Mechanisms of liver steatosis in rats with systemic carnitine deficiency due to treatment with trimethylhydraziniumpropionate

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Abstract **Rats with systemic carnitine deficiency induced by treatment with trimethylhydraziniumpropionate (THP) develop liver steatosis. This study aims to investigate the mechanisms leading to steatosis in THP-induced carnitine deficiency. Rats were treated with THP (20 mg/100 g) for 3 or 6 weeks and were studied after starvation for 24 h. Rats treated with THP had reduced in vivo palmitate metabolism and developed mixed liver steatosis at both time points. The hepatic carnitine pool was reduced in THP-treated rats by 65% to 75% at both time points. Liver mitochondria from THP-treated rats had increased oxidative metabolism of various substrates and of β-oxidation at 3 weeks, but reduced activities at 6 weeks of THP treatment. Ketogenesis was not affected. The hepatic content of CoA was increased by 23% at 3 weeks and by 40% at 6 weeks in THP treated rats. The cytosolic content of long-chain acyl-CoAs was increased and the mitochondrial content decreased in hepatocytes of THP treated rats, compatible with decreased activity of carnitine palmitoyltransferase I in vivo. THP-treated rats showed hepatic peroxisomal proliferation and increased plasma VLDL triglyceride and phospholipid concentrations at both time points. A reduction in the hepatic carnitine pool is the principle mechanism leading to impaired hepatic fatty acid metabolism and liver steatosis in THP-treated rats. Cytosolic accumulation of long-chain acyl-CoAs is associated with increased plasma VLDL triglyceride, phospholipid concentrations, and peroxisomal proliferation.—**Spaniol, M., P. Kaufmann, K. Beier, J. Wüthrich, M. Török, H. Scharnagl, W. März, and S. Krähenbühl. **Mechanisms of liver steatosis in rats with systemic carnitine deficiency due to treatment with trimethylhydraziniumpropionate. *J. Lipid Res.* 2003.** 44: **144–153.**

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Liver steatosis is a frequent finding in liver biopsies and a frequent cause of asymptomatic elevation of transaminases (1). Several risk factors have been identified, among them ingestion of certain drugs (2–5), alcohol abuse (6), viral hepatitis (7), diabetes (8), increased body weight (8, 9), and intoxications (10). While impaired mitochondrial β-oxidation is considered to be the principle cause for microvesicular steatosis (11), the mechanisms leading to macrovesicular steatosis have so far not been identified in detail. As shown in **Fig. 1**, important possibilities leading to this finding include a decrease in VLDL export and/or an increase in VLDL formation, which may result from impaired mitochondrial fatty acid metabolism or from other causes.

We have recently developed and characterized a rat model with systemic carnitine deficiency (12). In this model, carnitine deficiency is induced within 3 weeks by feeding trimethylhydraziniumpropionate (THP), which inhibits carnitine biosynthesis and increases renal excretion of carnitine. Interestingly, rats treated with THP develop combined micro- and macrovesicular liver steatosis within 3 weeks but have no macro- or microscopic accumulation of lipids in skeletal muscle or heart (12, 13). Since carnitine is essential for transport of long-chain fatty acids into the mitochondrial matrix (14), it can be speculated that at least the microvesicular part of liver steatosis in THP-treated rats could be caused by hepatic carnitine deficiency. In support of this assumption, both in children with primary systemic carnitine deficiency and in mice with systemic carnitine deficiency (JVS mice), microvesic-

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Abbreviations: AOX, acyl-CoA oxidase; Complex I, NADH:decylubiquinone-1 oxidoreductase; Complex II, succinate:dichlorindophenol oxidoreductase; Complex III, Ubiquinol:ferricytochrome c oxidoreductase; Complex IV, Cytochrome c oxidase; CPT, carnitine palmitoyltransferase; DAB, diaminobenzidine; FC, free cholesterol; jvs, juvenile visceral steatosis; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic] acid; PPAR, peroxisome proliferator-activated receptor; TC, total cholesterol; THP, 3-(2,2,2-trimethylhydrazinium)propionate.

