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Early Modulation of Genes Encoding Peroxisomal and Mitochondrial β-Oxidation Enzymes by 3-Thia Fatty Acids

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ABSTRACT. The aim of the present study was to elucidate the effects of a single dose of 3-thia fatty acids (tetradecylthioacetic acid and 3-thiadicarboxylic acid) over a 24-hr study period on the expression of genes related to peroxisomal and mitochondrial β-oxidation in liver of rats. The plasma triglyceride level decreased at 2–4 hr, 4–8 hr, and 8–24 hr, respectively, after a single dose of 150, 300, or 500 mg of 3-thia fatty acids/kg body weight. Four to eight hours after administration of 3-thia fatty acids, a several-fold-induced gene expression of peroxisomal multifunctional protein, fatty acyl-CoA oxidase (EC 1.3.3.6), fatty acid binding protein, and 2,4-dienoyl-CoA reductase (EC 1.3.1.43) resulted, concomitant with increased activity of 2,4-dienoyl-CoA reductase and fatty acyl-CoA oxidase. The expression of carnitine palmitoyltransferase-I and carnitine palmitoyltransferase-II increased at 2 and 4 hr, respectively, although at a smaller scale. In cultured hepatocytes, 3-thia fatty acids stimulated fatty acid oxidation after 4 hr, and this was both L-carnitine- and L-aminocarnitine-sensitive. The hepatic content of eicosapentaenoic acid and docosahexaenoic acid decreased throughout the study period. In contrast, the hepatic content of oleic acid tended to increase after 24 hr and was significantly increased after repeated administration of 3-thia fatty acids. Similarly, the expression of Δ^9-desaturase was unchanged during the 24-hr study, but increased after feeding for 5 days. To conclude, carnitine palmitoyltransferase-I expression seemed to be induced earlier than 2,4-dienoyl-CoA reductase and fatty acid binding protein, and not later than the peroxisomal fatty acyl-CoA oxidase. The expression of Δ^9-desaturase showed a more delayed response. BIOCHEM PHARMACOL **56**;12:1571–1582, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. mRNA; mitochondrial and peroxisomal β-oxidation; 3-thia fatty acids; acid-soluble CoAs; triglycerides; fatty acid composition

The effects of the 3-thia fatty acids TTA‖ (CH_3-$(CH_2)_{13}$-S-CH_2-COOH) and TD (HOOC-CH_2-S-$(CH_2)_{10}$-S-CH_2-COOH) on lipid metabolism have been extensively studied [1–4]. TTA is blocked for β-oxidation, while TD is blocked for both β- and ω-oxidation. When fed to rats, the 3-thia fatty acids are rapidly absorbed [2], activated to their CoA esters [3] and incorporated into hepatic lipids, especially phospholipids [2]. The 3-thia fatty acids reduce plasma triglycerides and cholesterol levels [4]. Both mitochondrial and peroxisomal β-oxidation are induced by 3-thia fatty acids [4] as are several enzymes involved in oxidation of fatty acids, for instance FAO (EC 1.3.3.6) [4]. On the contrary, the lipogenic enzymes are reported to be down-regulated after repeated administration of 3-thia fatty acids [4].

We have previously demonstrated that a single oral dose of 3-thia fatty acids (150 mg/kg body weight) increased the peroxisomal and mitochondrial β-oxidation and lowered the triglyceride level within 24 hr, while the activities of key enzymes involved in lipogenesis were mainly unchanged [2]. Thus, the triglyceride-lowering effect of the 3-thia fatty acids after acute administration was independent of decreased lipogenesis. Furthermore, there was a correlation between the increase in cytochrome P450IVA1 and FAO mRNA levels and the induction of their corresponding enzyme activities [5].

The effect of 3-thia fatty acids on the transcription of several enzymes in the fatty acid oxidation pathway remains to be elucidated. This study describes the effects of a single dose of 3-thia fatty acids on the expression of rat hepatic genes related to peroxisomal and mitochondrial β-oxidation and on the activity of the corresponding enzymes. The

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‖ *Abbreviations*: CMC, sodium carboxymethyl-cellulose; CPT, carnitine palmitoyltransferase; FABP, fatty acid binding protein; FAO, fatty acyl-CoA oxidase; PPAR, peroxisome proliferator-activated receptor; TD, 3-thiadicarboxylic acid; TTA, tetradecylthioacetic acid.

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effects of a single dose of 3-thia fatty acids on gene expression were compared to repeated administration of 3-thia fatty acids to rats.

MATERIALS AND METHODS

Chemicals

Palmitic acid was purchased from Sigma. TD and TTA were synthesized as described by Spydevold and Bremer [6]. [α-^{32}P] dCTP (specific activity 3000 Ci/mmol) was from Amersham. Other isotopes were purchased from New England Nuclear. Restriction enzymes were from Promega. Slot blot equipment and nylon membranes (NY 13N) were from Schleicher & Schuell. 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 150–190 g were housed in metal wire cages in a room maintained at 12-hr light-dark cycles (light 7 a.m-7 p.m) and a constant temperature of 20°. The animals were acclimatized for 1 week under these conditions before the start of the experiment. Palmitic acid and the 3-thia fatty acids were suspended in 0.5% CMC. The individual agents were administrated by oro-gastric intubation at a dose of 150, 300, or 500 mg/kg body weight in a volume of 1 mL. Where appropriate, the control animal group received either CMC or palmitic acid at a corresponding dose. All animals had free access to water and food (R34-EWOS-ALAB grower rat maintenance chow from Ewos, Sweden). There were three to six animals in each group. In the single dose experiment, the rats were anaesthetized with 0.2 mL/100 g of Hypnorm Dormicum® (Fentanyl/fluanisone-Midazolam) 2, 4, 8, 12, and 24 hr after administration of the fatty acids. In the repeated administration experiments, rats were fed TTA (150 mg/day/kg body weight) once a day for 5 days, 1 week, or 12 weeks. At the end of the feeding period, after overnight starvation, the animals were anaesthetized as described above. Cardiac puncture was performed to obtain blood samples. The livers were removed, one part of which was immediately frozen in liquid nitrogen and stored at −80°, and the other part chilled on ice. The protocol was approved by the Norwegian State Board of Biologic Experiments with Living Animals.

Lipid Analysis

Plasma was prepared by centrifugation of whole blood at 1,000 *g* for 10 min. Triglyceride, cholesterol, and phospholipid were measured using the Monotest triglyceride, cholesterol, and phospholipid enzymatic kit (Boehringer Mannheim).

Determination of Acid-Soluble CoA Esters

The amounts of acid-soluble CoA esters in liver were measured by reverse-phase HPLC as described by Demoz *et al.* [7].

