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Mitochondrial 3-Hydroxy-3-Methylglutaryl Coenzyme A Synthase and Carnitine Palmitoyltransferase II as Potential Control Sites for Ketogenesis during Mitochondrion and Peroxisome Proliferation

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ABSTRACT. 3-Thia fatty acids are potent hypolipidemic fatty acid derivatives and mitochondrion and peroxisome proliferators. Administration of 3-thia fatty acids to rats was followed by significantly increased levels of plasma ketone bodies, whereas the levels of plasma non-esterified fatty acids decreased. The hepatic mRNA levels of fatty acid binding protein and formation of acid-soluble products, using both palmitoyl-CoA and palmitoyl-L-carnitine as substrates, were increased. Hepatic mitochondrial carnitine palmitoyltransferase (CPT) -II and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase activities, immunodetectable proteins, and mRNA levels increased in parallel. In contrast, the mitochondrial CPT-I mRNA levels were unchanged and CPT-I enzyme activity was slightly reduced in the liver. The CoA ester of the monocarboxylic 3-thia fatty acid, tetradecylthioacetic acid, which accumulates in the liver after administration, inhibited the CPT-I activity *in vitro*, but not that of CPT-II. Acetoacetyl-CoA thiolase and HMG-CoA lyase activities involved in ketogenesis were increased, whereas the citrate synthase activity was decreased. The present data suggest that 3-thia fatty acids increase both the transport of fatty acids into the mitochondria and the capacity of the β-oxidation process. Under these conditions, the regulation of ketogenesis may be shifted to step(s) beyond CPT-I. This opens the possibility that mitochondrial HMG-CoA synthase and CPT-II retain some control of ketone body formation. BIOCHEM PHARMACOL **57**;9:1011–1019, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. 3-thia fatty acids; ketogenesis; fatty acid oxidation; carnitine palmitoyl transferase; mitochondrial HMG-CoA synthase; peroxisome proliferation

Hepatic ketogenesis in adult rats is mainly controlled by two systems: (i) entry of fatty acyl-CoA into mitochondria, catalysed by the CPT§ system [1]; and (ii) enzymatic activity of mitochondrial HMG-CoA synthase [2]. CPT-I, the outer form of mitochondrial CPT, seems to play a crucial role in the control of entry and β-oxidation of fatty acids in the mitochondria. CPT-I activity is regulated through inhibition by malonyl-CoA. In contrast, CPT-II, the inner form of mitochondrial CPT, is not sensitive to inhibition by malonyl-CoA [1]. The conversion of acetyl-CoA, produced by the β-oxidation process, to acetoacetate is conducted via the HMG-CoA cycle, which in turn is controlled by mitochondrial HMG-CoA synthase. Two control systems operate on this enzyme: a transcriptional control is exerted by nutritional and hormonal effects [3], and a posttranscriptional mechanism modulates the activity by succinylation and desuccinylation [4].

Moreover, the earlier findings of McGarry and coworkers, who showed a direct proportionality between ketogenesis from octanoate and L-octanoylcarnitine in perfused livers from rats treated in different ways, indicated that CPT-II may also be an important locus in the regulation of hepatic fatty acid oxidation and thus of ketogenesis [5, 6].

3-Thia fatty acids, such as C14-S-acetic acid (CH_3-$(CH_2)_{13}$-S-CH_2-COOH) and TD (COOH-CH_2-S-$(CH_2)_{10}$-S-CH_2-COOH), are not β-oxidisable, as a sulphur atom is located in the 3-position. However, they are both mitochondrion and peroxisome proliferators and have been reported to increase β-oxidation in treated animals and cultured hepatocytes [7–10]. The results from ten years of studies strongly suggest that increased mitochondrial fatty acid oxidation is the most important factor behind the

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§ *Abbreviations:* CPT, carnitine palmitoyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PPAR, peroxisome proliferator-activated receptor; NEFA non-esterified fatty acids; FABP, fatty acid binding protein; FAO, fatty acyl-CoA oxidase; C14-S-acetic acid, tetradecylthioacetic acid; TD, 1,10 bis(carboxymethylthio)decane; and CMC, 0.5% sodium carboxymethyl cellulose.

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hypotriglyceridemic effect of 3-thia fatty acids [11–13], fibrates [14], and fish oils [15, 16]. Unexpectedly, 3-thia fatty acid treatment decreased the plasma levels of NEFAs. As fatty acid oxidation in general is proportional to the plasma NEFA levels mobilised by hormone-sensitive lipase, we studied the activity and gene expression of enzymes involved in ketogenesis and fatty acid oxidation.

MATERIALS AND METHODS

Chemicals and Drugs

[^{32}Pα]dCTP (3000 mCi/mmol), L-[methyl-^{14}C]carnitine hydrochloride (54 mCi/mmol), [1-^{14}C] palmitoyl-L-carnitine (54 mCi/mmol), and [1-^{14}C] palmitoyl-CoA (54 mCi/mmol) were purchased from the Radiochemical Centre, Amersham. The CoA ester of C14-S-acetic acid was prepared as described by Kawaguchi *et al.* [17]. Nylon membranes were obtained from Schleicher & Schuell. All other chemicals and solvents were of reagent grade from common commercial sources. The sulphur-substituted fatty acids were prepared at the Department of Chemistry, University of Bergen, as described previously [18].

Treatment of Animals

Male Wistar rats, weighing 260–300 g, were obtained from Møllegard Breeding Laboratory, Ejby, Denmark. They were housed in pairs in wire cages and maintained on a 12-hr cycle of light and dark at 20 ± 3°. The rats had free access to standard rat pellet food and water during the experiment. They were acclimatised under these conditions for at least 1 week before the experiments. Each test and control group consisted of four animals. Palmitic acid and the 3-thia fatty acids were suspended in 0.5% CMC. The control animals received CMC or palmitic acid. Different doses (150 and 300 mg/kg body weight) were administered by gastric intubation once a day in a volume of 0.7–1.0 mL for 7 days. All animals had free access to water and standard rat pellet food during the experiment. After 12-hr fasting, the rats were anaesthetised with 0.2 mL Hypnorm–Dormicum® (fluanisone–fentylmidazolam)/100 g body weight. Cardiac puncture was performed and blood was collected in vacutainers and then centrifuged for 10 min (1300 *g*). The levels of free fatty acids were measured using an NEFA C ACS-ACOD kit from Wako Chemicals and levels of ketone bodies with the 310-A β-hydroxybutyrate kit from Sigma Diagnostics. The livers were removed, with one part freeze-clamped, weighed and stored at −80° for RNA isolation and the other parts immediately chilled on ice and weighed. The use of the animals was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Preparation of Subcellular Fractions and Protein Measurements