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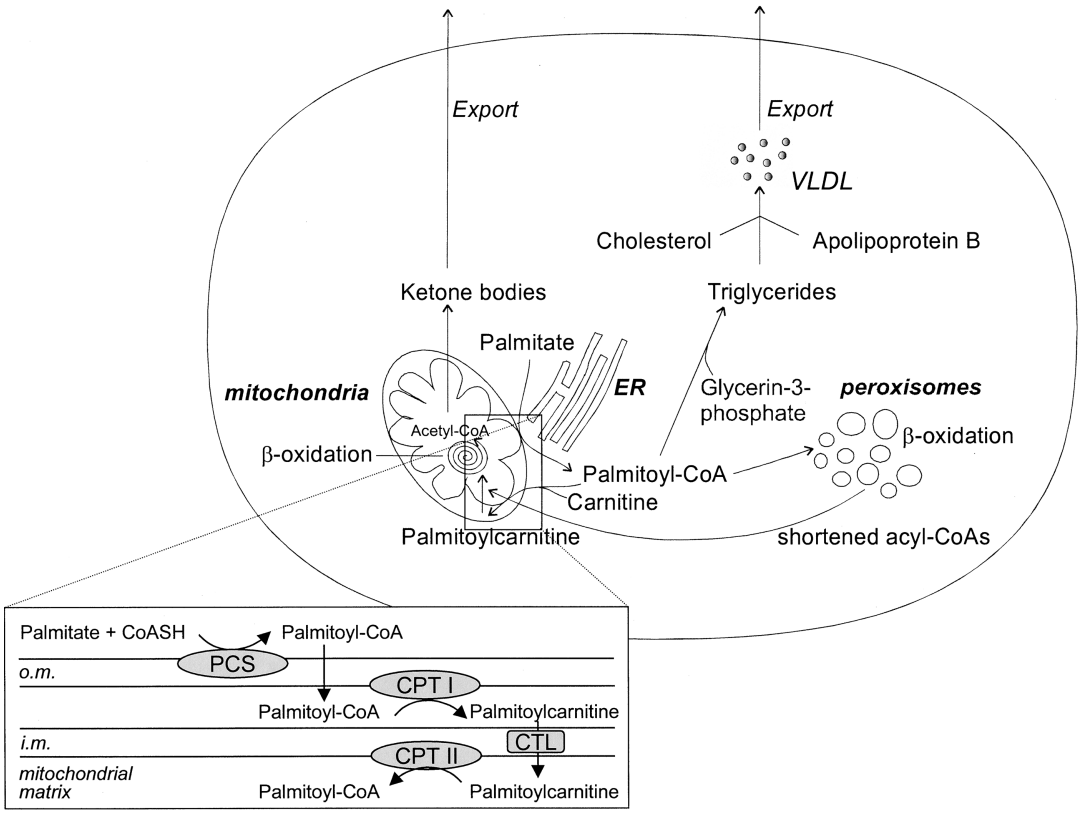

Fig. 1. Hepatic metabolism of long-chain fatty acids. Palmitate and other long-chain fatty acids are activated by palmitoyl-CoA (PCS) on the outer mitochondrial membrane, converted to palmitoylcarnitine by carnitine palmitoyltransferase I (CPT I), transported into the mitochondrial matrix and reconverted to palmitoyl-CoA by CPT II (see magnification). Palmitoyl-CoA is degraded to acetyl-CoA by the β-oxidation cycle. Acetyl-CoA can be converted to ketone bodies (major pathway) or be degraded in the Krebs cycle. Palmitoyl-CoA can also be β-oxidized by peroxisomes which produce medium-chain acyl-CoAs that are converted to the corresponding acyl-carnitines and can be metabolized to acetyl-CoA by mitochondria. In addition, cytosolic palmitoyl-CoA can also be used for the formation of triglycerides and phospholipids, which are both substrates for the formation of VLDL particles. See text for additional explanations. CTL, carnitine-acylcarnitine translocase, i.m., inner mitochondrial membrane, o.m., outer mitochondrial membrane.

ular liver steatosis has been reported (15, 16). However, at least the macrovesicular part of liver steatosis in rats treated with THP cannot be explained by an isolated defect in mitochondrial β-oxidation of fatty acids, suggesting additional mechanisms.