Preparation of Hybridization Probes

DNA fragments were labeled by random priming using the oligolabeling technique of Feinberg and Vogelstein [8]. The DNA probes were purified fragments of cloned rat genes. FAO cDNA: 1400 bp *Pst*I fragment of pMJ125 [9]; peroxisomal multifunctional protein cDNA: 1072 bp *Hind*III-insert in pGEM-4Z [10]; CPT-I cDNA: 1600 bp *Eco*RI fragment of pBK2-CPT I [11]; CPT-II cDNA: 1600 bp *Xho*I/*Xba*I fragment of pBKS-CPT II.4 [12]; 2,4-dienoyl-CoA reductase cDNA: 632 bp *Pst*I/*Eco*RI insert in pGEM-4Z [13]; FABP cDNA: 334 bp *Pvu*II-*Eco*RI fragment in pJG418 [14]; Δ^9-desaturase cDNA: 358 bp *Bgl*II-*Ava*I fragment of pDs3 [15]; Δ^3-Δ^2-Enoyl-CoA isomerase cDNA: 920 bp *Bam*HI-insert in pUEX [16]. Mouse PPARα cDNA: 1840 bp *Eco*RI-fragment (clone 54) in pSG5 [17]. Human 28S rRNA: 1400 bp *Bam*HI fragment of pA [18].

RNA Purification and Analysis

Total cellular RNA was isolated by the guanidinium-thiocyanate method described by Chomczynski and Sacchi [19]. The RNA concentrations were determined spectrophotometrically. Slot blotting of RNA onto nylon was carried out as described by Aasland *et al.* [20]. Hybridization to immobilized RNA was performed as described by Sambrook *et al.* [21], in the presence of 50% formamide, 5× 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 (1× SSC: 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7). Filters were washed to high stringency (0.2 × SSC, 0.1% (w/v) NaPPi, 0.1% (w/v) SDS at 65°). Kodak XAR-5 x-ray films were exposed to the membranes at −80° in the presence of intensifying screens. Suitably exposed autoradiograms were analyzed by densitometric scanning using the LKB Ultrogel laser-densitometer, or the hybridized membranes were analyzed by direct measurement of cpm on an Instant Imager, Electronic Autoradiography (Packard). 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 hybridization in the individual samples. Relative mRNA inductions of the different genes were then calculated.

Preparation of Subcellular Fractions

The livers were homogenized in ice-cold sucrose-medium (0.25 M sucrose in 10 mM HEPES buffer pH 7.4 and 1 mM EDTA) [22] and centrifuged at 600 *g* for 10 min to separate

postnuclear (E) from nuclear fraction. Further analytical differential centrifugation of the postnuclear fraction at 14,000 *g* for 30 min resulted in a mitochondrial- and peroxisomal-enriched fraction (M + L). A microsomal-enriched fraction (P) was isolated from the post- M + L fraction at 100,000 *g* for 1 hr. The remaining supernatant was used as cytosolic-enriched fraction (S) [22]. The procedure was performed at 0–4°, and the fractions were stored at −80°.

Determination of Enzyme Activities

FAO activity was determined in the M + L fraction of rat livers by the coupled assay described by Small *et al.* [23]. 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 M + L-fraction using palmitoyl-CoA and radiolabeled carnitine for production of butanol-soluble radiolabeled palmitoylcarnitine [24]. 2,4-dienoyl-CoA reductase activity was measured in the M + L fraction by the method of Kunau and Dommes [25] with some minor modifications. The assay solution contained 50 mM KH_2PO_4/K_2HPO_4, pH 7.4, 0.01% (w/v) Triton X-100, 100 μM NADPH, and 100 μg of protein. The assay was started by the addition of 100 μM 2-trans, 4-cis-decadienoyl-CoA.

Hepatocyte Isolation and Culture Conditions

Rat liver parenchymal cells were isolated as described by Berry and Friend [26] with modifications according to Seglen [27]. 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 containing 20 mM HEPES, 2% Ultroser G, 50 μg/mL of gentamicin, with or without 500 μM L-carnitine or 50 μM L-aminocarnitine. 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 Dulbecco's modified Eagle's medium containing 20 mM HEPES. The cells were incubated for 4 hr in the presence of 200 μM [1-^{14}C] labeled palmitic acid and 200 μM TTA or palmitic acid, with or without 500 μM L-carnitine or 50 μM L-aminocarnitine. The fatty acids were bound to BSA (fatty acid:BSA molar ratio was 2.5:1)

Determination of Fatty Acid Oxidation in Cultured Hepatocytes

The rate of β-oxidation was measured as oxidation of [1-^{14}C] palmitoyl-CoA to acid-soluble products. The medium was centrifuged at 2,500 *g* and 250 μL of cell-free medium was added to 100 μL of 6% (w/v) BSA and precipitated with 1.0 mL of 1 M ice-cold $HClO_4$ and 150 μL of 0.1 M KOH. The mixture was shaken vigorously and centrifuged at 2,500 *g* for 10 min and 500 μL of the supernatants were counted by a liquid scintillation counter. One set of samples containing the different media was precipitated immediately as described above and used as background activities.

Determination of Fatty Acid Composition

Total lipids were extracted from liver as described by Lie *et al.* [28]. The lipid fractions were evaporated, saponified, 19:0 added as internal standard, and the fatty acids esterified in 12% 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 (inner diameter 0.32 mm). The fatty acid composition was calculated using a Maxima 820 Chromatography Workstation software, installed in an IBM-AT, connected to the GLC and identification ascertained by standard mixtures of methyl esters (Nu-Chek, Elysian).

Presentation of Results

Data are reported as means ± SD from three to six animals. Statistical analysis was by one-way ANOVA. Fisher's least square difference was used to determine the significance of differences between the means at a 95% confidence interval.

TABLE 1. Changes in plasma triglycerides in rats fed 3-thia fatty acids or palmitic acid (percent of control)

Treatment	Dose (mg/kg body weight)	Time after administration (hr)				
		2	4	8	12	24
TD	150	50 ± 22*	36 ± 17*†	78 ± 32	115 ± 60	107 ± 16
TTA	150	40 ± 16*†	69 ± 22*†	93 ± 14	69 ± 3*†	110 ± 12
TTA	300	93 ± 26	40 ± 17*†	62 ± 6*†	69 ± 15	98 ± 22
TTA	500	104 ± 12	84 ± 12	85 ± 2†	47 ± 3*†	86 ± 15†
Palmitic acid	150	78 ± 2	101 ± 11	87 ± 48	102 ± 19	116 ± 33
Palmitic acid	300	118 ± 18	85 ± 21	129 ± 27	101 ± 36	114 ± 23
Palmitic acid	500	90 ± 23	86 ± 22	134 ± 26	84 ± 25	155 ± 9*

Data are given as percentages of the means ± SD of control (CMC-treated rats), and are from three rats in each experimental group and six rats in the control group. Means of the control values are 1.28 ± 0.49 mmol/L. *Denotes significantly different ($P < 0.05$) compared to CMC, and † denotes significantly different from palmitic acid at equal dose.