The livers from individual rats were homogenised in ice-cold sucrose medium (0.25 M sucrose, 10 mM HEPES [pH 7.4], and 1 mM EDTA). Postnuclear and mitochondrial fractions were prepared using preparative differential centrifugation as described earlier [19]. The fractionation process was carried out at 0–4°. The Bio-Rad protein kit (Bio-Rad) was used for protein measurement. BSA in distilled water was used as a standard. The absorbance was read on an LKB Bromma Ultrolab System 2074 Calculating Absorptiometer. Mitochondrial protein was separated on a 10% SDS gel, prior to Western blotting [20]. Autoradiograms were obtained using an LKB Ultrogel laser densiometer.

Measurement of Enzyme Activities

Acid-soluble products were measured using palmitoyl-CoA and palmitoyl-L-carnitine as substrates [15]. Briefly, the assay mix (0.3 mL) contained 12 mM HEPES buffer (pH 7.3), 11 mM $MgCl_2$, 12 mM dithiothreitol, 5.6 mM ADP, 0.2 mM NAD^+, 0.6 mM EDTA, 125 mM KCl, and 1.0 mg mitochondrial protein. Palmitoyl-L-carnitine oxidation was measured with 80 μM [1-^{14}C] palmitoyl-L-carnitine and the palmitoyl-CoA oxidation with 80 μM [1-^{14}C] palmitoyl-CoA supplemented with 1 mM L-carnitine. After 2-min incubation at 30°, the reaction was terminated with 150 μL 1.5 M KOH. Then, 2.5 mg BSA and 500 μL 4 M $HClO_4$ were added. The test tubes were centrifuged at 1880 g for 10 min and 500 μL of the supernatant was assayed for radioactivity. CPT-I and -II activity was measured essentially as described by Bremer [21]. 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). When included, malonyl-CoA was added prior to the start of the reaction. Assay conditions for CPT-II were identical, except that BSA was omitted and 0.01% Triton X-100 included. Tissue protein concentration was 0.1 mg/mL. Acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA lyase were measured according to Clinkenbeard *et al.* [22], and citrate syntase as described by Shepherd and Garland [23].

Purification of RNA and Hybridisation Analysis

Total RNA was isolated using the guanidinium thiocyanate–phenol method [24], and the RNA concentrations were determined by measuring the absorbance at 260 nm. The degree of RNA degradation was tested on gel electrophoresis using a 1% agarose minigel, followed by staining with ethidium bromide. RNA (28S and 18S) were then easily visualised under ultraviolet light. A Schleicher & Schuell aperture was used to transfer RNA to nylon filter for hybridisation as described earlier [25]. Three different RNA concentrations were applied. Hybridisation reactions were performed as described by Sambrook *et al.* [26] in the presence of 50% formamide, 5 × SSC (0.15 M sodium chloride + 0.015 M sodium citrate), 200 mg/mL heat-denatured herring sperm DNA, 0.1% SDS, 25 mM sodium

TABLE 1. Effect of 3-thia fatty acids at a dose of 300 mg/day/kg body weight on acid soluble products in liver and ketone bodies and the levels of free fatty acids in plasma of rats

	Liver				Plasma	
	Acid-soluble products (nmol/min/mg protein)					
	Palmitoyl-CoA as substrate		Palmitoyl-L-carnitine as substrate		Ketone bodies (mEq/L)	NEFA (mmol/L)
Treatment	E	M	E	M		
Palmitic acid	0.22 ± 0.07	1.28 ± 0.18	0.69 ± 0.57	2.53 ± 0.57	4.6 ± 0.3	0.84 ± 0.08
C14-S-acetic acid	0.70 ± 0.13*	2.20 ± 0.24*	1.72 ± 0.14*	4.98 ± 0.45*	7.4 ± 1.1*	0.45 ± 0.07*
TD	0.84 ± 0.21*	2.07 ± 0.53*	1.65 ± 0.27*	5.35 ± 0.70*	7.2 ± 0.8*	0.42 ± 0.09*

The formation of acid-soluble products was measured in postnuclear homogenate (E) and in isolated mitochondria (M). The values represent means ± SD for four animals in each experimental group.
*$P < 0.05$ for differences between control and treated rats.

phosphate, pH 6.5, and 8.25% dextran sulphate at 42° for 48 hr. The membranes were stringently washed three times in 0.2 × SSC, 0.1% SDS, and 0.1% $Na_4P_2O_7$ at 65°. When the membranes were to be rehybridised, the bound probe was first stripped off by incubation of the filters in 0.1% SDS for 7 min at 95–100°. Kodak XAR-5 x-ray films were exposed to the membranes at −80° in the presence of intensifying screens for an adequate exposure (3 days to 2 weeks). Autoradiograms were obtained using an LKB Ultrogel laser densiometer. The relative level of mRNA expression was estimated as the amount of radioactive probe hybridised to each sample of RNA relative to the level of 28S rRNA in each sample.

Preparation of Hybridisation Probes

The following cDNA fragments were used as probes: A partial fragment of 0.7 kb *Bam*HI–*Sal*I of rat CPT I cDNA, covering the region corresponding to positions 1272–2020 of the rat mRNA CPT I [27] was amplified by polymerase chain reaction as described [28]. A 1.4 kb *Xho*I–*Bam*HI fragment from cDNA clone (pBKS-CPTII.4) for rat CPT II [29], a specific 1.5 kb probe corresponding to the *Kpn*I–*Kpn*I fragment of the cDNA for rat mitochondrial HMG-CoA synthase, which had been subcloned in Bluescript [30], a fragment from the cDNA (pRSa13) clone for rat serum albumin [31], and the 28S rRNA or rat β-actin cDNA [32] were used. The FABP cDNA probe was a 506 bp *Eco*RI fragment of pTZ18R [33]. All DNA probes were generated by labelling with [^{32}Pα]dCTP to a specific radioactivity of 1.5×10^9 cpm/mg of DNA by random priming with Klenow polymerase [34].