Since the morphology of liver steatosis in THP-treated rats resembles that found in certain types of steatotic livers in humans, e.g., certain types of alcohol-induced liver steatosis (17) or steatosis observed during administration of amiodarone (18), knowledge about the mechanisms leading to liver steatosis in THP-treated rats may also be relevant for understanding this disease in humans. We therefore decided to study the mechanisms leading to liver steatosis in rats treated with THP for 3 or 6 weeks. Beside the mechanisms leading to liver steatosis, we also investigated adaptive changes secondary to a decrease in the hepatic carnitine pool and to impaired in vivo mitochondrial β-oxidation. Our studies demonstrate that hepatic carnitine deficiency is the most important cause for liver steatosis in THP-treated rats and suggest that reduced mitochondrial fatty acid oxidation may be partially compensated by increased peroxisomal fatty acid metabolism due to proliferation of peroxisomes.

MATERIALS AND METHODS

Induction of carnitine deficiency and in vivo palmitate metabolism

The experiments have been reviewed and accepted by the State Ethics Committee of animal research. Carnitine deficiency was induced in male Sprague-Dawley rats by feeding vegetarian food poor in carnitine (Kliba Futter 2435, Basel, Switzerland) and THP (20 mg/100 g/day) for 3 or 6 weeks (n = 6 rats for each time point) (12). Control rats were kept for the same periods of time with the same rat chow ad libitum (n = 12).

In order to investigate a potential toxic effect on mitochondrial metabolism by THP itself in vivo, rats (n=3 in each group) were treated with THP (20 mg/100 g/day), with L-carnitine (50 mg/100 g/day), or with the combination of THP and L-carnitine (same dosages) for 3 weeks.

Metabolism of palmitate was determined in vivo after 3 weeks of treatment with THP by intraperitoneal injection of [^{14}C]palmitate and determination of exhaled $^{14}CO_2$ as described previously (19).

Isolation of rat liver mitochondria

Rats were killed by decapitation and mitochondria were isolated from the liver by differential centrifugation according to a previously described method (20). This method yields mitochondria of