TABLE 2. Changes in plasma cholesterol in rats fed 3-thia fatty acids or palmitic acid (percent of control)

Treatment	Dose (mg/kg body weight)	Time after administration (hr)				
		2	4	8	12	24
TD	150	85 ± 12*†	99 ± 17†	92 ± 11	87 ± 23	75 ± 18*†
TTA	150	92 ± 3†	98 ± 17	89 ± 10	96 ± 4	88 ± 6*†
TTA	300	74 ± 12*†	108 ± 16	84 ± 12*†	98 ± 29	87 ± 10
TTA	500	114 ± 24	125 ± 16*	88 ± 8	91 ± 14	78 ± 23*
Palmitic acid	150	117 ± 13*	125 ± 22	107 ± 6	106 ± 3	109 ± 8
Palmitic acid	300	112 ± 18	109 ± 5	116 ± 16	104 ± 19	90 ± 15
Palmitic acid	500	96 ± 12	103 ± 7	114 ± 8*	91 ± 7	98 ± 13

Data are given as percentages of the means ± SD of control (CMC-treated rats), and are from three rats in each experimental group and six rats in the control group. Means of the control values are 1.68 ± 0.18 mmol/L. *Denotes significantly different ($P < 0.05$) compared to CMC, and † denotes significantly different from palmitic acid at equal dose.

RESULTS

Effect on Plasma Lipid Levels

Table 1 shows that administration of a single dose of TTA reduced the plasma triglycerides to 50–60% of control values at different time points, depending on the dose. With increasing doses of TTA, i.e. 150, 300, or 500 mg/kg of body weight, the plasma triglyceride level was decreased at 2 and 4 hr, at 4 and 8 hr, and at 12 hr, respectively. In rats fed TD (150 mg/kg of body weight), the triglyceride level was significantly reduced 2 and 4 hr after feeding. Plasma triglyceride concentrations were not significantly changed compared to control or palmitic acid throughout the time period studied. An exception was observed at 24 hr, where palmitic acid at a dose of 500 mg/kg body weight increased plasma triglycerides.

There were only small changes in plasma cholesterol and phospholipid levels after TD and TTA feeding, but generally the plasma cholesterol and phospholipid levels were decreased compared to controls (Tables 2 and 3, respectively).

Total Acid-Soluble CoA Esters in Liver

TTA is converted to its CoA ester in liver [29], and thereby incorporated into hepatic cellular membranes. Table 4 shows the level of total acid-soluble CoA esters in the liver of rats fed a single dose of 3-thia fatty acids at three different doses. Generally, the level of the acid-soluble CoA esters was elevated at all time points studied in rats fed TD. However, when the dose of TTA was increased, the observed increase in acid-soluble CoA esters changed with respect to time. Interestingly, at a low dose (150 mg/kg of body weight), a medium dose (300 mg/kg of body weight), and a high dose (500 mg/kg of body weight), an increased level of CoA esters was observed after 2, 4, and 8–24 hr, respectively (Table 4). Thus, this delayed effect was very similar to the delayed hypolipidemic effect of higher doses of 3-thia fatty acids (Table 1).

Effect on FABP and PPAR mRNA

We observed a two-fold increase in the FABP mRNA level 8 hr after TD and TTA administration. After 24 hr, the mRNA level was increased almost five-fold (Fig. 1D). PPARα expression was not changed after administration of TTA (Fig. 2A).

Effect on Peroxisomal Enzymes and mRNA Levels

In a previous report, we demonstrated that the activity and mRNA level of FAO increased in rat liver after administration of a single dose (150 mg/kg of body weight) of TD and TTA [5]. Treatment of rats with a higher dose of TTA (500 mg/day/kg of body weight) resulted in a significant

TABLE 3. Changes in plasma phospholipids in rats fed 3-thia fatty acids or palmitic acid (percent of control)

Treatment	Dose (mg/kg body weight)	Time after administration (hr)				
		2	4	8	12	24
TD	150	88 ± 7*	82 ± 14*†	89 ± 17	85 ± 18*	83 ± 17*
TTA	150	85 ± 3*†	94 ± 14†	93 ± 11	88 ± 3*†	96 ± 5
TTA	300	81 ± 6*†	96 ± 2	92 ± 14†	91 ± 15	95 ± 8
TTA	500	107 ± 14	118 ± 7*	85 ± 9*†	88 ± 3*	82 ± 16*†
Palmitic acid	150	102 ± 11	122 ± 13*	105 ± 14	105 ± 5	87 ± 7*
Palmitic acid	300	109 ± 11	108 ± 5	111 ± 9	102 ± 7	96 ± 8
Palmitic acid	500	95 ± 12	105 ± 5	114 ± 4*	100 ± 10	112 ± 11

Data are given as percentages of the means ± SD of control (CMC-treated rats), and are from three rats in each experimental group and six rats in the control group. Means of the control values are 1.56 ± 0.22 mmol/L. *Denotes significantly different ($P < 0.05$) compared to CMC, and † denotes significantly different from palmitic acid at equal dose.

TABLE 4. Total hepatic acid-soluble CoA level (nmol/g wet liver) in rats after a single dose of TD, TTA or palmitic acid

Treatment	Dose (mg/kg body weight)	Time after administration (hr)				
		2	4	8	12	24
TD	150	105 ± 6*†	79 ± 21	104 ± 18*†	96 ± 20*†	122 ± 20*†
TTA	150	82 ± 9*	54 ± 1†	53 ± 10	60 ± 5	70 ± 11
TTA	300	56 ± 6	82 ± 6†	61 ± 21	73 ± 2	66 ± 9†
TTA	500	65 ± 19	67 ± 9	90 ± 18*†	81 ± 1†	87 ± 20*†
Palmitic acid	150	69 ± 10	63 ± 5	68 ± 10	48 ± 12	58 ± 10
Palmitic acid	300	56 ± 2	46 ± 5*	64 ± 8	62 ± 10	47 ± 7*
Palmitic acid	500	76 ± 8	64 ± 13	68 ± 12	55 ± 9	59 ± 2
CMC	—	61 ± 12	66 ± 12	56 ± 7	57 ± 11	66 ± 6

The results are means ± SD of 3 animals in each group. *Denotes significantly different compared to CMC ($P < 0.05$), and † denotes signficantly different from palmitic acid at equal dose at each time point.

