				
Activity	(nmol/min/mg protein)	1.22 ± 0.07	1.39 ± 0.07	1.47 ± 0.24
mRNA	(relative values)	1.00 ± 0.34	0.77 ± 0.11	0.46 ± 0.06*
CPT-II				
Activity	(nmol/min/mg protein)	6.74 ± 1.40	7.60 ± 1.79	6.68 ± 1.44
mRNA	(relative values)	1.00 ± 0.20	1.21 ± 0.26	1.14 ± 0.26
Mitochondrial β-oxidation				
Palmitoyl-CoA	(nmol/min/mg protein)	0.66 ± 0.08	0.56 ± 0.16	0.62 ± 0.21
Palmitoyl-L-carnitine	(nmol/min/mg protein)	0.62 ± 0.14	0.52 ± 0.15	0.56 ± 0.16
Plasma lipids				
Triglycerides	(mmol/L)	0.84 ± 0.21	0.54 ± 0.12	0.55 ± 0.10
Cholesterol	(mmol/L)	1.45 ± 0.30	1.51 ± 0.34	1.32 ± 0.11
Phospholipids	(mmol/L)	1.36 ± 0.09	1.42 ± 0.38	1.37 ± 0.21
Fatty acyl-CoA oxidase				
Activity	(nmol/min/mg protein)	9.03 ± 0.76	11.04 ± 1.49	17.16 ± 1.02*

The rats were fed TTA or 2-methyl-TTA (15 mg/day/kg body weight) for 7 days. Rats fed CMC were used as control.
The values are presented as means ± SD (N = 4).
*Denote significantly different from CMC ($P < 0.05$).

depends on the methylation and bulkiness, which reduces susceptibility to undergo β-oxidation. Thus, the length of the alkyl group (ethyl instead of methyl) and the bulkiness of the group (two methyl groups instead of one) attached to the EPA molecule in the 2-position was of vital importance for the EPA derivatives as lipid-lowering agents. 3-Methyl-EPA, with a methyl branching in the 3 (β)-position, theoretically cannot be degraded by β-oxidation, but is probably first oxidatively decarboxylated by α-oxidation that yields a 2-methyl branched fatty acid [38]. Several groups have suggested that branched fatty acids are mainly degraded in peroxisomes [39–42], and a specific acyl-CoA oxidase has been identified in the peroxisomes, which metabolize 2-methyl branched fatty acids [40, 41, 43]. These metabolism routes for branched fatty acids may contribute to the degradation of the different methylated EPA derivatives and the 2-methylated TTA. However, it is obvious that branched fatty acids trigger the peroxisomal degradation of fatty acids without branches, as palmitoyl-CoA oxidase is up-regulated. The lipid-lowering effect also depends on the doses of these modified fatty acids, as we have shown earlier that 2-methyl-EPA reduced plasma triglycerides at high doses, but not at 250 mg/day/kg body weight [8].

Stimulated fatty acid oxidation may contribute to the lipid-lowering effect of the EPA derivatives. Indeed, 3-methyl-EPA, and especially 2,2-dimethyl-EPA, induced mitochondrial and peroxisomal β-oxidation, concomitant with the lipid-lowering effect. Increased activities of mitochondrial CPT and possibly the mitochondrial auxiliary enzyme 2,4-dienoyl-CoA reductase were found, and the mRNA levels of these enzymes as well as for FAO were up-regulated. It is therefore likely that the hypolipidemic effect results from increased fatty acid oxidation, which reduces the pool of hepatic fatty acids available for esterification. The increased activity of total CPT in rats fed 2,2-dimethyl-EPA seemed to be due to an increased gene expression of CPT-II, as the CPT-I mRNA level was unchanged.

Increased fatty acid oxidation after administration of methylated EPA was confirmed *in vitro* in cultured hepatocytes. However, *in vitro*, in contrast to the *in vivo* experi-

TABLE 3. Fatty acid composition (mol % of total fatty acids) of phospholipids in liver E fraction

Fatty acid	18:1 n-9	20:5 n-3	22:5 n-3	22:6 n-3	EPA derivatives
2-Methyl-EPA	3.9 ± 0.3	1.0 ± 0.2	1.2 ± 0.1†	9.3 ± 1.1	Trace
2-Ethyl-EPA	3.8 ± 0.3	0.9 ± 0.2	1.1 ± 0.2†	8.8 ± 0.2	Trace
2,2-Dimethyl-EPA	4.2 ± 0.5	0.9 ± 0.6	1.1 ± 0.2†	8.6 ± 0.9	Trace
3-Methyl-EPA	4.5 ± 0.4†	0.8 ± 0.1†	2.1 ± 0.9*	8.8 ± 0.6	0.7 ± 0.1
EPA	3.4 ± 0.5	1.6 ± 0.2	2.5 ± 0.1*	9.0 ± 0.9	
Omacor	3.6 ± 0.3	1.3 ± 0.3	1.7 ± 0.5	8.7 ± 1.5	
DHA	3.5 ± 0.1	1.1 ± 0.2	1.6 ± 0.4	9.2 ± 0.8	
CMC	3.9 ± 0.5	0.9 ± 0.2	1.0 ± 0.0†	8.5 ± 0.6	

