In contrast with docosahexaenoic acid, eicosapentaenoic acid and hypolipidaemic derivatives decrease hepatic synthesis and secretion of triacylglycerol by decreased diacylglycerol acyltransferase activity and stimulation of fatty acid oxidation

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Hypolipidaemic fatty acid derivatives and polyunsaturated fatty acids decrease concentrations of plasma triacylglycerol by mechanisms that are not fully understood. Because poor susceptibility to β- and/or ω-oxidation is apparently a determinant of the peroxisome proliferating and hypolipidaemic capacity of fatty acids and derivatives, the relative importance of activation of the peroxisome-proliferator-activated receptor α (PPARα), fatty acid oxidation and triacylglycerol synthesis were examined. We have compared the effects of differentially β-oxidizable fatty acids on these parameters in primary cultures of rat hepatocytes. Tetradecylthioacetic acid (TTA), 2-methyleicosapentaenoic acid and 3-thia-octadecatetraenoic acid, which are non-β-oxidizable fatty acid derivatives, were potent activators of a glucocorticoid receptor (GR)-PPARα chimaera. This activation was paradoxically reflected in an substantially increased oxidation of [1-^{14}C]palmitic acid and/or oleic acid. The incorporation of [1-^{14}C]palmitic acid and/or oleic acid into cell-associated and secreted triacylglycerol was decreased by 15–20% and 30% respectively with these non-β-oxidizable fatty acid derivatives. The CoA ester of TTA inhibited the esterification of 1,2-diacylglycerol in rat liver microsomes. Both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) activated GR–PPARα. EPA increased the oxidation of [1-^{14}C]palmitic acid but DHA had no effect. The CoA ester of EPA inhibited the esterification of 1,2-diacylglycerol, whereas DHA-CoA had no effect. The ratio between synthesized triacylglycerol and diacylglycerol was lower in hepatocytes cultured with EPA in the medium compared with DHA or oleic acid, indicating a decreased conversion of diacylglycerol to triacylglycerol. Indeed, the incorporation of [1-^{14}C]oleic acid into secreted triacylglycerol was decreased by 20% in the presence of EPA. In conclusion, a decreased availability of fatty acids for triacylglycerol synthesis by increased mitochondrial β-oxidation and decreased triacylglycerol formation caused by inhibition of diacylglycerol acyltransferase might explain the hypolipidaemic effect of TTA and EPA.

Key words: mitochondria, peroxisome-proliferator-activated receptor, peroxisomes, tetradecylthioacetic acid.

INTRODUCTION

It has been generally accepted that both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are the major fatty acid constituents of fish oil, have similar effects on plasma triacylglycerols. There is, however, growing evidence that EPA and DHA possess different hypolipidaemic properties. We have reported that EPA is the fatty acid responsible for the triacylglycerol-lowering effect of fish oil in rats [1–3], but the underlying mechanism is not yet understood. However, it is interesting that feeding with EPA but not DHA increases mitochondrial mass [3].

Several studies have indicated that the secretion of triacylglycerol-rich particles is dependent on triacylglycerol synthesis and availability within hepatocytes [4,5]. Because mitochondrial fatty acid oxidation and triacylglycerol biosynthesis are major competitors for the use of fatty acids as substrates in the liver, it is conceivable that plasma triacylglycerol might be altered by factors influencing this balance [6]. In rats, the triacylglycerol-lowering effect of tetradecylthioacetic acid (TTA) [7,8] and EPA [9] is established within hours of feeding; this is accompanied by stimulated mitochondrial fatty acid oxidation, thereby decreasing the hepatic synthesis and secretion of triacylglycerol.

The present study was undertaken to examine whether the β-oxidation stimulated by EPA or TTA influences fatty acid incorporation into cell-associated and secreted triacylglycerol in rat hepatocytes. To evaluate this relationship further, the effect of EPA, TTA and other fatty acids and derivatives were studied under different conditions: without L-carnitine supplement and with a supplement of L-carnitine or L-aminocarnitine, which are known to stimulate or inhibit fatty acid oxidation respectively [8,10,11].

Moreover, TTA [12] and the $n-3$ fatty acids EPA and especially DHA [3] are reported to be peroxisome proliferators. It seems that the minimal structural constraints for a peroxisome proliferator consist of a carboxy group linked to a hydrophobic backbone [12,13]. Poor susceptibility to β- and/or ω-oxidation is apparently a determinant of peroxisome-proliferating capacity. To test the hypothesis that hypolipidaemic fatty acids act because they are poorly oxidized, we have introduced a methyl group at the 2-position of EPA, thereby decreasing its susceptibility to β-oxidation [14]. We have also synthesized polyunsaturated fatty acids, derived from EPA and DHA, that are blocked for β-

Abbreviations used: AP, alkaline phosphatase; DGAT, diacylglycerol acyltransferase; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; GR, glucocorticoid receptor; PPAR, peroxisome-proliferator-activated receptor; THEPEA, 3-thia-heneicosa-6,9,12,15,18-pentaenoic acid; TODTEA, 3-thia-octadeca-6,9,12,15-tetraenoic acid; TTA, tetradecylthioacetic acid.