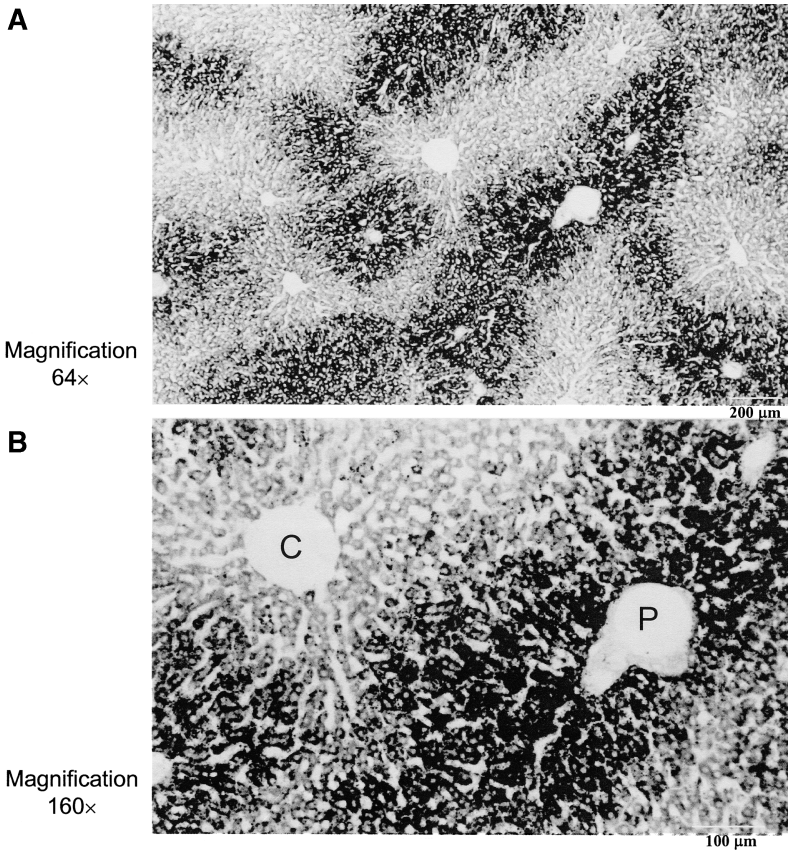

Fig. 2. Liver steatosis in rats treated with THP for 3 weeks. Cryosections stained with Sudan Black for specific labeling of lipids. As shown in A (enlargement 64×), lipids accumulate predominantly in zones 1 and 2 of the liver lobules, while zone 3 is spared. A higher enlargement (Fig. 2B, enlargement 160×) shows that the size of the lipid droplets varies, small and larger droplets can be detected in most hepatocytes, in particular in the periportal regions. Similar findings were present after 6 weeks of treatment with THP. In comparison, livers from control animals contained no fat (not shown). P, portal vein; C, central vein.

high purity with only minor contamination by peroxisomes or lysosomes (21). The mitochondrial protein content was determined using the biuret method with BSA as a standard (22). The content of mitochondrial protein/g liver was determined by correcting for the recovery of the mitochondria isolated using the activities of citrate synthase and succinate dehydrogenase (21).

Oxidative metabolism of intact mitochondria

Oxygen consumption by freshly isolated liver mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30°C as described previously (23). The concentrations of the substrates used were 20 mmol/l for L-glutamate and succinate, 40 μmol/l for palmitoyl-L-carnitine, 20 μmol/l for palmitoyl-CoA, and 80 μmol/l for palmitate. All incubations with fatty acids contained 5 mmol/l L-malate, incubations with palmitoyl-CoA and palmitate contained in addition 2 mmol/l L-carnitine, and incubations with palmitate contained in addition 250 μmol/l ATP and 250 μmol/l CoASH.

In vitro mitochondrial β-oxidation and formation of ketone bodies

The β-oxidation of [1-^{14}C] palmitic acid by liver mitochondria was assessed as described by Fréneaux et al. (24) with some modifications described previously (25). This assay measures the formation of acid-soluble products from mitochondrial palmitate metabolism, which equals production of ketone bodies and citric acid cycle intermediates (24).

Ketone body formation was measured using freeze-thawed mitochondria according to Chapman et al. (26) with some modifications as described previously (25). The reactions were stopped by adding 100 μl of 30% perchloric acid (w/v). After having removed the precipitate by centrifugation, the supernatants were analyzed for acetoacetate according to Olsen (27).

Activities of the enzyme complexes of the respiratory chain

Complex I (NADH:decylubiquinone-1 oxidoreductase) was determined spectrophotometrically as described by Veitch et al. (28) with some modifications. Briefly, 0.1 mg mitochondria were preincubated in 35 mmol/l potassium phosphate buffer pH 7.4, 5 mmol/l magnesium chloride, 2 mmol/l potassium cyanide, and 60 μmol/l decylubiquinone at 30°C. The reaction was started by the addition of 0.13 mmol/l NADH and the decrease of absorption was recorded spectrophotometrically at 340 nm using rotenone as inhibitor.

Complex II (succinate:dichloroindophenol oxidoreductase) was determined according to a previously described method (23). This method is based on the reduction of dichloroindophenol by complex II using succinate as a substrate. The reaction is followed spectrophotometrically at 600 nm in the presence and absence of the inhibitor thenoyltrifluoroacetone.

Complex III (ubiquinol:ferricytochrome c oxidoreductase) was determined spectrophotometrically at 550 nm by the conversion of ferricytochrome c to ferrocytochrome c using decylubiquinol as substrate (29). The reaction velocity was assessed as the difference in absorption with and without antimycine as inhibitor.