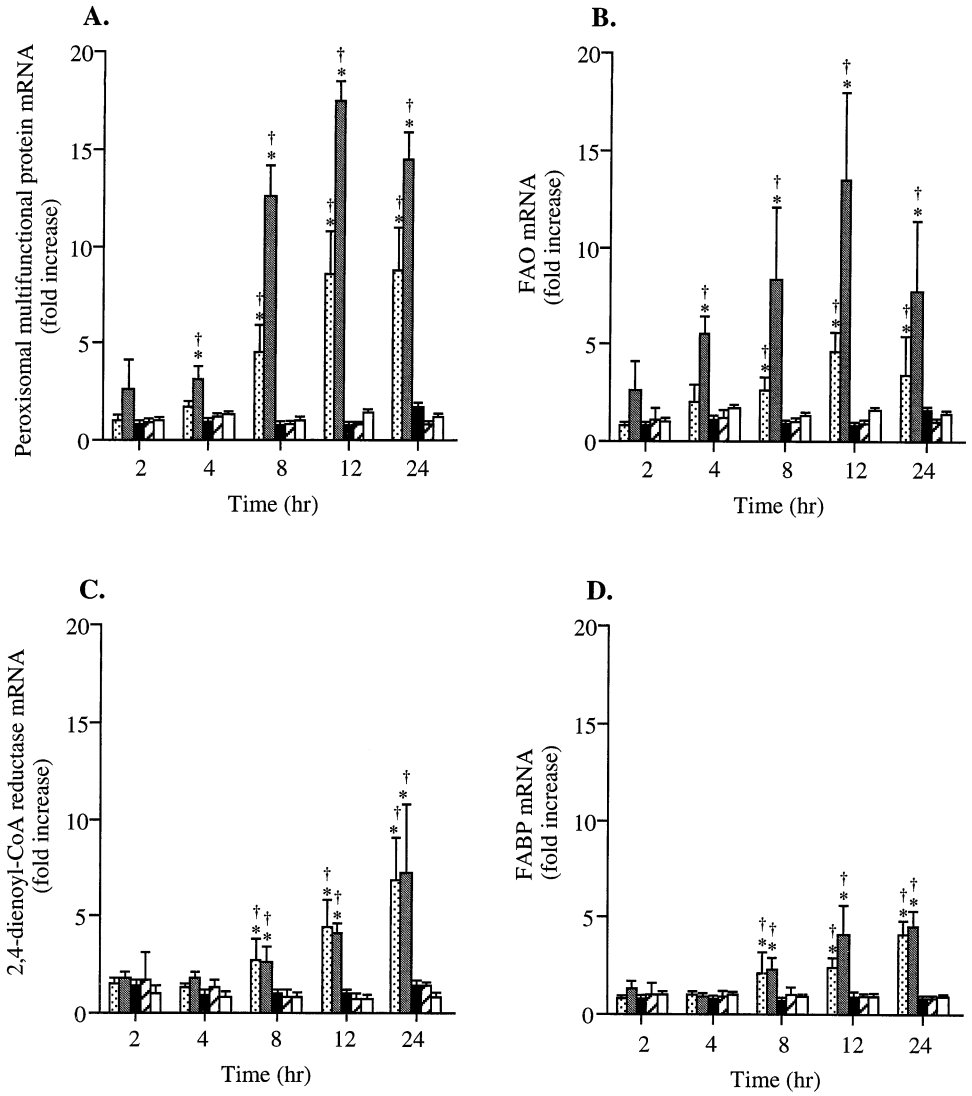

FIG. 1. Time courses on the effect of TD and TTA on relative mRNA levels of: A) peroxisomal multifunctional protein; B) FAO; C) 2,4-dienoyl-CoA reductase; and D) FABP. (▒) TTA 500 mg/kg of body weight; (▨) TD 150 mg/kg of body weight; (■) palmitic acid 150 mg/kg of body weight; (▨) palmitic acid 500 mg/kg of body weight; (□) CMC. Total RNA was purified, and data were obtained from hybridizations of slot blot membranes, as described in Materials and Methods. The relative mRNA levels were determined by densitometric scanning of the autoradiograms and the different mRNA levels were normalized to 28S rRNA. Means of 2-hr CMC treatment are set to 1. Data are given as means ± SD and are from three different rats. *Denotes significantly different from palmitic acid at equal dose. †Denotes significantly different from CMC.

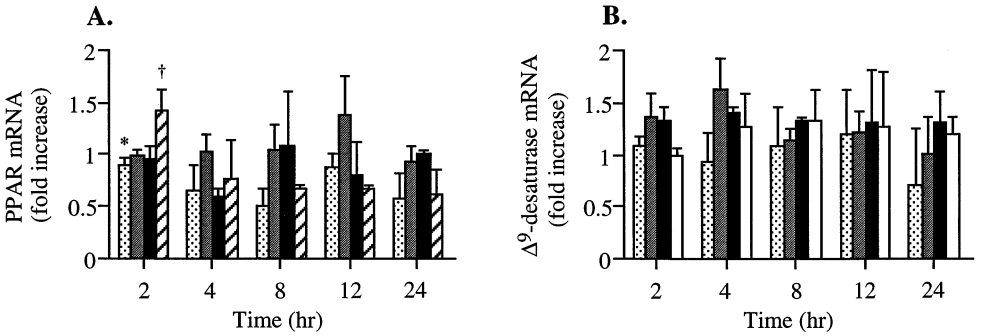

FIG. 2. Time courses on the effect of TD and TTA on relative mRNA levels of: A) PPARα; B) Δ^9-desaturase. (▨) TTA 500 mg/kg of body weight; (▩) TD 150 mg/kg of body weight; (■) palmitic acid 150 mg/kg of body weight; (▨) palmitic acid 500 mg/kg of body weight; (□) CMC. Experiments were performed as explained in Fig. 1. Data are given as means ± SD and are from three different rats. *Denotes significantly different from palmitic acid at equal dose. †Denotes significantly different from CMC.

increase in FAO activity (Table 5) and its mRNA level (Fig. 1B) after 8 hr. The effect on enzyme activity was much more pronounced in rats fed 500 mg/kg of body weight than 150 mg/kg of body weight TTA. FAO activity increased significantly 4 hr after administration of TD. After 24 hr, FAO activity (Table 5) was increased 3.2-fold compared to 2 hr with both TD (150 mg/kg of body weight) and a high dose (500 mg/kg of body weight) of TTA, while FAO activity was normalized after treatment with a low dose (150 mg/kg of body weight) of TTA. After 12 hr, FAO-specific mRNA was increased *ca.* 13-fold in TD-, and *ca.* five-fold in TTA-fed rats, compared to controls and palmitic acid-treated rats at similar doses.

Feeding rats 3-thia fatty acids resulted in an increased mRNA level of the peroxisomal multifunctional protein [2-enoyl-CoA hydratase (EC 4.2.1.17)/L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)/Δ^3-Δ^2-enoyl-CoA isomerase (EC 5.3.3.8)], reaching a maximum of 8.5-fold and 17.5-fold after 12 hr with TTA and TD, respectively (Fig. 1A).

FAO enzyme activity and the mRNA concentrations of FAO and peroxisomal multifunctional protein remained unchanged in rats fed palmitic acid at different doses (Table 5 and Fig. 1A and B).

Mitochondrial Enzyme Activities and mRNA Levels

As shown in Table 5, there was a small increase in total CPT activity at 2 hr in rats fed low doses of TD and TTA. TD caused a rapid (2 hr) increase in the CPT-I mRNA level to *ca.* 3 times that of the control and palmitic acid group (Fig. 3A). Moreover, the CPT-II mRNA level (Fig. 3B) increased early, significantly 4 hr after TD treatment with a maximum threefold induction after 8 hr.