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oxidation by a sulphur atom at the 3-position. The role of peroxisome-proliferator-activated receptor α (PPARα) activation was tested by comparing the effects of different fatty acids and derivatives on their ability to activate PPARα with their ability to decrease the synthesis and secretion of triacylglycerol. A chimaeric construct of cDNA encoding the ligand-binding domain of rat PPARα and the N-terminal and DNA-binding domains of the human glucocorticoid receptor (GR), together with the gene for placental alkaline phosphatase (AP), was used for transfection studies.

Because it has been demonstrated that EPA and EPA-CoA inhibit diacylglycerol acyltransferase (DGAT) activity, it has been proposed that diets rich in fish oil decrease the concentration of plasma triacylglycerol by inhibition of this enzyme [15–17]. However, no information on the effects of DHA and DHA-CoA on DGAT has been provided. It was of importance to investigate whether EPA-CoA and DHA-CoA affect DGAT differently. The partitioning of phosphatidate phosphohydrolase activity between cytosol and microsomes has been implicated in the regulation of glycerolipid synthesis [18]. We have, however, previously demonstrated that neither fish oil nor pure EPA had any effect on the activity or translocation of phosphatidate phosphohydrolase [9,17].

In the present study we investigate the effect of DHA, EPA and hypolipidaemic derivatives on fatty acid oxidation, triacylglycerol synthesis and secretion, and their ability to activate PPARα.

EXPERIMENTAL

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was obtained from Flow Lab. (Irvine, Ayrshire, U.K.). The medium was supplemented with 20 mM Hepes from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and 2 % (v/v) Ultroser G (a serum substitute) from Gibco, Life Technologies (Gaithersburg, MD, U.S.A.). When indicated, 500 μM L-carnitine from Sigma or 50 μM L-aminocarnitine (L-3-amino-4-trimethylaminobutyrate), a gift from Sigma-Tau S.p.A. (Rome, Italy), was added.

[1-^{14}C]Palmitic acid (58 mCi/mmol), [1-^{14}C]oleic acid (52 mCi/mmol) and L-3-phosphatidylcholine,1,2-di[1-^{14}C]oleoyl (114 Ci/mol) were purchased from Amersham Pharmacia Biotech (Rainham, Essex, U.K.); [1-^{14}C]oleoyl-CoA (60 Ci/mol) and [1,2,3-^{3}H]glycerol (5.0 mCi/mmol) were purchased from New England Nuclear. EPA and DHA were purchased from Sigma; WY 14,643 was obtained from Chemsyn (Lenexa, KA, U.S.A.). TTA [19], 2-methyl-EPA and 3-methyl-EPA [20] were synthesized as described previously. 3-Thia-octadeca-6,9,12,15-tetraenoic acid (TODTEA) and 3-thia-heneicosa-6,9,12,15,18-pentaenoic acid (THEPEA) were a gift from Professor Skattebøl (Department of Organic Chemistry, University of Oslo, Oslo, Norway), synthesized from EPA and DHA respectively as described by Willumsen et al. [21]. Silica-gel F 1500 TLC plates were obtained from Schleicher & Schuell (Dassel, Germany). The CoA esters of TTA, EPA and DHA were synthesized as described by Kawaguchi et al. [22]. All other chemicals and solvents were of reagent grade from common commercial sources.

Animals

Male Wistar rats, weighing 260–300 g, were obtained from Møllegard Breeding Laboratory (Ejby, Denmark). They were housed in pairs in metal wire cages and maintained on a 12 h cycle of light and dark at 20 ± 3 °C (light from 08:00h to 20:00h). The rats had free access to standard rat pellet food and water. They were acclimatized under these conditions for at least 1 week before the experiments. The rats were anaesthetized with 0.2 ml of Hypnorm-Dormicum® (fluanisone-fentylmidazolam) per 100 g body weight before the experiment (between 09:00h and 10:00h). The use of the animals was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Preparation of cultured hepatocytes

Rat liver parenchymal cells were isolated as described by Berry and Friend [23], and as modified by Seglen [24]. The hepatocytes were plated at a density of 2.0×10^6 per dish (1–1.5 mg of cell protein) in 2 ml of DMEM containing 20 mM Hepes and 2 % (v/v) Ultroser G. When indicated, 500 μM L-carnitine or 50 μM L-aminocarnitine was added. Cultures were maintained in a humidified incubator at 37 °C in an air/CO_2 (19:1) atmosphere. After incubation overnight, the medium was replaced with DMEM (2 ml per dish) containing 1-^{14}C-labelled fatty acids (0.25 μCi/ml) bound to BSA. Additions of other fatty acids or [1,2,3-^{3}H]glycerol (5 μCi/ml) to cell cultures are stated in the legends to figures and tables. The molar ratio between fatty acids and BSA was 2.5:1. The cells were incubated for a further 4 or 9 h.

Termination of incubation and harvesting of cells

Incubations were terminated by cooling the culture dishes on ice. Incubation medium was collected and centrifuged at 600 ***g*** for 5 min; the cell-free supernatant was treated as described below. The cells were scraped off the dish with a rubber 'policeman' into 2 ml of PBS, pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4. The culture dishes were washed once with 2 ml of PBS. The cell suspensions were centrifuged and the cells were resuspended in 0.5 or 1.0 ml of distilled water and frozen. A Bio-Rad protein kit (Bio-Rad, Richmond, CA, U.S.A.) was used for protein measurement in the cell suspensions, with BSA dissolved in distilled water as a standard.