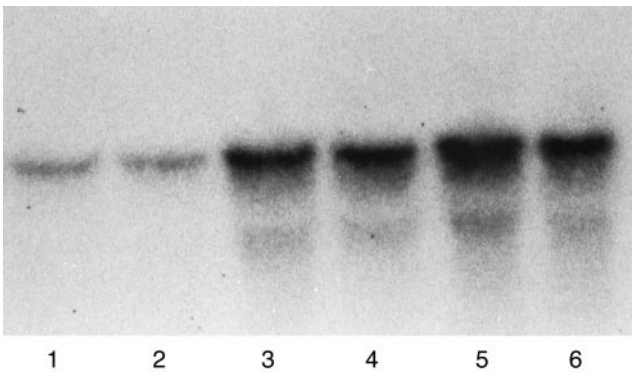

FIG. 1. Effect of 3-thia fatty acids on the mRNA levels of FABP in rat liver. Rats were fed 3-thia fatty acids suspended in 0.5% CMC at a dose of 300 mg/day/kg body weight for 7 days. The mRNA was purified and measured in four individual rats as described under Materials and Methods. The Northern blot shows the following (from left to right): lanes 1 and 2 represent control rats, lanes 3 and 4 C14-S-acetic acid-treated rats, and lanes 5 and 6 TD-treated rats.

Statistical Analysis and Presentation of Data

The data are presented as means ± standard deviation (SD) from 4 animals and were evaluated by a two-sample variance Student's *t*-test (two-tailed distribution) where relevant. The level of statistical significance was set at $P < 0.05$.

RESULTS

Confirming earlier studies, feeding rats with 3-thia fatty acids for one week resulted in an increase in hepatic ^{14}C-labeled acid-soluble products, using both palmitoyl-CoA and palmitoyl-L-carnitine as substrates [7, 8] (Table 1). A concomitant increase in ketone body concentration was observed in serum, whereas the levels of free fatty acids were reduced (Table 1). The hepatic mRNA levels of FABP increased 4.6- and 5.8-fold after C14-S-acetic acid

TABLE 2. Effect of 3-thia fatty acids at a dose of 300 mg/day/kg body weight on the mRNA levels of FABP and 28S rRNA in rat liver

Treatment	FABP mRNA (%)	28S rRNA (%)
Palmitic acid	100 ± 16	100 ± 11
C14-S-acetic acid	456 ± 50*	107 ± 8
TD	584 ± 61*	115 ± 13

The values represent means ± SD for four animals in each experimental group.
*$P < 0.05$ for differences between control and treated rats.

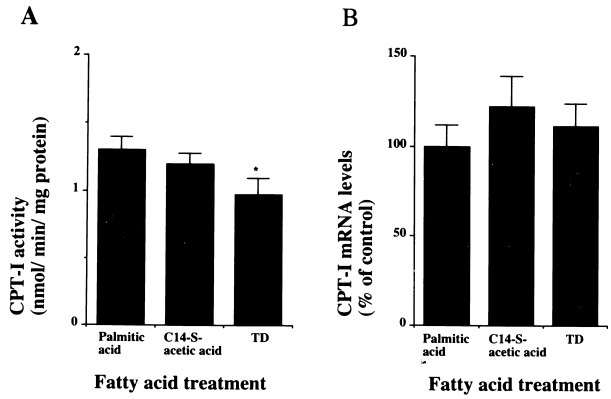

FIG. 2. Effect of palmitic and 3-thia fatty acids on mitochondrial CPT-I activity and mRNA levels in rat liver. Rats were fed palmitic or 3-thia fatty acids suspended in 0.5% CMC at a dose of 300 mg/day/kg body weight for 7 days. CPT-I activity (A) and mRNA levels (B) were measured in four individual rats as described under Materials and Methods. The relative mRNA levels were normalised to the corresponding 28S rRNA levels. CPT-I mRNA/28S rRNA levels in palmitic acid-treated rats were set to 100%. The values are presented as means ± SD.

and TD treatment, respectively (Fig. 1 and Table 2). As shown in Fig. 2, both the C14-S-acetic acid and especially the dithiadicarboxylic acid TD decreased hepatic CPT-I activity in the mitochondrial fraction, while no significant change was observed in mitochondrial CPT-I mRNA levels, compared to palmitic acid treatment. The CoA ester of C14-S-acetic acid was poorly converted to the carnitine ester and inhibited CPT-I activity (Fig. 3). Malonyl-CoA did not modify the inhibition of CPT-I activity during 3-thia fatty acid treatment (Fig. 4).

The earlier finding of a direct proportionality between ketogenesis from octanoate and (−)-octanoylcarnitine in perfused livers from rats treated in different ways suggested that CPT II may also be an important enzyme in the regulation of hepatic fatty acid oxidation [5, 6]. In the present study, both doses of C14-S-acetic acid and TD significantly increased CPT-II activity (Fig. 5A). Treatment of the rats with 3-thia fatty acids also resulted in a significant induction of rat liver mitochondrial CPT-II gene expression (Fig. 5B), with concomitant increases in immunodetectable protein (Table 3 and Fig. 6B). CPT-II activity was not inhibited by C14-S-acetyl-CoA (data not shown).

The conversion of acetyl-CoA produced by the β-oxidation process to acetoacetate is conducted via the HMG-CoA cycle, which in turn is controlled by HMG-CoA synthase. The activity and mRNA levels of mitochondrial HMG-CoA synthase increased 3- to 4-fold after C14-S-acetic acid and TD treatment (Fig. 7). A parallel increase in immunodetectable protein (Table 3) was observed, suggesting a coordinate mechanism between transcription and translation. Mitochondrial HMG-CoA lyase activity was only marginally affected, whereas thiolase activity was increased about 4-fold, after treatment with 3-thia fatty acids (Table 4).