Complex IV (cytochrome c oxidase) activity was measured by following the oxidation of ferrocytochrome c at 550 nm as described by Wharton and Tzagaloff (30).

Determination of CoA and carnitine

Liver samples (about 50 mg/1 ml, prepared without thawing) were homogenized in 3% perchloric acid and centrifuged for 5 min at 10,000 *g*. Mitochondria (100 μl corresponding to about 10 mg protein) were mixed with 20 μl 0.2 mol/l dithiothreitol and precipitated with 1.88 ml 3.2% perchloric acid. The suspension was vortexed, kept on ice for 5 min, and then centrifuged for 10 min at 10,000 *g*. This perchloric acid treatment yields an

acid soluble (supernatant) and an acid insoluble fraction (pellet). The acid soluble fraction is used to measure free and acetyl-CoA and, after alkaline hydrolysis, total acid soluble CoA. The difference between these values represents short chain acyl-CoA. Long chain CoA is determined in the pellet after alkaline hydrolysis. Total CoA refers to the sum of total acid soluble CoA and long-chain acyl-CoA.

In these fractions, CoASH and acetyl-CoA, total acid soluble CoA (supernatant), and long-chain acyl-CoA (pellet) were determined using the CoA recycling assay of Allred and Guy (31), with some modifications in the work-up as described before (32). Since the CoA recycling assay does not differentiate CoASH and acetyl-CoA, acetyl-CoA was determined specifically using a radioenzymatic assay as described previously (33).

For the determination of carnitine, liver tissue was worked up as described above for CoA. Plasma was also treated with perchloric acid (final concentration 3%) to obtain a supernatant and a pellet. The determination of carnitine in these fractions was performed using the radioenzymatic assay described by Brass and Hoppel (34). Direct analysis of the supernatant yields free carnitine, and, after alkaline hydrolysis, total acid soluble carnitine. The difference between free and total acid soluble carnitine is the short-chain acylcarnitine fraction (up to a chain-length of the acyl-group of about 10). Long-chain acylcarnitines were determined in the pellet after alkaline hydrolysis. Addition of total acid soluble and long-chain acylcarnitine yields total carnitine.

Lipid determination in livers

Lipids from rat livers were extracted according to the method described by Bligh and Dyer (35). Briefly, about 100 mg of liver tissue were homogenized in 2 ml 20 mmol/l potassium phosphate buffer, pH 7.4, and the lipids were extracted by the addition of 5.0 ml chloroform-methanol (1:1, v/v). The samples were vortexed and incubated for 60 min under periodical stirring. Then, 2.5 ml chloroform and 0.8 ml 0.74% potassium chloride were added and the mixture centrifuged for 5 min at 500 *g*. The lower phase was separated and washed with 1.5 ml of a mixture containing 0.74% potassium chloride-chloroform-methanol (94:96:6, v/v/v). The organic phase was then evaporated to dryness and the lipid extract stored at −20°C until analysis.

For lipid determination, liver extracts were resuspended in 250 μl isopropanol. Total cholesterol, triacylglycerides, and free fatty acids were measured using enzymatic methods and reagents from Wako (Neuss, Germany). The measurements were calibrated using standards from Roche Diagnostics (Mannheim, Germany).

Determination of plasma lipids

Total cholesterol (TC), free cholesterol (FC), triacylglycerides (TG), phospholipids (PL), HDL cholesterol (HDL-C), and free fatty acids (FFA) were measured using enzymatic methods and reagents from Wako (Neuss, Germany). The measurements were performed on a Wako 30R automatic analyzer (Wako) and were calibrated using standards from Roche Diagnostics (Mannheim, Germany). Esterified cholesterol was calculated as the difference between TC and FC.