Determination of acid-soluble products and CO_2

The cell-free supernatant (250 μM) was precipitated with 1.0 ml of ice-cold 0.75 M $HClO_4$. To secure the complete precipitation of free fatty acids and lipids, 0.45 % BSA was added. The extract was centrifuged at 1800 ***g*** for 10 min; 0.5 ml of the supernatant was assayed for radioactivity by liquid-scintillation counting. $^{14}CO_2$ was trapped from sealed culture flasks essentially as described by Christiansen and Davies [25].

Lipid extraction and quantification

Cellular lipids were extracted from the cell suspensions by the method of Folch et al. [26]. The cell suspension was mixed with 20 vol. of chloroform/methanol (2:1, v/v); 4 vol. of 0.9 % NaCl, pH 2, was added and the mixture was allowed to separate into two phases. The organic phase was evaporated under N_2; the extracted lipids were dissolved in n-hexane and separated by TLC on silica-gel plates developed in hexane/diethyl ether/acetic acid (80:20:1, by vol.). The bands were detected with iodine vapour, cut into pieces and assayed for radioactivity by scintillation counting. To 1 ml of the cell-free medium were added 4 vol. of chloroform/methanol (2:1, v/v) and 2 % (v/v) serum as lipid carrier. The water phase of the medium was re-extracted once with 4 vol. of chloroform/methanol (2:1, v/v), and the combined organic phases were further treated in the same way as the cells.

DGAT activity

DGAT activity was measured with labelled 1,2-dioleoylglycerol as described by Coleman and Bell [27] and by Rustan et al. [16]. The assay was performed at 23 °C in a shaking water bath in a total volume of 0.5 ml of Tris/HCl buffer (175 mM, pH 7.8) containing BSA (15 μM, fatty acid-free), $MgCl_2$ (8 mM) and oleoyl-CoA in the presence or absence of different acyl-CoA derivatives. After 10 min of preincubation, the reaction was started by the addition of rat liver microsomes (10–20 μg/ml protein) followed immediately by 1,2-di[1-^{14}C]oleoylglycerol (125 μM) [dissolved in ethanol, 10 % (v/v) final concentration] and blending on a vortex mixer. The reaction was terminated after 10 min by the addition of 10 ml of chloroform/methanol (2:1, v/v) and the lipids were extracted and separated by TLC as described above. The triacylglycerol band was cut out and the amount of ^{14}C was determined by scintillation counting. 1,2-Di[1-^{14}C]oleoylglycerol was prepared from L-3-phosphatidylcholine,1,2-di[1-^{14}C]oleoyl by treatment with purified phospholipase C, as described elsewhere [28]. More than 98 % of the labelled phosphatidylcholine,1,2-dioleoyl was converted to 1,2-dioleoylglycerol (0.92–1.1 μCi/ml) by this method. The dioleoylglycerol was extracted twice with diethyl ether, redissolved in ethanol and stored at −20 °C.

Transfected reporter cells and transactivation assay

The reporter cell line, culture conditions and cell treatment were as described [29]. In brief, the cDNA for a secreted form of human placental AP under the control of the GR-dependent MMTV promotor was integrated into Chinese hamster ovary (CHO) cells as a reporter gene. Additionally, a chimaeric receptor encompassing the N-terminal transactivating and DNA-binding domains of GR and the ligand-binding domain of PPARα was expressed. Thus the activation of the PPARα ligand binding domain was detectable by increased reporter enzyme activity in the cell culture supernatant. Polyunsaturated fatty acids were added as liquids to culture medium containing 10 % (v/v) fetal bovine serum (FBS) and were suspended by sonication. Serial dilutions were prepared in cell culture medium. Sulphur-substituted fatty acids were added to the cell culture medium as 400–1000-fold concentrated stock solutions in DMSO. H_2O_2 was added to the culture medium as a 30 % (v/v) solution in water. After 48 h of exposure to the test compounds, heat-stable placental AP activity in the cell culture supernatant was determined in a colorimetric assay, as described previously [29].

Activation of the GR–PPARα chimaera in primary rat hepatocytes

Primary hepatocytes were seeded on 60 mm diameter culture dishes at a density of 10^6 per dish in 3 ml of DMEM/Ham's F12 medium (1:1). The medium was supplemented with insulin (0.1 μg/ml), 3,3′,5-tri-iodothyronine (1 nM), vitamin C (280 μM), penicillin (100 i.u./ml), streptomycin (100 μg/ml) and 5 % (v/v) FBS. After the first 24 h in culture, the FBS content was decreased to 2.5 % and after an additional 24 h to 1.25 %. Cultures were maintained at 37 °C in a humidified air/CO_2 (19:1) atmosphere. After 48 h in culture, cells were transfected with 1 μg of the reporter plasmid pMMTV-AP (in which MMTV stands for murine-mammary-tumour virus) and 3 μg of the expression vector pMT-GR–PPARα [29] with 25 μl of the cationic lipid DOTAP liposomal transfection reagent (Boehringer Mannheim, Germany) in 3 ml of cell culture medium containing 1.25 % (v/v) FBS, 0.1 % BSA, 0.1 % (v/v) DMSO and the fatty acids. AP activity in the cell culture supernatant was determined 30 h after the addition of fatty acids [13].