In contrast to the ketogenic pathway, citrate synthase activity was decreased in C14-S-acetic acid-treated rats (Table 4). Fatty acid oxidation, measured as acid-soluble

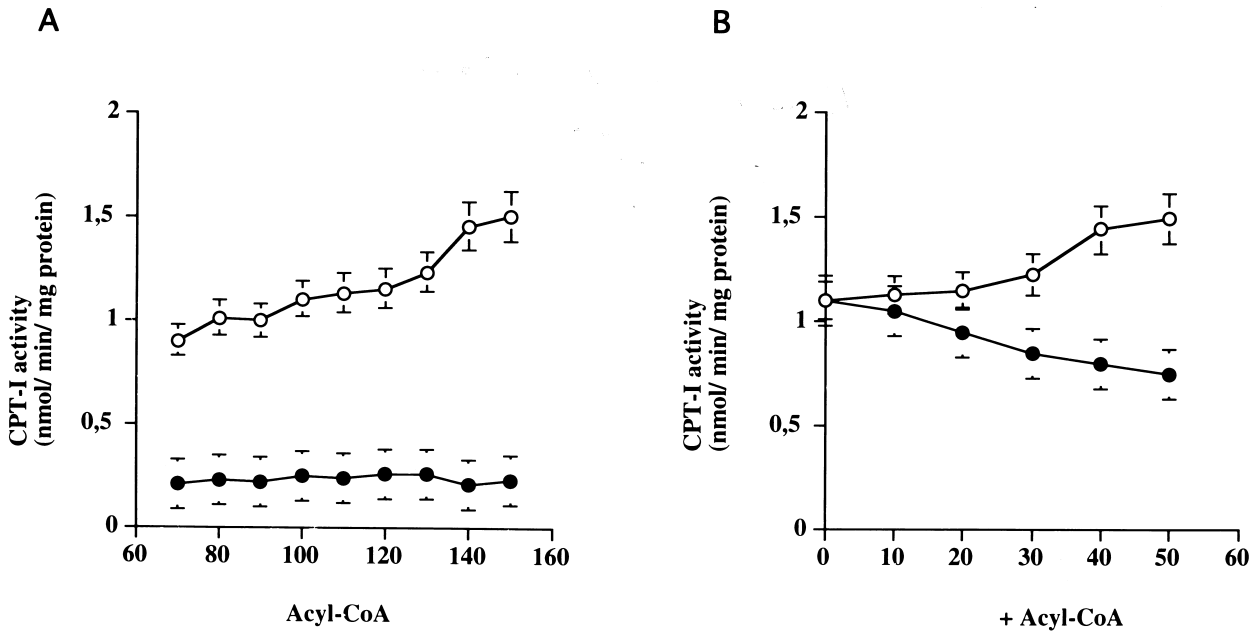

FIG. 3. Effect of C14-S-acetyl-CoA on mitochondrial CPT-I activity. (A) CPT I activity was measured in isolated mitochondria from four different control rat livers as described under Materials and Methods, using palmitoyl-CoA —○— and C14-S-acetyl-CoA —●— as substrates at different concentrations. The values are presented as means ± SD. (B) CPT I activity was measured in isolated mitochondria from four different control rat livers as described under Materials and Methods using 100 μM palmitoyl-CoA as a substrate. Additional acyl-CoA (palmitoyl-CoA —○— or C14-S-acetyl-CoA —●—) was added at 10, 20, 30, 40, and 50 μM. The values are presented as means ± SD.

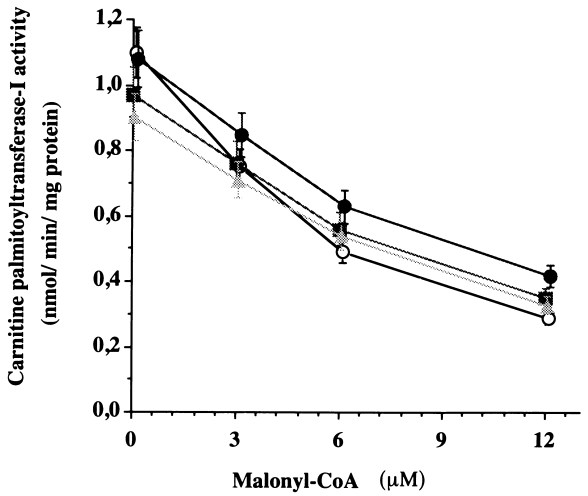

FIG. 4. Effect of palmitic acid —●—, C14-S-acetic acid —△—, and TD —■— on mitochondrial CPT-I activity in the presence and absence of malonyl-CoA. Rats were fed palmitic or 3-thia fatty acids suspended in 0.5% CMC at a dose of 300 mg/day/kg body weight for 7 days. Control animals received CMC —○— only. CPT I activity was measured in isolated mitochondria in four individual rat livers as described under Materials and Methods in the absence and presence of 3, 6, and 12 mM malonyl-CoA. The values are presented as means ± SD.

products, increased as a function of chain length of the 3-thia fatty acids, in agreement with earlier findings [35] (Table 5). Interestingly, C12-S-, C13-S-, and C14-S-acetic acid administration increased the levels of both HMG-CoA synthase and CPT-II, but not CPT-I (Table 5).

DISCUSSION

Feeding rats with C14-S-acetic acid resulted in a reduction of plasma and hepatic triglycerides with a concomitant increased mitochondrial β-oxidation (Table 1) [12]. The rate of fatty acid oxidation and ketogenesis is normally regulated by CPT-I in relation to the nutritional state of the animal and plasma concentration of NEFA released from adipose tissue into the blood. The finding that the serum NEFA levels were decreased, whereas the fatty acid oxidation increased in C14-S-acetic acid-treated animals (Table 1), therefore suggests a different regulation. The

TABLE 3. Effect of 3-thia and palmitic acids at a dose of 300 mg/day/kg body weight on the immunodetectable mitochondrial HMG-CoA synthase and CPT-II in rat liver

	HMG-CoA synthase	CPT-II
Treatment	(fold increase)	
Control	1.00 ± 0.08	1.00 ± 0.06
Palmitic acid	1.08 ± 0.14	1.13 ± 0.12
C14-S-acetic acid	2.15 ± 0.29*	2.45 ± 0.23*
TD	2.07 ± 0.25*	2.11 ± 0.21*

The values represent means ± SD for four animals in each experimental group.
*$P < 0.05$ for differences between control and treated rats.

recently identified fatty acid transport protein can facilitate rapid uptake and coordinate the import of NEFAs in harmony with the metabolic demand [36]. Increased mRNA levels of liver FABP (Fig. 1, Table 2), which can act as a cytoplasmic sink for fatty acids, suggest an increased transport of NEFA into hepatocytes for oxidation.