Interestingly, the mRNA level of 2,4-dienoyl-CoA reductase (EC 1.3.1.34), a mitochondrial enzyme needed for β-oxidation of unsaturated fatty acids, showed a delayed response compared to CPT-I and CPT-II. Figure 1C shows that 2,4-dienoyl-CoA reductase-specific mRNA increased significantly after 8 hr of TD or TTA treatment. After 24 hr, the 2,4-dienoyl-CoA reductase mRNA level was increased *ca.* seven-fold. Figure 4 shows an example of the slot

TABLE 5. Enzyme activities of FAO, CPT and 2,4-dienoyl-CoA reductase in rats fed 3-thia fatty acids or palmitic acid

Treatment	Dose (mg/kg body weight)	Time after administration (hr) 2	4	8	12	24
FAO (nmol/min/g liver)						
TD	150	368.0 ± 21.5	487.2 ± 97.9†	731.7 ± 66.5†	1137.0 ± 65.2†	1184.6 ± 82.3†
TTA	150	343.2 ± 14.1	366.3 ± 59.4†	341.4 ± 22.8	464.1 ± 18.1†	359.5 ± 80.5
TTA	500	247.7 ± 12.0	307.0 ± 8.7	415.9 ± 119.8†	453.2 ± 24.4†	797.0 ± 123.5†
Palmitic acid	150	295.8 ± 31.2	256.3 ± 32.2	306.0 ± 71.6	307.7 ± 35.8	300.5 ± 10.4
Palmitic acid	500	321.1 ± 15.5	305.6 ± 47.6	287.3 ± 31.2	311.9 ± 47.6	214.7 ± 15.0
CPT (nmol/min/mg protein)						
TD	150	9.0 ± 1.4†	6.7 ± 0.9	6.9 ± 0.3	7.1 ± 0.1	6.5 ± 0.6
TTA	150	9.1 ± 0.4†	8.7 ± 1.7†	7.7 ± 0.5	7.6 ± 0.9	7.3 ± 0.6
TTA	500	6.7 ± 1.2	7.3 ± 1.0	6.7 ± 0.4	7.4 ± 0.5	7.7 ± 1.2
Palmitic acid	150	6.9 ± 0.3	5.9 ± 0.2	7.8 ± 0.9	7.1 ± 1.1	7.5 ± 1.5
Palmitic acid	500	6.9 ± 0.3	7.0 ± 1.3	7.9 ± 0.9	7.9 ± 0.4	8.3 ± 0.3
2,4-dienoyl-CoA reductase (nmol/min/mg protein)						
TD	150	1.2 ± 0.2	1.5 ± 0.4	1.6 ± 0.4†	2.4 ± 0.7†	3.8 ± 0.2†
Palmitic acid	150	1.3 ± 0.2	1.2 ± 0.4	1.0 ± 0.5	0.9 ± 0.1	1.0 ± 0.1

The values are given as means ± SD (N = 3). †Denotes significantly different from palmitic acid at equal concentration.

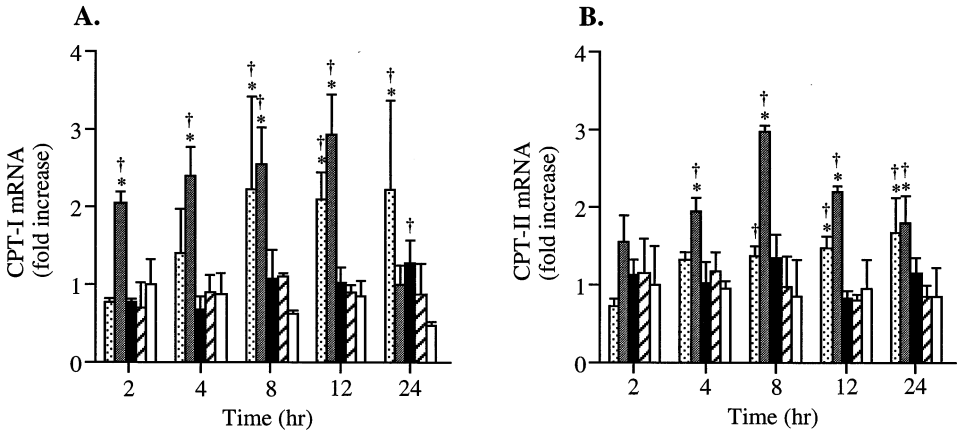

FIG. 3. Time-courses on the effect of TD and TTA on relative mRNA levels of: A) CPT-I; B) CPT-II. (▨) TTA 500 mg/kg of body weight; (▩) TD 150 mg/kg of body weight; (■) palmitic acid 150 mg/kg of body weight; (▨) palmitic acid 500 mg/kg of body weight; (□) CMC. Experiments were performed as explained in Fig. 1. Data are given as means ± SD and are from three different rats. *Denotes significantly different from palmitic acid at equal dose. †Denotes significantly different from CMC.

blot hybridization experiments with cDNA probes for 2,4-dienoyl-CoA reductase and 28S rRNA. The transcriptional induction of 2,4-dienoyl-CoA reductase is evident. In addition, the enzyme activity of 2,4-dienoyl-CoA reductase was increased, as shown for TD compared to palmitic acid in Table 5.

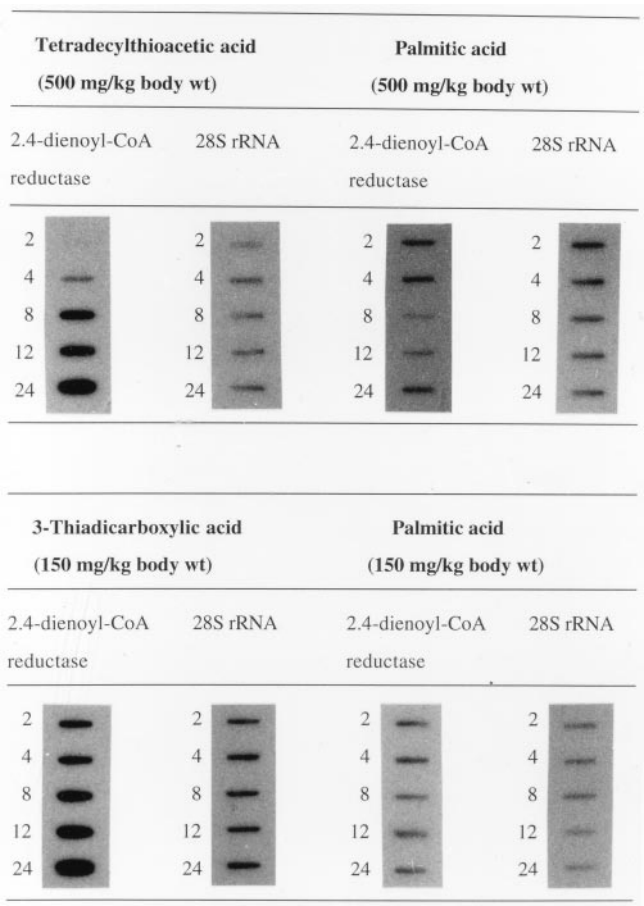

FIG. 4. Example of the mRNA kinetics in Figures 1, 2, and 3, here shown for 2,4-dienoyl-CoA reductase and 28S rRNA after acute treatment with TTA (500 mg/kg of body weight), TD (150 mg/kg of body weight), and palmitic acid (150 and 500 mg/kg of body weight). 2, 4, 8, 12, and 24 denotes the respective hours after treatment. One representative slot blot from each of the time points is shown. Experiments were performed as explained in Fig. 1.