Values are expressed as means ± SD for 3–5 animals treated for 5 days with the different fatty acids at a dose of 250 mg/day/kg body weight.
*Significantly different from CMC ($P < 0.05$).
†Significantly different from EPA.

ments, 2-methyl-EPA was the most potent of the EPA derivatives tested. Supplementation of 2-methyl-EPA, but not 2-ethyl-EPA or 3-methyl-EPA, significantly increased the oxidation of radiolabeled palmitic acid compared to EPA or oleic acid supplementation. Furthermore, a negative correlation was obtained between palmitic acid oxidation and synthesis of palmitic acid-labeled triglycerides. Evidently, the increased oxidation of palmitic acid by 2-methyl-EPA was most likely due to increased mitochondrial β-oxidation, as the FAO activity was unchanged (results not shown). In these cells, grown with L-carnitine, 2-methyl-EPA, but not 2-ethyl- or 3-methyl-EPA, significantly lowered the secretion of palmitic acid-labeled triglycerides. The differences between the potency of the various EPA derivatives in the *in vivo* versus the *in vitro* experiments could possibly be due to differences in uptake of these fatty acids in cultured cells versus *in vivo*. Reduced transport over the cell membrane in isolated hepatocytes may be the reason 3-methyl-EPA is not acting *in vitro*, as it is one of the most potent drugs *in vivo*. A recent report by Larsen *et al.* [44], where different EPA derivatives were added to hepatoma cells, further confirms our results that 2-methyl-EPA was more potent than 3-methyl-EPA *in vitro*.

An inhibition of one of the enzymes involved in fatty acid synthesis is consistent with retarded lipogenesis. Acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis, and fatty-acid synthase are both down-regulated by hypolipidemic EPA derivatives. Thus, decreased fatty acid synthesis might also be of importance for the lipid-lowering effect of the EPA derivatives. Whether elevated fatty acid oxidation or down-regulated lipogenesis is the main cause of the hypolipidemic effect of the EPA derivatives was not determined in this study. However, earlier experiments by our group have shown that, at least within 24 hr, the hypolipidemic effect was independent of the lipogenesis [45].

TTA is a peroxisome proliferator with a sulphur atom inserted at the 3-position of the carbon chain, thereby preventing it from being β-oxidized. TTA induces hypolipidemia, and it is possibly the blocking of β-oxidation of this compound that causes fatty acid oxidation to increase. To further potentiate the effects of TTA on peroxisomal β-oxidation, TTA was methylated in the 2-position of the carbon chain. In an attempt to differentiate between the induction of mitochondrial and peroxisomal β-oxidation, we fed rats a very low dose of 2-methyl TTA (15 mg/day/kg body weight). TTA and 2-methyl-TTA at this low dose did not have any effect on mitochondrial β-oxidation, CPT-I or CPT-II activity, or on plasma lipids. On the other hand, peroxisomal FAO activity increased significantly after administration of 2-methyl-TTA but not TTA, compared to control. Thus, the increased peroxisomal β-oxidation (measured as FAO activity) in rats fed 2-methyl-TTA did not cause any hypolipidemic effect compared to TTA-fed rats. According to these findings, it is reasonable to believe that the peroxisomes are of minor importance for the lipid-lowering effect. This study thereby supports the hypothesis by our group that increased mitochondrial β-oxidation, and not peroxisomal β-oxidation, is responsible for the triglyceride-lowering effect [4, 46, 47].

One of the EPA derivatives, 3-methyl-EPA, was incorporated into hepatic phospholipids and changed the hepatic fatty acid composition. *In vitro*, it has recently been shown that 2-methyl- and 2-ethyl-EPA are also able to be incorporated into phospholipids and triglycerides [44]. However, the percentage incorporation was higher for 3-methyl-EPA than for 2-methyl- or 2-ethyl-EPA. It is likely that these EPA derivatives were also incorporated into hepatic phospholipids in rats, but at undetectable levels. The hepatic content of 20:5 n-3 tended to decrease in rats fed 3-methyl-EPA. In contrast, there was a small increase in the amount of 18:1 n-9 in liver. The change in 18:1 n-9 did not correlate to Δ^9-desaturase gene expression. The increased content of 22:5 n-3 by EPA feeding may result from elongation of 20:5 n-3. Elevation of 22:5 n-3 was also observed following 3-methyl-EPA feeding. TTA feeding, but not EPA feeding, increases the content of 18:1 n-9 [47–49]. Furthermore, EPA, but not TTA, has been found to increase the hepatic content of 22:5 n-3 [47–49]. With regard to fatty acid composition, 3-methyl-EPA thereby seems to have the same property as EPA to increase the content of 22:5 n-3, and the same property as a peroxisome proliferator to increase the content of 18:1 n-9.

In summary, we have demonstrated that methylated EPA is more potent than EPA as a hypolipidemic agent. The position and number of the methyl groups on EPA had a great impact on its ability to cause hypolipidemia. The hypolipidemic EPA derivatives simultaneously inhibited lipid synthesis and stimulated oxidation within the hepatocytes, concomitant with lowering of the plasma lipids. 2-Methyl-TTA, which induced peroxisomal β-oxidation but rendered mitochondrial β-oxidation unchanged, caused no lipid-lowering effect. The 2-methyl-TTA experiment supports the hypothesis that the mitochondrion is the primary site for the hypolipidemic effect.

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