Statistical analysis

Each experiment was run with triplicate culture dishes for each determination, and results are means ± S.D. for three to five independent experiments, as indicated in the legends to figures and tables. Where relevant, results are considered statistically significant when $P < 0.05$ (analysis of variance or t test).

RESULTS

Activation of the GR–PPARα chimaera by $n-3$ fatty acids and hypolipidaemic derivatives

The triacylglycerol-lowering effect of peroxisome proliferators seems to be mediated via PPARα, because the peroxisome proliferator WY 14.643 fails to decrease plasma triacylglycerol in PPARα-deficient mice [30]. Polyunsaturated fatty acids fulfil the structural requirement for PPARα activation; it was therefore of interest to test the ability of the $n-3$ fatty acids and their derivatives to activate the GR–PPARα chimaera. Although both EPA and DHA were able to activate the GR–PPARα chimaera at similar concentrations, lower concentrations of 2-methyl EPA were required (Figure 1). In agreement with earlier findings, the non-β-oxidizable fatty acid analogue TTA induced the reporter activity at concentrations comparable to those of WY 14.643 (Figure 1). Interestingly, the saturated sulphur-substituted fatty acid TTA activated the GR–PPARα chimaera as least as effectively as the polyunsaturated 3-sulphur-substituted fatty acid, TODTEA, prepared from EPA (Figure 1).

Effect of $n-3$ fatty acids and hypolipidaemic derivatives on total metabolism and oxidation of [1-^{14}C]palmitic acid and [1-^{14}C]oleic acid

Treatment of rats with PPARα activators influence hepatic fatty acid metabolism [31]. The metabolism of [1-^{14}C]palmitic acid and [1-^{14}C]oleic acid was therefore studied in the presence of PPARα-

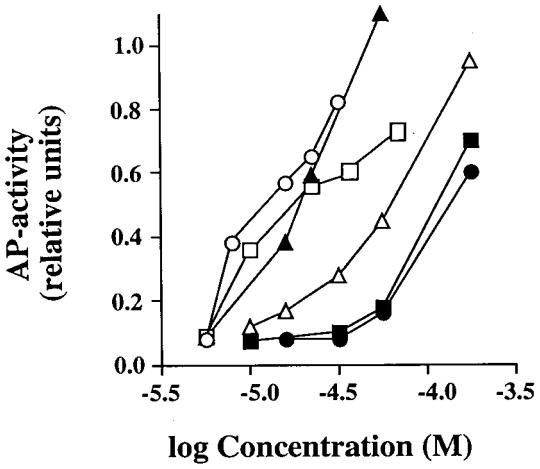

Figure 1 Effect of different fatty acids on the activation of the GR–PPARα chimaeric receptor

Activation of the GR–PPARα chimaeric receptor by DHA (■), EPA (●), 2-methyl-EPA (△) and TODTEA (□) and TTA (○) compared with that by WY 14.643 (▲). Primary hepatocytes were transfected with 1 μg of the reporter plasmid pMMTV-AP and 3 μg of the expression vector pMT-GR–PPARα by using 25 μl of the cationic lipid DOTAP in 3 ml of cell culture medium containing 1.25% (v/v) FBS, 0.1% BSA, 0.1% (v/v) DMSO and the fatty acids, as explained in the Experimental section. AP activity in the cell culture supernatant was determined 30 h after the addition of fatty acids. SDs were less than 15%.