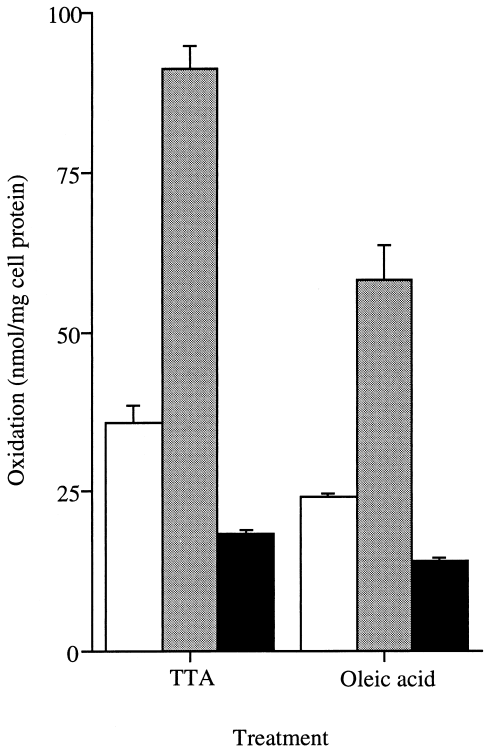

FIG. 5. Oxidation of [^{14}C]palmitic acid (200 μM) in the presence of 200 μM oleic acid or TTA in cultured rat hepatocytes. The cells were plated and incubated in medium containing L-carnitine (0.5 mM) or L-aminocarnitine (50 μM). After overnight incubation, the medium was removed and new medium containing L-carnitine or L-aminocarnitine, labeled palmitic acid (200 μM) was added, and the cells were incubated for 4 hr. (□) No addition, (▨) L-carnitine, (■) L-aminocarnitine. Acid-soluble activity was determined as described under Materials and Methods. Data are expressed as means ± SEM.

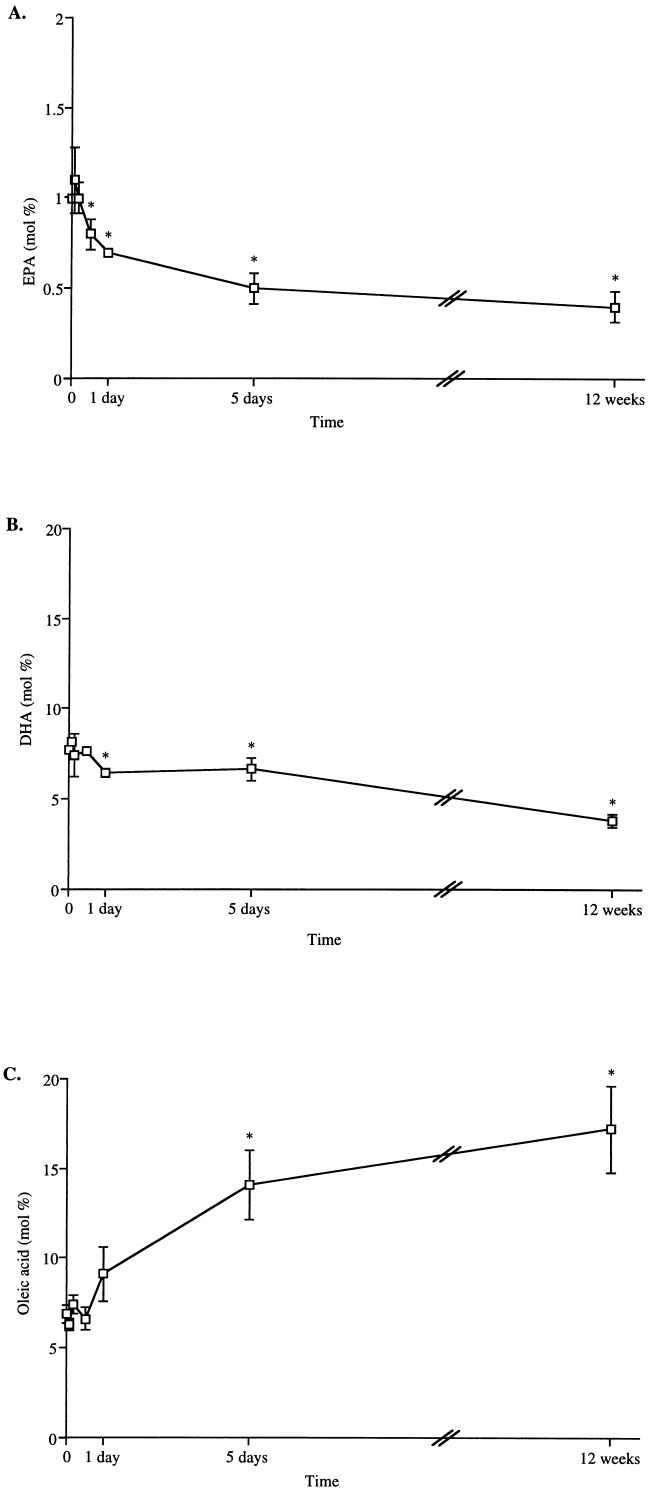

FIG. 6. Time-course effect of TTA (150 mg/kg of body weight) on the amount (mol %) of A) EPA, B) DHA, and C) oleic acid in total homogenates of rat liver. The fatty acid composition was determined as described under Materials and Methods. Results are expressed as means ± SD of three to six animals.

Fatty Acid Oxidation in Cultured Rat Hepatocytes

Oxidation of palmitic acid was more than 2 times higher in rat hepatocytes grown with L-carnitine than in cells grown without addition of L-carnitine (Fig. 5). Moreover, TTA significantly stimulated oxidation of palmitic acid in absence and presence of L-carnitine. In the presence of L-aminocarnitine, a potent CPT-II inhibitor, oxidation of palmitic acid was decreased in the presence and absence of TTA when compared to cells grown in the presence as well as absence of L-carnitine.

Short-term versus Long-term Administration of 3-Thia Fatty Acids

TTA treatment alters fatty acid composition in long-term studies (Fig. 6). Repeated administration of this 3-thia fatty acid increased the content of oleic acid in a time-dependent manner. In contrast, DHA and especially EPA decreased. In the short-term study, however, the content of oleic acid was unchanged whereas a significant decrease in EPA concentration was seen at 12 and 24 hr. Similarly, in this time period the percentage of TTA of the total hepatic lipids decreased (Fig. 7). In agreement with earlier findings, long-term feeding caused induction of Δ^9-desaturase mRNA. In contrast, the expression of Δ^9-desaturase was unchanged after a single dose of 3-thia fatty acids (Fig. 8). As shown in Table 6, the expression of different genes involved in fatty acid oxidation were more pronounced after administration of TTA (150 mg/day/kg of body weight) for 12 weeks than for 1 week, especially 2,4-dienoyl-CoA reductase, enoyl-CoA isomerase, and FAO.