Increased fatty acid oxidation is generally associated with increased CPT-I activity and/or decreased sensitivity to malony-CoA. It was therefore unexpected that neither the activity of CPT-I nor the malonyl-CoA sensitivity of CPT-I was changed after C14-S-acetic acid treatment, compared to palmitic acid treatment (Figs. 2 and 5). Similarly, the mRNA levels of CPT-I were marginally changed (Fig. 2). It is difficult to explain the increased mitochondrial palmitoyl-CoA oxidation without any concomitant increased CPT-I activity. A possible explanation, however, would be that palmitic acid is partially oxidised in the peroxisome and enters the mitochondria as a medium chain fatty acid via peroxisomal CPT, as do phytanic and pristanic acid [37]. We demonstrated earlier that the specific CPT-I activity measured in the peroxisomes was approximately 50% of the activity measured in the mitochondrial fraction [38]. In contrast to the mitochondrial CPT-I activity, the peroxisomal activity increased 2- and 3-fold after C14-S-acetic acid and TD treatment, respectively. The role of CPT-I in peroxisomes is not elucidated, but unlike in mitochondria, CoA esters do not need carnitine for their penetration into the peroxisomes [39]. It is possible that palmitoyl-L-carnitines formed in peroxi-

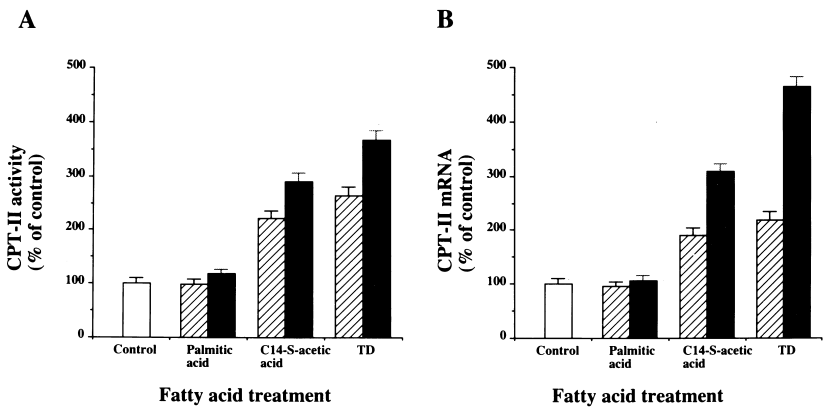

FIG. 5. Effect of palmitic and 3-thia fatty acids on mitochondrial CPT II activity and mRNA levels in rat liver. Rats were fed palmitic or 3-thia fatty acids suspended in 0.5% CMC at a dose of 150 (hatched bars) or 300 (solid bars) mg/day/kg body weight for 7 days. Control animals received CMC only. CPT-II activity (A) and mRNA levels (B) were measured in four individual rats as described under Materials and Methods. The relative mRNA levels were normalised to the corresponding 28S rRNA levels. CPT-II activity and CPT-II mRNA/28S rRNA levels in control rats were set to 100%. CPT-II activity in control rats was 22 ± 3 nmol/min/mg protein. The values are presented as means ± SD.

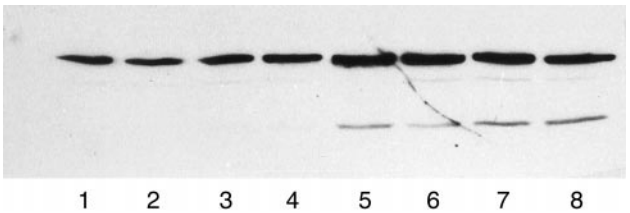

FIG. 6. Effect of palmitic and 3-thia fatty acids on the immunodetectable CPT II. Rats were fed palmitic (lanes 3 and 4), C14-S-acetic acid (lanes 5 and 6), or TD (lanes 7 and 8) suspended in 0.5% CMC at a dose of 300 mg/day/kg body weight for 7 days. Control animals received CMC only (lanes 1 and 2). Mitochondrial proteins were isolated and subjected to SDS-PAGE followed by immunoblotting anti-CPT-II antiserum, as described under Materials and Methods.

somes are delivered to the mitochondria for further oxidation. Such a mechanism could also explain why the increased oxidation of palmitoyl-CoA is more pronounced when measured in the postnuclear homogenate than when measured in isolated mitochondria (Table 1).

It has been suggested that 3-thia fatty acids act through the PPARs, as it was demonstrated that the trancriptional activity of C14-S-acetic acid correlated to its ability to bind PPAR α [40]. 3-Thia fatty acids, which cannot undergo β-oxidation, inhibit fatty acyl-CoA dehydrogenase [41] and the CoA ester of C14-S-acetic acid inhibits CPT-I activity (Fig. 3). Consequently, C14-S-acetic acid seems to have a dual function effect. As a ligand, it activates PPAR α directly, while as a metabolic inhibitor it may indirectly lead to accumulation of endogenous fatty acid ligands. It is also worth noting that C14-S-acetic acid might also act as a fatty acid. As C14-S-acetic acid is non-oxidisable by β-oxidation, accumulation of its respective CoA ester in the mitochondria might give a "fatty acid overload" signal leading to increased mitochondrial fatty acid oxidation. Moreover, it was recently demonstrated that PPARα is required for induction of FAO and cytochrome P450 4A2, but not for suppression of fatty acid synthase by polyunsaturated fatty acids [42]. These findings suggest that PPAR activation upregulates both mitochondrial and peroxisomal fatty acid oxidation and provide evidence for two distinct pathways for polyunsaturated fatty acid control of hepatic

TABLE 4. Effect of 3-thia- and palmitic acid at a dose of 300 mg/day/kg body weight on citrate synthase, acetoacetyl-CoA thiolase, and HMG-CoA lyase activities in the hepatic mitochondrial fractions of rats

	Citrate synthase	Acetoacetyl-CoA thiolase	HMG-CoA lyase
Treatment	(nmol/min/mg protein)		
Control	54 ± 3	14 ± 4	154 ± 39
Palmitic acid	53 ± 7	16 ± 4	180 ± 62
C14-S-acetic acid	44 ± 4*	56 ± 20*	205 ± 17*
TD	49 ± 9	60 ± 12*	279 ± 55*

The values represent means ± SD for four animals in each experimental group.
*$P < 0.05$ for differences between control and treated rats.

lipid metabolism. As administration of the 3-thia fatty acids seem to force eicosapentaenoic acid to the mitochondria [38], an additional "polyunsaturated fatty acid effect" might be seen.