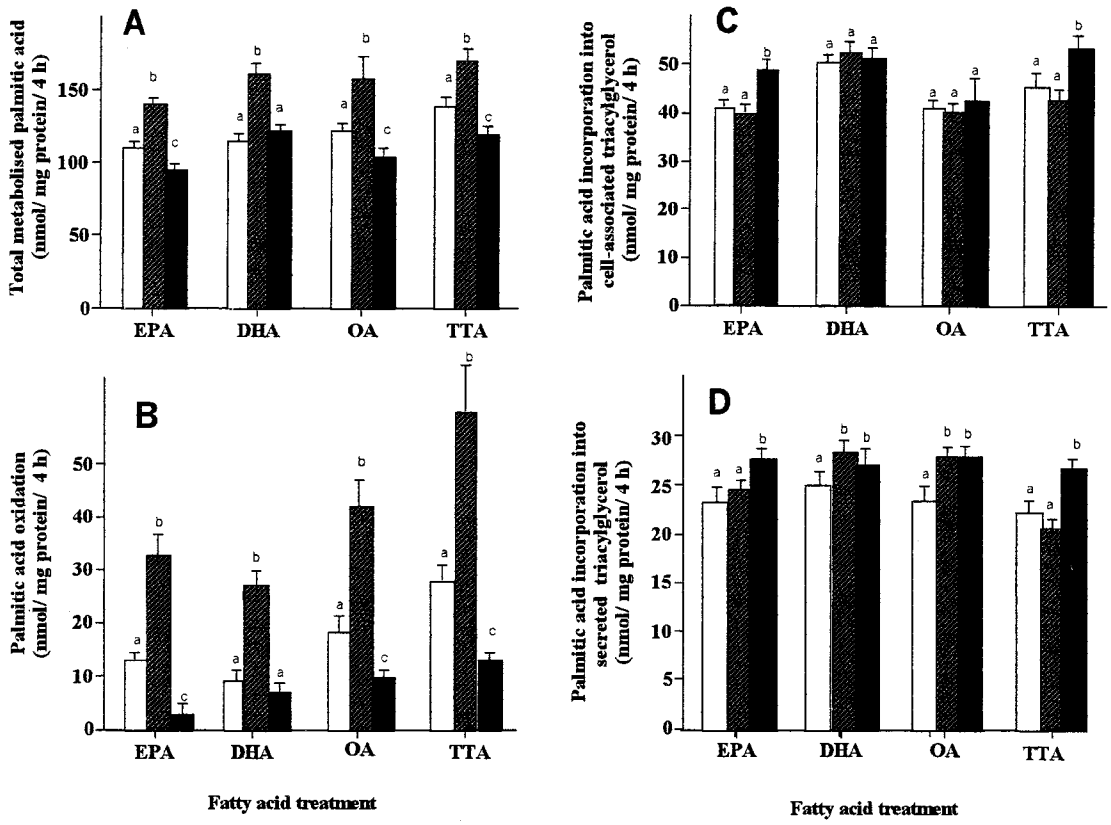

Figure 2 Effects of different fatty acids on total metabolism (sum of acid-soluble products and incorporation into glycerolipids) (A), the formation of acid-soluble products of fatty acids (B), and the incorporation of [1-^{14}C]palmitic acid into cell-associated (C) and secreted (D) triacylglycerol in the presence of L-carnitine or L-aminocarnitine

To cultured hepatocytes was added 200 μM [1-^{14}C]palmitic acid (0.25 μCi/ml) in the presence of 200 μM EPA, DHA, oleic acid (OA) or TTA, then incubated for 4 h as described in the Experimental section. The medium contained no addition (open bars), 0.5 mM L-carnitine (grey bars) or 50 μM L-aminocarnitine (black bars). The ratio of fatty acid to BSA was 2.5:1. Each experiment was run with triplicate culture dishes; results are means $\pm$ S.D. for three to five independent experiments. Values without a common superscript within an experimental group were significantly different at $P < 0.05$ (analysis of variance).

Table 1 Effect of fatty acids and derivatives on metabolism of [1-^{14}C]oleic acid in hepatocytes cultured with L-carnitine

Cells were plated overnight in DMEM supplemented with 0.5 mM L-carnitine, then incubated for 4 h with 100 μM [1-^{14}C]oleic acid (0.25 μCi/ml) in the absence and the presence of 100 μM additional fatty acids and derivatives, as explained in the Experimental section. The ratio of fatty acid to BSA was kept constant at 2.5:1. Each experiment was run with triplicate culture dishes for every value determined; results are means $\pm$ S.D. for three to five independent experiments. *$P < 0.05$ (t test) compared with control (no addition).

	[1-^{14}C]Oleic acid metabolized (nmol/4 h per mg of cell protein)						
	No addition	Palmitic acid	EPA	2-methyl-EPA	TTA	TODTEA	THEPEA
[1-^{14}C]Oleic acid metabolized	105 $\pm$ 10	102 $\pm$ 13	88 $\pm$ 10	94 $\pm$ 7	95 $\pm$ 17	96 $\pm$ 17	83 $\pm$ 12
Acid-soluble products	6.4 $\pm$ 0.5	6.2 $\pm$ 1.1	9.2 $\pm$ 0.5*	22.4 $\pm$ 1.2*	21.0 $\pm$ 0.9*	19.7 $\pm$ 2.3*	12.9 $\pm$ 1.3*
[1-^{14}C]Oleic acid-labelled cell associated lipids							
Triacylglycerol	46 $\pm$ 3	46 $\pm$ 5	44 $\pm$ 2*	34 $\pm$ 2*	39 $\pm$ 3*	37 $\pm$ 2*	22 $\pm$ 1*
Phospholipids	8.9 $\pm$ 0.8	8.2 $\pm$ 0.9	11.1 $\pm$ 0.9*	13.4 $\pm$ 0.7*	9.4 $\pm$ 0.4	12.0 $\pm$ 0.5*	12 $\pm$ 0.3*
[1-^{14}C]Oleic acid-labelled secreted lipids							
Triacylglycerol	31 $\pm$ 4	28 $\pm$ 2	22 $\pm$ 1*	18 $\pm$ 2*	19 $\pm$ 1*	18 $\pm$ 2*	21 $\pm$ 2*

activating fatty acids. In the presence of L-carnitine, the total metabolism (the sum of the incorporated radioactivity into acid-soluble products and glycerolipids) of [1-^{14}C]palmitic acid (Figure 2) and [1-^{14}C]oleic acid (Table 1) was comparable in all experiments.