DISCUSSION

In the present study, we have shown that the expression of several mitochondrial and peroxisomal fatty acyl-CoA ester metabolizing enzymes were rapidly increased in rats fed 3-thia fatty acids, but the increase seemed to follow different time-courses.

The mechanism by which 3-thia fatty acids regulate the expression of genes is not fully elucidated. PPARα regulates lipid homeostasis by adjusting the expression of enzymes involved in fatty acid degradation (β- and ω-oxidation) [30]. Inhibitors of subsequent steps of the β-oxidation pathway, for instance TTA [31], activate PPARα [32] and have recently been found to be PPARα ligands [33]. Thus, TTA represents a dual-function activator. As a ligand it activates PPARα directly, while as a metabolic inhibitor it may indirectly lead to accumulation of endogenous fatty acid ligands. In rats treated with 3-thia fatty acids, we found no difference in the specific mRNA level of PPARα compared to the control level of PPARα mRNA during the first 24 hr. However, repeated administration of TTA (12 weeks) elevated the PPARα mRNA, although at a small scale.

PPARα regulates the gene expression of FAO and peroxisomal multifunctional protein by binding to a responsive element present in the genes (reviewed in [30]). In rats fed TD, hepatic FAO activity was significantly increased after 4 hr. An increase in the transcription of genes encoding FAO and peroxisomal multifunctional protein

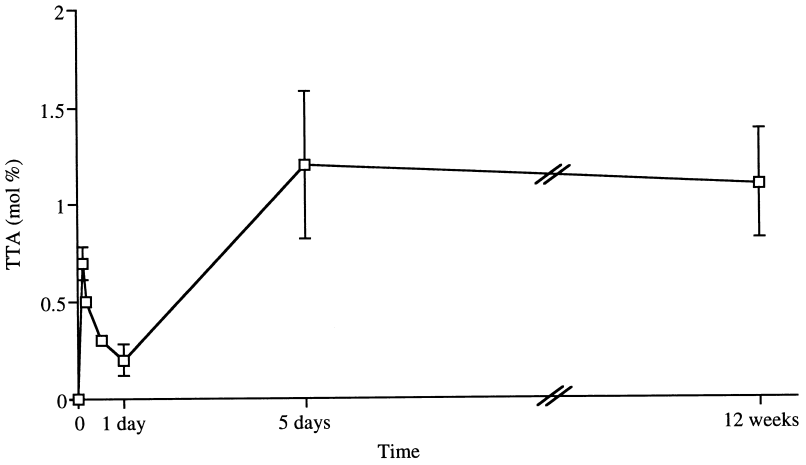

FIG. 7. Effect of TTA (150 mg/kg of body weight) on the amount (mol %) of TTA in total homogenates of rat liver, determined as described under Materials and Methods. Results are expressed as means ± SD of three to six animals.

seemed to occur already 2 hr after TD administration, although this increase was not significant. The major increase in the mRNA levels of these peroxisomal enzymes did not appear until 8–12 hr after administration of 3-thia fatty acids. These time points are in accordance with the results obtained earlier by Reddy *et al.* [34]. FABP, whose gene contains a PPAR-responsive element [30], also increased its mRNA content, significantly 8 hr after treatment with 3-thia fatty acids. Up-regulated gene transcription indicates a need for more FABP protein to transport fatty acids to the mitochondria and peroxisomes for oxidation. Repeated administration of low doses of TTA upregulated the transcription of these genes in a time-dependent manner. In addition, increasing doses of TTA have an effect on the transcription of genes involved in mitochondrial and peroxisomal β-oxidation.*

The present experiment shows that a single oral administration of 3-thia fatty acids to rats leads to specific upregulation of liver CPT-I and CPT-II gene expression. Interestingly, CPT-II mRNA and, especially, CPT-I mRNA were induced early, that is, at 4 and 2 hr, respectively, after administration of 3-thia fatty acids. The early induction of CPT-I transcription is in agreement with the results on CPT-I transcription obtained recently by Assimacopoulos–Jeannet *et al.* [35]. The CPT activity was also increased at 2 hr, although marginally, with TD and TTA at the lowest dose. Whether or not this represents *de novo* synthesis of the enzyme, should be considered.

The gene expression of several enzymes involved in the peroxisomal and mitochondrial β-oxidation pathways are regulated through PPAR (reviewed in [30]). To our knowledge, no responsive element for PPAR in the genes for CPT-I or CPT-II has yet been identified. We found that the CPT-I expression seemed to be induced earlier than the other enzymes involved in fatty acid oxidation. It is therefore interesting to speculate if PPAR is responsible for mediating the effects of peroxisome proliferators on CPT-I or if there is another mechanism controlling its transcription, as has been suggested earlier [35, 36].

In rat hepatocytes cultured for 4 hr, the oxidation of palmitic acid was stimulated by TTA. The finding that the fatty acid oxidation was both L-carnitine- and L-aminocarnitine-sensitive suggested that the mitochondrial fatty acid oxidation was affected early by TTA. No increased FAO activity was observed in the cultured cells at this early time point (results not shown). Evidently, there is a longer time-lag (compared to stimulation of mitochondrial β-oxidation) between the administration of 3-thia fatty acids and the stimulation of peroxisomal β-oxidation both *in vitro* and *in vivo*. Generally, CPT-I is believed to be the regulatory enzyme in mitochondrial β-oxidation, controlled by inhibition with malonyl-CoA [37]. Indeed, *in vivo*, CPT-I expression was induced early. Conversely, CPT-II is not usually associated with the control of fatty acid oxidation. Because the fatty acid oxidation *in vitro* was L-aminocarnitine-sensitive, it is tempting to suggest that

* Madsen L and Berge RK, manuscript submitted for publication.

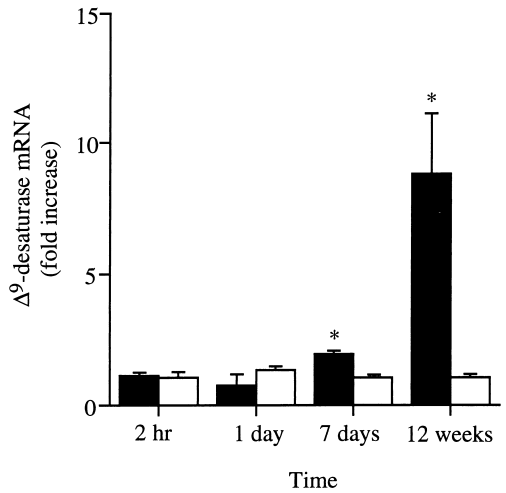

FIG. 8. Effect of TTA on the expression of Δ^9-desaturase at different time points. Data are given as means ± SD and are from three to six rats. TTA (solid bars); CMC (open bars). *Denotes significantly different from CMC. Experiments were performed as explained in Fig. 1.