C14-S-acetic acid is converted to its CoA ester in different subcellular fractions [38, 43]. In addition, C14-S-acetic acid incorporates in the cellular membranes and C14-S-acetic acid treatment alters the fatty acid composition and consequently membrane fluidity [35, 44, 45]. Consequently, conversion of substrates along major metabolic routes may be changed, as activation of several enzymes and protein factors may depend on the cellular fatty acid content and molecular species [46]. If the integral membrane protein CPT I does exhibit different conformational structures, changed fatty acid composition might determine the structure/function relationship of this complex enzyme, thereby explaining our results. In addition, C14-S-acetyl-CoA inhibits CPT-I activity (Fig. 3).

The first committed step of ketone body formation is catalyzed by acetoacetyl-CoA thiolase. Feeding with 3-thia fatty acids increased thiolase activity but decreased that of citrate synthase in rat liver (Table 4). Accordingly, it is most plausible that 3-thia fatty acids increase β-oxidation of long-chain acyl-CoA esters to acetyl-CoA with a subsequent conversion to ketone bodies. How these fatty acid analogues alter the tricarboxylic acid cycle flux has yet to be established. As the mitochondrial β-oxidation was increased (Table 1) with a concomitant increased ketone body formation (Ta-

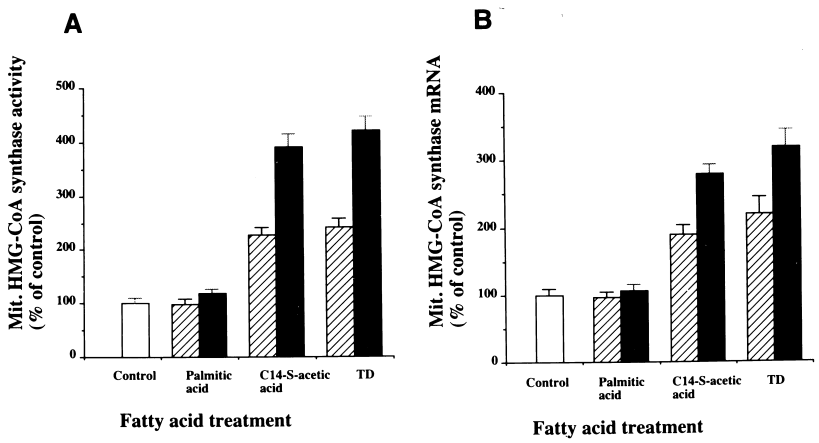

FIG. 7. Effect of palmitic and 3-thia fatty acids on mitochondrial HMG-CoA synthase activity and mRNA levels in rat liver. Rats were fed palmitic or 3-thia fatty acids suspended in 0.5% CMC at a dose of 150 (hatched bars) or 300 (solid bars) mg/day/kg body weight for 7 days. Control animals received CMC only. HMG-CoA synthase activity (A) and mRNA levels (B) were measured in four individual rats as described under Materials and Methods. The relative mRNA levels were normalised to the corresponding 28S rRNA levels. Mitochondrial HMG-CoA synthase activity mRNA/28S rRNA levels in control rats were set to 100%. The HMG-CoA synthase activity in control rats was 11 ± 2 nmol/min/mg protein. The values are presented as means ± SD.

TABLE 5. The effect of 3-thia fatty acids at a dose of 150 mg/day/kg body weight with different chain length on acid-soluble products and the mRNA levels of CPT-I, CPT-II, and HMG-CoA synthase

	Acid-soluble products	mRNA levels		
	Palmitoyl-CoA as substrate	CPT-I	CPT-II	HMG-CoA synthase
Treatment		(fold increase)		
Palmitic acid	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.2
C8-S-acetic acid	0.9 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	0.8 ± 0.2
C11-S-acetic acid	1.1 ± 0.1	1.0 ± 0.3	1.5 ± 0.3	1.1 ± 0.2
C12-S-acetic acid	1.4 ± 0.2*	1.2 ± 0.2	2.4 ± 0.5*	1.3 ± 0.2*
C13-S-acetic acid	1.5 ± 0.2*	1.2 ± 0.2	2.5 ± 0.3*	1.9 ± 0.3*
C14-S-acetic acid	2.9 ± 0.4*	1.2 ± 0.2	2.3 ± 0.4*	2.0 ± 0.4*
C15-S-acetic acid	1.0 ± 0.1	1.0 ± 0.1	1.6 ± 0.4	1.5 ± 0.3*
C16-S-acetic acid	1.1 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.2

The values represent means ± SD for four animals in each experimental group.
*$P < 0.05$ for differences between control and treated rats.

ble 1) and a decreased citrate synthase activity (Table 4), it is most likely that the acetyl-CoA produced by the β-oxidation process is converted into ketone bodies.

As mitochondrial HMG-CoA synthase [47] activity has been shown to be induced via PPAR α, it is likely that C14-S-acetic acid binds and activates PPAR α, which mediates the induction of the mitochondrial HMG-CoA synthase gene, as do other peroxisome proliferators and fatty acids [40]. In contrast to CPT-I, the mRNA levels of CPT-II (Fig. 7) increased in parallel with HMG-CoA synthase (Fig. 5). In this context, it is interesting to note that Chatelain *et al.* [48] have demonstrated that the peroxisome proliferator, clofibrate, stimulates both CPT-I and CPT-II gene expression, whereas long-chain fatty acids increased only the CPT-I mRNA levels in cultured hepatocytes. We have also recently demonstrated that after single-dose C14-S-acetic acid administration, CPT-I mRNA levels are increased after only 2 hr, whereas CPT-II and FAO mRNA levels are increased after 4 hr [49]. This suggests both that CPT-I and CPT-II mRNA levels are increased by different mechanisms and that peroxisome proliferators and fatty acids act through distinct modalities. This implies that the induction of CPT-II may be related to peroxisome proliferation. This notion was recently supported by the finding that neither FAO nor CPT-II mRNA were induced by the classical peroxisome proliferator Wy-14,643 in PPARα knockout mice [50]. Unfortunately, these authors did not measure CPT-I mRNA.