As previous work has suggested that increased mitochondrial fatty acid oxidation is an important factor in the hypotriacylglycerolaemic effect of TTA [8,32–34] and EPA [2,3,9], it was of further interest to study the metabolism of [1-^{14}C]palmitic acid and [1-^{14}C]oleic acid in the presence of EPA and TTA in cultured

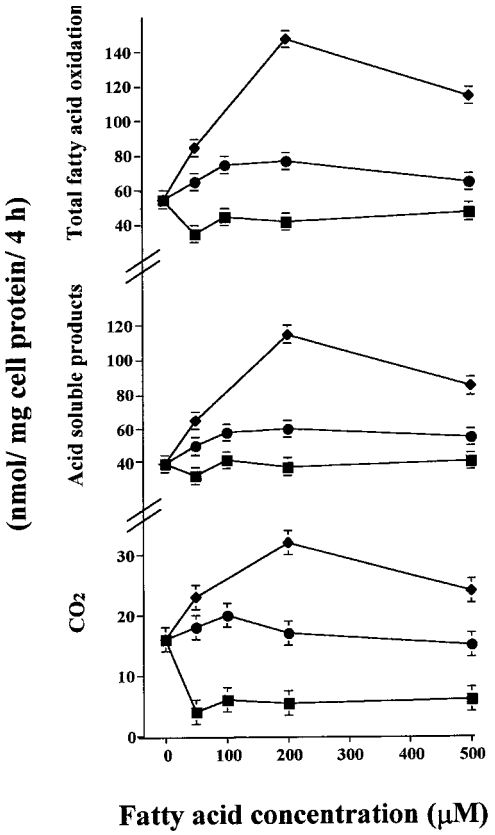

Figure 3 Effect of different concentrations of fatty acids on the formation of acid-soluble products and CO_2 in the presence of L-carnitine

The formation of acid-soluble products, CO_2 and the sum of the acid-soluble products and CO_2 from 200 μM [1-^{14}C]palmitic acid (0.25 μCi/ml) in the presence of 50, 100, 200 and 500 μM EPA (●), DHA (■) or TTA (◆) by cultured hepatocytes incubated for 4 h as described in the Experimental section. The medium contained 0.5 mM L-carnitine. The ratio of fatty acid to BSA was 2.5:1. Each experiment was run with triplicate culture dishes; results are means ± S.D. for three to five independent experiments.

hepatocytes. Transport of fatty acids into the mitochondria before oxidation is dependent on L-carnitine [10]. Thus the removal of carnitine from the medium leads to decreased mitochondrial β-oxidation and total metabolism of [1-^{14}C]palmitic acid (Figures 2B and 2A). In the presence of 200 μM DHA, the addition of the mitochondrial carnitine palmitoyltransferase II inhibitor L-aminocarnitine did not further decrease either β-oxidation or the metabolism of [1-^{14}C]palmitic acid (Figures 2B and 2A). However, the addition of L-aminocarnitine to the cells decreased both β-oxidation and the metabolism of [1-^{14}C]palmitic acid in the presence of EPA or TTA at 200 μM (Figure 2As and 2B). In the presence of L-carnitine, the oxidation of [1-^{14}C]palmitic acid to CO_2 and acid-soluble products increased after the addition of EPA and TTA compared with DHA (Figure 3).

The sulphur 3-substituted fatty acid THEPEA, prepared from EPA, TODTEA and DHA, increased the oxidation of [1-^{14}C]oleic acid more than EPA (Table 1). Moreover, the 2-alkyl-substituted EPA derivative potentiated the oxidation of oleic acid compared with EPA (Table 1).

We have previously shown that the effects of fatty acids on fatty acid oxidation and the synthesis and secretion of triacylglycerol are saturated by 100–200 μM [8].

Table 2 Effect of different acyl-CoA derivatives on the esterification of 1,2-diacylglycerol in rat liver microsomes in the presence of oleoyl-CoA

The esterification of diacylglycerol was assayed with labelled diacylglycerol as described in the Experimental section. The incubation mixture contained Tris/HCl (175 mM, pH 7.8), BSA (15 μM, fatty acid-free), $MgCl_2$ (8 mM) and oleoyl-CoA (20 μM) in the presence of different acyl-CoA derivatives (20 μM). After a 10 min preincubation, rat liver microsomes (10 μg/ml protein) and 1,2-di[1-^{14}C]oleylglycerol (approx. 0.1 μCi/ml; 125 μM) were added and incubation was continued for 10 min. The lipids were extracted and separated by TLC; the triacylglycerol band was cut out and scintillation counted. Results, expressed as a percentage of control, are means ± S.D. for triplicate samples for at least three separate experiments. The control value was 35.7 ± 1.9 nmol triacylglycerol/10 min per mg of microsomal protein. $^*P < 0.05$ (*t* test) compared with control.