TABLE 6. mRNA expression in liver of rats fed TTA or palmitic acid (150 mg/kg body weight) for 1 or 12 weeks

	Palmitic acid		TTA		CMC	
	1 week	12 weeks	1 week	12 weeks	1 week	12 weeks
CPT-I	1.1 ± 0.3	1.3 ± 0.2	1.1 ± 0.1	4.4 ± 0.9*	1.0 ± 0.1	1.0 ± 0.5
CPT-II	1.1 ± 0.2	0.7 ± 0.1	2.0 ± 0.2*	4.5 ± 0.4*	1.0 ± 0.1	1.0 ± 0.2
2,4-dienoyl-CoA reductase	0.8 ± 0.2	1.0 ± 0.4	1.6 ± 0.2*	10.9 ± 1.6*	1.0 ± 0.2	1.0 ± 0.1
Enoyl-CoA isomerase	1.2 ± 0.1	1.0 ± 0.2	2.5 ± 0.4*	13.7 ± 1.5*	1.0 ± 0.2	1.0 ± 0.1
FAO	1.2 ± 0.3	1.6 ± 0.4*	4.1 ± 0.7*	14.0 ± 2.2*	1.0 ± 0.1	1.0 ± 0.2
FABP		0.8 ± 0.1		3.5 ± 0.5*		1.0 ± 0.2
PPARα		1.0 ± 0.3		1.8 ± 0.4*		1.0 ± 0.4

Total RNA was purified, and data were obtained from hybridization of slot blot membranes, as described in Materials and Methods. The relative mRNA levels were determined by densitometric scanning of the autoradiograms and the different mRNA were levels normalized to 28S rRNA. Means of CMC treatment are set to 1. Data are given as means ± SD and are from 4–6 different rats. *Significantly different from CMC ($P < 0.05$).

CPT-II may be a regulator of fatty acid oxidation during treatment with 3-thia fatty acids.

The decreased hepatic content of EPA and DHA was observed at 12 and 24 hr, respectively, after a single dose of TTA. The further reductions in these n-3 polyunsaturated fatty acids during repeated administration of TTA were in accordance with earlier reports by our group [38, 39]. Conversion of EPA to DHA could not explain the decreased content of EPA, as the DHA content was also decreased. Hagve *et al.* [40] have suggested that EPA is initially rapidly esterified in the phospholipids and then liberated by hydrolysis due to lipases with specificity to liberate EPA in preference to other fatty acids. Furthermore, the activity of phospholipase A_2, which preferentially hydrolyzes fatty acids esterified at the *sn*-2 position of phospholipids, increased after administration of 3-thia fatty acids to rats [38]. Evidently, this effect renders polyunsaturated fatty acids available for further metabolism. We have recently obtained evidence that EPA stimulates mitochondrial β-oxidation, while DHA is a more effective peroxisome proliferator and stimulates FAO activity [41]. It is therefore likely that EPA is forced to the mitochondria at an early point of time, where it could directly influence the transcription of 2,4-dienoyl-CoA reductase and be β-oxidized, thereby causing the observed reduction in hepatic EPA. Indeed, mitochondrial 2,4-dienoyl-CoA reductase activity was significantly induced 8 hr after administration of 3-thia fatty acids, concomitant with an increase in its mRNA concentration. Similarly, the decreased amount of DHA may be partly due to an induced peroxisomal β-oxidation as the FAO activity and gene expression increased, in accordance with recent evidence that DHA, and not EPA, is a peroxisome proliferator [41]. Contrary to EPA and DHA, the hepatic content of oleic acid increased in a time-dependent manner after administration of TTA. Oleic acid tended to increase after 24 hr, although not significantly as compared to control. However, no change was observed in the Δ^9-desaturase mRNA level after a single dose of 3-thia fatty acids. Thus, the changes in the fatty acid composition after a single dose of 3-thia fatty acids may be linked to induced fatty acid oxidation, and not to Δ^9-desaturase activity. After repeated administration of TTA, there was a more pronounced increase in the content of oleic acid, compared to 24 hr, simultaneously with an elevation of the Δ^9-desaturase mRNA expression. TTA is reported to be a good substrate for Δ^9-desaturase [42], and in rats fed this compound for one or several weeks, Δ^9-desaturated TTA accumulated and become incorporated into the phospholipids [43]. However, in the present short-term experiment, no Δ^9-desaturated TTA could be detected,* indicating that there was not any up-regulation of Δ^9-desaturase activity within 24 hr. The increased Δ^9-desaturase mRNA, therefore, shows a delayed induction compared to the expression of the mitochondrial and peroxisomal β-oxidation enzymes. In fact, we have recently proposed that the modification of Δ^9-desaturation was unrelated to peroxisome proliferation [43]. It is possible that the increased oleic acid content is an attempt to keep the degree of unsaturation in the cell stable. A responsive element for polyunsaturated fatty acids has been found on the Δ^9-desaturase gene, which down-regulates the expression of Δ^9-desaturase [44], thereby regulating the production of oleic acids. It thus seems as if the up-regulation of oleic acid and Δ^9-desaturase appears after the decreased content of EPA and DHA.

As shown in Table 1, the triglyceride-lowering effect was already established 2 hr after administration of 3-thia fatty acids. We found earlier that mitochondrial β-oxidation was upregulated early after a single dose of 3-thia fatty acids [2, 45], whereas peroxisomal β-oxidation increased at a later point of time [45]. Indeed, CPT activity was also upregulated after 2 hr. By increasing the dose of TTA, the triglyceride-lowering effect was delayed. This tendency was also seen for the total hepatic acid-soluble CoA esters. There is no explanation for this phenomenon as yet. However, as TTA was incorporated into the hepatic phospholipids at 2 hr, TTA must have been activated to its CoA ester. The amount of TTA was reduced throughout the entire 24-hr study period. After repeated administration of TTA, TTA accumulated in the hepatic tissue. Recent results show that both TTA and especially its CoA ester

* Grav H, personal communication.

inhibit CPT-I enzyme activity.* It seems as if TTA has both an inhibitory and a stimulatory effect on CPT, and there might be a balance between these two opposing effects of TTA. Therefore, it is possible that the higher doses of TTA temporarily inhibit mitochondrial β-oxidation, causing a delay in the triglyceride-lowering effect. In accordance with these hypotheses, experiments *in vitro* have shown that 3-thia fatty acids inhibit both mitochondrial and peroxisomal β-oxidation in a dose-dependent manner [31].

From the present study, it can be concluded that feeding rats 3-thia fatty acids results in increased expression of the enzymes involved both in peroxisomal and mitochondrial β-oxidation. This is in agreement with the changes in the activities of the corresponding peroxisomal and mitochondrial enzymes. This study shows that the expression of CPT-I and CPT-II are induced at a much smaller scale, and that CPT-I expression is induced earlier than the enzymes that are actually metabolizing the fatty acids in the mitochondria. Expression of the peroxisomal β-oxidation enzymes FAO and peroxisomal multifunctional protein are also induced early. In contrast, the expression of Δ^9-desaturase showed a delayed response.

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