In conclusion, the present data show that CPT-II and HMG-CoA synthase enzyme activity and mRNA levels are up-regulated after 3-thia fatty acid treatment. Whether these enzymes retain some control over the relevant fluxes of fatty acid oxidation to ketones should be investigated in future.

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References

1. McGarry JD, Woeltje KF, Kuwajima M and Foster DW, Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase. *Diabetes Metab Rev* **5:** 271–284, 1989.
2. Quant PA, Robin D, Robin P, Girard J and Brand MD, A top-down control analysis in isolated rat liver mitochondria: Can the 3-hydroxy-3-methylglutaryl-CoA pathway be rate-controlling for ketogenesis? *Biochim Biophys Acta* **1156:** 135–143, 1993.
3. Casals N, Roca N, Guerrero M, Gil-Gomez G, Ayte J, Ciudad CJ and Hegardt FG, Regulation of the expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene. *Biochem J* **283:** 261–264, 1992.
4. Quant PA, Robin D, Robin P, Ferre P, Brand MD and Girard J, Control of hepatic mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase during the foetal/neonatal transition, suckling and weaning in rat. *Eur J Biochem* **195:** 449–454, 1991.
5. McGarry JD and Foster DW, The metabolism of (−)-octanoylcarnitine in perfused livers from fed and fasted rats. *J Biol Chem* **249:** 7984–7990, 1974.
6. McGarry JD, Wright PH and Foster DW, Rapid activation of hepatic ketogenetic capacity in fed rats by anti-insulin serum and glucagon. *J Clin Invest* **55:** 1202–1209, 1975.
7. Berge RK, Aarsland A, Kryvi H, Bremer J and Aarsaether N, Alkylthioacetic acids (3-thia fatty acids)-A new group of non-β-oxidizable peroxisome-inducing fatty acid analogues I. A study on the structural requirements for proliferation of peroxisomes and mitochondria in rat liver. *Biochim Biophys Acta* **1004:** 345–356, 1989.
8. Berge RK, Aarsland A, Kryvi H, Bremer J and Aarsaether N, Alkylthio acetic acids (3-thia fatty acids)-A new group of non-β-oxidizable peroxisome-inducing fatty acid analogues II. Dose–response studies on hepatic peroxisomal and mitochondrial changes and long-chain fatty acid-metabolizing enzymes in rats. *Biochem Pharmacol* **38:** 3969–3979, 1989.
9. Skrede S, Bremer J, Berge RK and Rustan AC, Stimulation of fatty acid oxidation by a 3-thia fatty acid reduces triacylglyc-