Addition to 20 μM oleyl-CoA	Triacylglycerol produced (% of control)
None (control)	100.0 ± 5.4
Palmitoyl-CoA (20 μM)	87.8 ± 2.4
Stearoyl-CoA (20 μM)	78.9 ± 2.8
Oleyl-CoA (20 μM)	99.7 ± 8.7
Linoleyl-CoA (20 μM)	74.7 ± 5.0
Linolenoyl-CoA (20 μM)	74.5 ± 0.2
Arachidonoyl-CoA (20 μM)	75.8 ± 9.7
EPA-CoA (20 μM)	23.8 ± 8.5*
DHA-CoA (20 μM)	88.3 ± 1.7
TTA-CoA (20 μM)	42.3 ± 12.4*

Effect of *n* − 3 fatty acids and derivatives on synthesis and secretion of triacylglycerol from [1-^{14}C]palmitic acid, [1-^{14}C]oleic acid and [^{3}H]glycerol

Increased oxidation of [1-^{14}C]palmitic acid by the addition of EPA and TTA, but not DHA (Figure 3), might decrease the availability of palmitic acid for triacylglycerol synthesis and secretion. The finding that EPA and TTA, but not DHA or oleic acid, also decreased the incorporation of [^{3}H]glycerol by 40–50% suggests that triacylglycerol synthesis was inhibited by these two fatty acids (results not shown). Moreover, TTA, EPA, 2-methyl EPA and the sulphur-substituted polyunsaturated fatty acids TODTEA and THEPEA, which led to an increased oxidation of [1-^{14}C]oleic acid, caused a statistically significant decrease in the synthesis and secretion of oleic acid-labelled triacylglycerol compared with the addition of palmitic acid (Table 1). It is also noteworthy that the inhibition of mitochondrial β-oxidation by L-aminocarnitine increased the incorporation of [1-^{14}C]palmitic acid into both cell-associated (Figure 2C) and secreted (Figure 2D) triacylglycerol in the presence of TTA and EPA but not of DHA.

Inhibition of DGAT by TTA-CoA and EPA-CoA but not by DHA-CoA

Because DGAT is the only enzyme exclusively involved in triacylglycerol synthesis, it possesses considerable potential for specific regulation [35,36]. The present study demonstrates that TTA-CoA and EPA-CoA, but none of the other acyl-CoA derivatives examined, including DHA-CoA, decreased the formation of triacylglycerol from 1,2-[^{14}C]diacylglycerol by 55–75% in isolated microsomes (Table 2). Similarly, the incorporation of [1-^{14}C]oleoyl-CoA into triacylglycerol was decreased by 50–60% when EPA-CoA or TTA-CoA, but not DHA-CoA, was added to isolated rat liver microsomes (results not shown). Moreover, Figure 4 shows that the ratio of triacyl[^{3}H]glycerol to

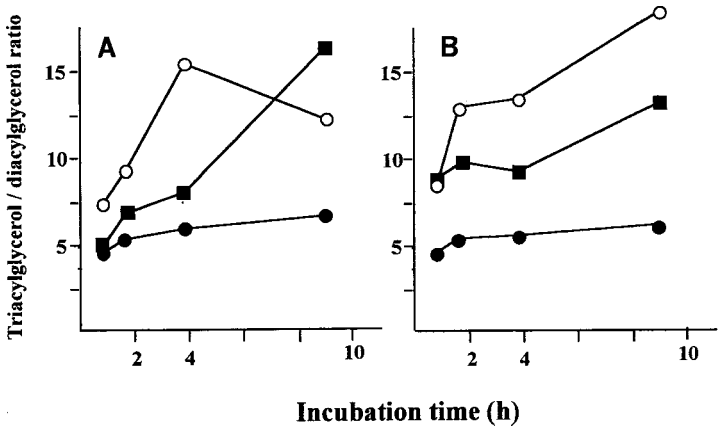

Figure 4 Effect of different fatty acids on the ratio of triacyl[^{3}H]glycerol to diacyl[^{3}H]glycerol

Cells were plated overnight in DMEM supplemented with 0.5 mM L-carnitine and incubated for 1–9 h with 200 μM palmitic acid and 25 μM [^{3}H]glycerol (5 μCi/ml) in the presence of 50 μM (**A**) or 500 μM (**B**) EPA (●), DHA (■) or oleic acid (○), as described in the Experimental section. The ratio of fatty acid to BSA was 2.5:1. Results are presented as the ratio of triacyl[^{3}H]glycerol to diacyl[^{3}H]glycerol. Each experiment was run with triplicate culture dishes; results are means ± S.D. for three independent experiments.

diacyl[^{3}H]glycerol was lower in the presence of EPA than with DHA and oleic acid. This ratio was also markedly decreased by TTA (results not shown), suggesting that the last step in triacylglycerol synthesis might be inhibited by EPA and TTA.

DISCUSSION

This study demonstrates that EPA and hypolipidaemic fatty acid derivatives, such as the non-β-oxidizable sulphur-substituted fatty acid TTA, activated the GR–PPARα chimaera, stimulated the oxidation of [1-^{14}C]palmitic acid and [1-^{14}C]oleic acid and decreased the secretion of oleic acid- and palmitic acid-labelled triacylglycerol in cultured rat hepatocytes. Moreover, EPA-CoA and TTA-CoA, but not DHA-CoA, decreased the incorporation of 1,2-di[1-^{14}C]oleoylglycerol and [1-^{14}C]oleoyl-CoA into triacylglycerol in isolated rat liver microsomes. DHA activated the GR–PPARα chimaera as effectively as EPA but did not influence triacylglycerol metabolism.