The determination of the lipoprotein fractions was performed by a combined ultracentrifugation-precipitation method (36, 37). VLDL were removed quantitatively by ultracentrifugation using a TFT 56.6 rotor (Kontron, Germany) with adapters for 0.8 ml polycarbonate tubes. Five hundred microliters of plasma was pipetted into tubes and 0.1 ml of 0.9% sodium chloride solution was layered on top of the plasma. After centrifugation (18 h at 30,000 rpm, 10°C), the floating VLDL fraction was aspired with a 2 ml syringe until the supernatant was completely clear. The volume was reconstituted to the original weight with 0.9% saline. LDL were precipitated in the infranatant using phosphotungstic acid/magnesium chloride (PTA, Roche, Mannheim, Germany), and HDL lipids were measured in the supernatant after LDL precipitation. Lipids in the LDL fraction were calculated as the difference between the concentrations in the density fraction d > 1.006 kg/l and the HDL fraction.

Cytochemical localization of catalase in liver sections

Livers from control rats and rats treated with THP for 3 weeks were fixed by perfusion through the portal vein with a fixative containing 0.25% glutaraldehyde and 2% sucrose in 0.1 M PIPES buffer, pH 7.4. The tissue was cut into 70 μm sections with a DSK-Microslicer (Dosaka EM Co., Kyoto, Japan). The sections were incubated in alkaline diaminobenzidine (DAB) medium for cytochemical visualization of catalase (38), followed by osmication and embedding in Epon 812.

SDS-PAGE and immunoblotting

For Western blotting, tissues were homogenized in a buffer containing 250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% ethanol, pH 7.4, using an Ultra-Turrax (IKA Labortechnik, Staufen, Germany). Equal amounts of protein (20 μg per sample) were subjected to SDS-PAGE. After electrotransfer of the polypeptides onto nitrocellulose, the sheets were incubated overnight with the primary antibody at a concentration of 1 μg protein/ml. The polyclonal antibody against acyl-CoA oxidase (AOX) was a generous gift of A. Völkl, Institute of Anatomy and Cell Biology, University of Heidelberg. Its specificity was assessed as described previously (39). After repeated washing, a peroxidase conjugated goat anti-rabbit antibody (Sigma, München, Germany) was added for 1 h at room temperature. The immunoreactive bands were visualized by enhanced chemoluminescence (ECL, Amersham International, Little Chalfont, England) according to the manufacturer's protocol.

The blots for subunit IV of cytochrome c oxidase and apolipoprotein B (apoB) were performed according to the method described above. The polyclonal antibody against subunit IV of cytochrome c oxidase was obtained from Molecular Probes (Juro Supply, Lucerne, Switzerland) and used at a dilution of 0.5 μg/ml. The polyclonal antibody against apoB was obtained from LabForce AG (4208 Nunningen, Switzerland) and used at a dilution of 0.5 μg/ml.

Determination of acyl-CoA oxidase activity

Acyl-CoA oxidase activity was determined according to a previously described method (40). This method is based on the production of hydrogen peroxide by the action of acyl-CoA oxidase, which is measured spectrophotometrically at 502 nm using dichlorofluorescein diacetate as a chromophore. Briefly, 50 mg of frozen liver tissue were homogenized in 20 mmol/l potassium phosphate buffer, pH 7.4, and the volume made up to 2.0 ml. One hundred microliters of this homogenate were preincubated in 1.9 ml of 10 mmol/l potassium phosphate buffer pH 7.4 containing 0.05 mg 2′,7′dichlorfluorescein diacetate, 5.2 mg sodium azide, 74 mU horseradish peroxidase, and 0.01% TritonX at 37°C for 5 min. The reaction was started by the addition of 15 μmol/l palmitoyl-CoA and the increase in absorbance was recorded spectrophotometrically over 3 min at 502 nm. Activities were calculated using an extinction coefficient of 91,000 $l \times mol^{-1} \times cm^{-1}$.

Statistics

Data are presented as mean ± SD. Groups were compared using the Student's *t*-test (comparison of two groups) or ANOVA followed by Tukey's protected Student's *t*-test (>2 groups). Since the values of control animals were generally not different between 3 and 6 weeks, they were pooled unless indicated otherwise.