erol secretion in cultured rat hepatocytes. *J Lipid Res* **35:** 1395–1404, 1994.
10. Frøyland L, Helland K, Totland GK, Kryvi H and Berge RK, A hypolipidemic peroxisome proliferating fatty acid induces polydispersity of rat liver mitochondria. *Biol Cell* **87:** 105–112, 1996.
11. Skorve J, Al-Shurbaji A, Asiedu D, Björkhem I, Berglund L and Berge RK, On the mechanism of the hypolipidemic effect of sulfur-substituted hexadecanedoic acid (3-thiadicarboxylic acid) in normolipidemic rats. *J Lipid Res* **34:** 1177–1185, 1993.
12. Berge RK and Hvattum E, Impact of cytochrome P450 system on lipoprotein metabolism. Effect of abnormal fatty acids (3-thia fatty acids). *Pharmacol Ther* **61:** 345–383, 1994.
13. Asiedu DK, Al-Shurbaji A, Rustan AC, Björkhem I, Berglund L and Berge RK, Hepatic fatty acid metabolism as a determinant of plasma and liver triacylglycerol levels. *Eur J Biochem* **227:** 715–722, 1995.
14. Frøyland L, Madsen L, Vaagenes H, Totland GK, Auwerx J, Kryvi H, Staels B and Berge RK, Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism. *J Lipid Res* **38:** 1851–1858, 1997.
15. Willumsen N, Hexeberg S, Skorve J, Lundquist M and Berge RK, Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats. *J Lipid Res* **34:** 13–22, 1993.
16. Willumsen N, Vaagenes H, Asiedu D, Lie Ø, Rustan AC and Berge RK, Eicosapentaenoic acid but not docosahexaenoic acid (both as ethyl esters) increases mitochondrial fatty acid oxidation and upregulates 2,4-dienoyl-CoA reductase gene expression. A potential mechanism for the hypolipidemic action of fish oil in rats. *Lipids* **31:** 579–592, 1996.
17. Kawaguchi A, Tsubotani S, Seyama Y, Yamakawa T, Osumi T, Hashimoto T, Ando M and Okuda S, Stereochemistry of dehydrogenation catalyzed by Acyl-CoA oxidase. *J Biochem* **88:** 1481–1486, 1980.
18. Spydevold Ø and Bremer J, Induction of peroxisomal β-oxidation in 7800 C1 Morris hepatoma cells in steady state by fatty acids and fatty acid analogues. *Biochim Biophys Acta* **1003:** 72–79, 1989.
19. Berge RK, Flatmark T and Osmundsen H, Enhancement of long-chain acyl-CoA hydrolase activity in peroxisomes and mitochondria of rat liver by peroxisomal proliferators. *Eur J Biochem* **141:** 637–644, 1984.
20. Martinez A, Knappskog PM, Olafsdottir S, Døskeland AP, Eiken HG, Svebak RM, Bozzini ML, Apold J and Flatmark T, Expression of recombinant human phenylalanine hydroxylase as fusion protein in *Escherichia coli* circumvents proteolytic degradation by host cell proteases. *Biochem J* **306:** 589–597, 1995.
21. Bremer J, The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. *Biochim Biophys Acta* **665:** 628–631, 1981.
22. Clinkenbeard KD, Reed WD, Mooney RA and Lane MD, Intracellular localization of the 3-hydroxy-3-methylglutaryl coenzyme A cycle enzymes in liver. *J Biol Chem* **250:** 3108–3116, 1975.
23. Shepherd D and Garland PB, *Methods in Enzymology*, Vol. XIII. Academic Press, New York and London, 1969.
24. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
25. Aasland R, Akslen L, Varhaug JE and Lillehaug JR, Co-expression of the genes encoding transforming growth factor-α and its receptor in papillary carcinomas of the thyroid. *Int J Cancer* **46:** 382–387, 1990.
26. Sambrook J, Fritsch E and Maniatis T, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, 1989.
27. Esser V, Britton CH, Weis BC, Foster DW and McGarry JD, Cloning, sequencing, and expression of a cDNA encoding rat liver carnitine palmitoyltransferase I. *J Biol Chem* **268:** 5817–5822, 1993.
28. Asins G, Rosa JL, Serra D, Gil-Gomez G, Ayté J, Batrons R, Tauler A and Hegardt FG, Gene expression of enzymes regulating ketogenesis and fatty acid metabolism in regenerating rat liver. *Biochem J* **299:** 65–69, 1994.
29. Woeltje KF, Esser V, Weis BC, Sen A, Cox WF, McPhaul MJ, Slaughter CA, Foster DW and McGarry JD, Cloning, sequencing, and expression of a cDNA encoding rat liver mitochondrial carnitine palmitoyltransferase II. *J Biol Chem* **265:** 10720–10725, 1990.
30. Ayté J, Gil-Gomez G, Haro D, Marrero PF and Hegardt FG, Rat mitichondrial and cytocolic 3-hydroxy-3-methylglutaryl-CoA synthases are encoded by two different genes. *Proc Natl Acad Sci* USA **87:** 3874–3878, 1990.
31. Sargent TD, Wu JR, Sala-Trepat JM, Wallace RB, Reyes AA and Bonner J, The rat serum albumin gene: analysis of cloned sequences. *Proc Natl Acad Sci* USA **76:** 3256–3260, 1979.
32. Serra D, Asins G and Hegardt FG, Ketogenic mitochondrial 3-hydroxy 3-methylglutaryl-CoA synthase gene expression in intestine and liver of suckling pigs. *Arch Biochem Biophys* **301:** 445–448, 1993.
33. Kaikaus RM, Sui Z, Lysenko N, Yan Wu N, Oritz de Montellano PR, Ockner RK and Bass NM, Regulation of pathways of extramitochondrial fatty acid oxidation and liver fatty acid-binding protein by long-chain monocarboxylic fatty acids in hepatocytes. *J Biol Chem* **268:** 26866–26871, 1993.
34. Feinberg A and Vogelstein P, A technique for radiolabeling DNA restriction fragments to high specific activity. *Anal Biochem* **132:** 6–13, 1983.
35. Frøyland L, Madsen L, Sjursen W, Garras A, Lie Ø, Songstad J, Rustan AC and Berge RK, Effect of 3-thia fatty acids on the lipid composition of rat liver, lipoproteins, and heart. *J Lipid Res* **38:** 1522–1534, 1997.
36. Scaffer JE and Lodish HF, Expression cloning and characterisation of a novel long-chain fatty acid transport protein. *Cell* **79:** 427–436, 1994.
37. Verhoeven NM, Roe DS, Kok RM, Wanders RJA, Jacobs C and Roe CR, Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J Lipid Res* **39:** 66–74, 1998.
38. Madsen L, Frøyland L, Dyrøy E, Helland K and Berge RK, Docosahexaenoic and eicosapentaenoic acid are differently metabolized in rat liver during mitochondria and peroxisome proliferation. *J Lipid Res* **39:** 583–593, 1998.
39. Mannaerts GP, Debeer LJ, Thomas J and DeShepper PJ, Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and clofibrate-treated rats. *J Biol Chem* **254:** 4585–4595, 1979.
40. Forman BM, Chen J and Evans RM, Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferaor-activated receptors α and δ. *Proc Natl Acad Sci* USA **94:** 4312–4317, 1997.
41. Lau S-M, Brantley RK and Thorpe C, The reductive half-reaction in acyl-CoA dehydrogenase from pig kidney: Studies with thiaoctanoyl-CoA and oxaoctanoyl-CoA analogues. *Biochemistry* **27:** 5089–5094, 1988.
42. Ren B, Thelen AP, Peters JM, Gonzalez FJ and Jump DB, Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor α. *J Biol Chem* **272:** 26827–26832, 1997.
43. Skorve J, Rosendal J, Vaagenes H, Knudsen J, Lillehaug JR and Berge RK, Fatty acyl-CoA oxidase activity is induced

before long-chain acyl-CoA hydrolase activity and acyl-CoA binding protein in liver of rat treated with peroxisome proliferating 3-thia fatty acids. *Xenobiotica* **25:** 1181–1194, 1995.
44. Grav H, Asiedu D and Berge RK, Gas chromatographic measurement of 3- and 4-thia fatty acids incorporated into various classes of rat liver lipids during feeding experiments. *J Chromatog B* **658:** 1–10, 1994.
45. Madsen L, Frøyland L, Grav H and Berge RK, Upregulated Δ^9-desaturase gene expression by hypolipidemic peroxisome proliferating fatty acids resulted in increased oleic acid content in liver and VLDL: accumulation of a Δ^9-desaturated metabolite of tetradecylthioacetic acid. *J Lipid Res* **38:** 554–563, 1997.
46. Helleland C, Eiane SA, Berge RK, Holmsen H and Lillehaug JR, 1-(Carboxymethylthio)tetradecane attenuates PDGF- and TPA-induced c-*fos* mRNA expression and increases the formation of phosphatidylethanolamine with a shift from less to more polar molecular species in C3H/10T1/2 cells. *Exp Cell Res* **236:** 330–340, 1997.
47. Rodríguez JC, Gil-Gómez G, Hegardt FG and Haro D, Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids. *J Biol Chem* **269:** 18767–18772, 1994.
48. Chatelain F, Kohl C, Esser V, McGarry JD, Girard J and Pegorier J-P, Cyclic AMP and fatty acids increase carnitine palmitoyltransferase I gene transcription in cultured fetal rat hepatocytes. *Eur J Biochem* **235:** 789–798, 1996.
49. Vaagenes H, Madsen L, Asiedu DK, Lillehaug JR and Berge RK, Early modulation of genes encoding peroxisomal and mitochondrial β-oxidation enzymes by 3-thia fatty acids. *Biochem Pharmacol* **56:** 1571–1582, 1998.
50. Aoyama T, Peters JM, Iritani N, Nakajima T, Furihata K, Hashimoto T and Gonzalez FJ, Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor α (PPARα). *J Biol Chem* **273:** 5678–5684, 1998.