It has been demonstrated that the PPARα activator WY 14.643 fails to decrease plasma triacylglycerol in PPARα-deficient mice [30], suggesting that PPARα activation might be necessary to decrease plasma triacylglycerol concentrations. A functional carboxy group connected to a hydrophobic backbone that is not easily oxidized characterizes activators of PPARα. Thus polyunsaturated fatty acids that are poorly oxidizable compared with saturated fatty acids fulfil the structural and metabolic requirements as activators of PPARα [13]. Both EPA and DHA did indeed activate the GR–PPARα chimaera (Figure 1). Introducing a methyl group in the 2-position of EPA would theoretically decrease its ability to undergo β-oxidation; thus a lower concentration to activate the GR–PPARα chimaera was required when EPA was methylated (Figure 1). Moreover, sulphur substitution in the 3-position, which blocked β-oxidation, decreased the required concentration further (Figure 1). The finding that TTA was as potent as the sulphur-substituted polyunsaturated fatty acid TODTEA (Figure 1) suggests that the ability of fatty acids and derivatives to activate PPARα depends on a poor susceptibility to β-oxidation rather than polyunsaturation.

The activation of PPARα increases the transcriptional rates of several genes involved in both peroxisomal and mitochondrial β-oxidation [31]. Thus, the treatment of rats with peroxisome proliferators such as fibrates and TTA increases the hepatic rate of fatty acid oxidation [3]. This study demonstrates that sulphur-substituted fatty acids and poorly oxidizable fatty acids that activate PPARα, such as EPA and its derivatives, increase the oxidation of oleic acid and palmitic acid in cultured hepatocytes (Figures 2 and 3 and Table 1). However, it was interesting to note that the sulphur-substituted saturated fatty acid TTA increased fatty acid oxidation more effectively than the sulphur-substituted polyunsaturated fatty acids (Table 1). DHA also activated the GR–PPARα chimaera as effectively as EPA but did not influence fatty acid oxidation, suggesting that this acute effect might be caused by additional mechanisms.

In the presence of L-carnitine, EPA and TTA increased the oxidation of palmitic acid compared with DHA (Figures 2 and 3). Because the potent carnitine palmitoyltransferase II inhibitor, L-aminocarnitine, prevented EPA and TTA from stimulating the oxidation of palmitic acid, it is likely that EPA and TTA stimulated mitochondrial fatty acid oxidation rather than peroxisomal oxidation (Figure 2B). In contrast, in the presence of DHA, neither the total metabolism nor the oxidation of palmitic acid was influenced by addition of L-aminocarnitine (Figures 2A and 2B). Because peroxisomal fatty acid oxidation, in contrast with mitochondrial oxidation, is independent of carnitine [37], it is likely that DHA influences the peroxisomal rather than the mitochondrial system. This is in agreement with previous findings showing that DHA is a more potent peroxisome proliferator than EPA [3].

It has previously been shown that the secretion of very-low-density lipoprotein particles is dependent on the synthesis and availability of triacylglycerol within the hepatocytes [4,5]. Thus the acute stimulation of fatty acid oxidation might decrease the availability of fatty acids for the synthesis and secretion of triacylglycerol. In the presence of L-carnitine, the distribution of [1-^{14}C]oleic acid and [1-^{14}C]palmitic acid was shifted from incorporation into triacylglycerol towards oxidation, when TTA, EPA and its derivatives were added to cells, compared with the addition of DHA (Figures 2 and 3 and Table 1). This effect was prevented by L-aminocarnitine, suggesting that increased mitochondrial β-oxidation and triacylglycerol synthesis might be inversely co-ordinated. The addition of L-aminocarnitine did not influence neither the oxidation of [1-^{14}C]palmitic acid nor the

incorporation of [1-^{14}C]palmitic acid into secreted triacylglycerol in the presence of DHA (Figure 2). Thus, increased peroxisomal β-oxidation and decreased plasma triacylglycerol concentrations seem to be unrelated events [1,3,7], and DHA shows no hypotriacylglycerolaemic effect in rats [1,3]. Moreover, the synthesis of triacylglycerol from [^{3}H]glycerol was also decreased in the presence of EPA and TTA, compared with DHA and oleic acid.

EPA and TTA might further decrease the availability of triacylglycerol for very-low-density lipoprotein secretion owing to their ability to inhibit triacylglycerol synthesis. The finding that the addition of EPA and TTA to the cells decreased the ratio of synthesized triacylglycerol to diacylglycerol compared with oleic acid and DHA (Figure 4), suggests that the last step in triacylglycerol synthesis was inhibited. The finding that TTA-CoA and EPA-CoA, but not DHA-CoA, also decreased DGAT activity in isolated liver microsomes (Table 2) further supports this hypothesis.

We conclude that an acute stimulation of mitochondrial fatty acid oxidation, thereby decreasing the availability of fatty acids for synthesis and secretion of triacylglycerol, might explain the hypotriacylglycerolaemic effect of TTA [8,32–34], EPA [9], 2-methyl-EPA [20] and TODTEA [21]. The activation of PPARα might be necessary, but not sufficient, to decrease plasma triacylglycerol levels. The inhibition of DGAT by EPA and TTA and their CoA derivatives further decreases hepatic triacylglycerol synthesis. In contrast, DHA did not influence fatty acid oxidation capacity, triacylglycerol metabolism or DGAT-activity. It is therefore likely that the triacylglycerol-lowering effect of fish oil occurs independently of DHA.

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