RESULTS

The studies were carried out to elucidate the principle mechanisms leading to liver steatosis in rats with systemic carnitine deficiency due to treatment with THP.

Rats were treated with THP for 3 or 6 weeks and studied after starvation for 24 h. As shown in **Fig. 2**, and in agreement with previous studies (12, 13), rats treated with THP for 3 weeks developed micro- and macrovesicular liver steatosis predominantly in zone I and II of the liver lobules. Similar findings were obtained after 6 weeks of treatment with THP (results not shown).

Palmitate metabolism was assessed in vivo in rats treated with THP for 3 weeks and corresponding control rats by injecting l-[^{14}C]palmitate ip and measuring the amount of $^{14}CO_2$ exhaled over the next 2 h. The percentage of radioactivity exhaled was decreased in THP treated rats (28.2 ± 6.3% vs. 54.8 ± 8.2% of the dose injected, $P < 0.05$), compatible with reduced hepatic metabolism of long-chain fatty acids. Accordingly, the hepatic lipid content was increased significantly ($P < 0.05$) in rats treated with THP for 6 weeks for all lipid classes investigated [cholesterol 0.77 ± 0.09 vs. 0.58 ± 0.09 mg/g liver (THP-treated vs. control), triglycerides 4.63 ± 2.78 vs. 1.54 ± 0.46 mg/g liver, and free fatty acids 0.80 ± 0.09 vs. 0.58 ± 0.02 μmol/g liver]. As can be seen in Fig. 1, reduced metabolism of long-chain fatty acids can be due to impaired activation of fatty acids, impaired transport of fatty acids into the mitochondrial matrix, impaired β-oxidation and/or an impaired function of the respiratory chain.

As expected from the mechanism by which THP reduces the body carnitine content, namely inhibition of carnitine biosynthesis and increased renal excretion of carnitine (12), the liver carnitine pool was reduced in THP-treated rats, showing a drop by ~70% in comparison to control rats (**Table 1**). Since carnitine is essential for the function of CPT I (Fig. 1), this finding may explain liver steatosis in THP-treated rats. Interestingly, both the short-chain acylcarnitine to free carnitine and the long-chain acylcarnitine to free carnitine ratios were increased in livers of rats treated for 6 weeks, compatible with accumulation of β-oxidation intermediates due to impaired β-oxidation of fatty acids (32).

Since β-oxidation is primarily a mitochondrial function, these findings prompted us to study the function of the respiratory chain and oxidative metabolism of fatty acids in isolated mitochondria (**Table 2**). Surprisingly, state-3 oxidation rates tended to be higher in rats treated with THP for 3 weeks but were generally decreased in rats treated for 6 weeks. This decrease was observed particularly for succinate, palmitic acid, palmitoyl-CoA, and palmitoyl-carnitine, suggesting a defect in the activity of complex II of the electron transport chain and of β-oxidation. It is noteworthy that the experiments with palmitate and palmitoyl-CoA were performed in the presence of exogenous carnitine. When no exogenous carnitine was added to these substrates, state-3 oxidation rates were not different between mitochondria of control and THP-treated rats, and were approximately only 10% of the rates obtained with control mitochondria in the presence of exogenous carnitine.

In order to exclude a major effect of carnitine deficiency on the mitochondrial electron transport chain, the activity of the enzyme complexes of the electron transport chain was determined. As shown in **Table 3**, the activities of complexes I to IV were unchanged or increased after 3 weeks of treatment with THP, when compared with con-

TABLE 1. Liver and plasma carnitine pools

	Control (n = 12)	THP 3 weeks (n = 6)	THP 6 weeks (n = 6)
Liver (nmol/g liver wet weight)			
Free Cn	266 ± 73	74 ± 31[a]	75 ± 16[a]
SCA Cn	127 ± 52	38 ± 20[a]	51 ± 17[a]
LCA Cn	50 ± 21	14 ± 12[a]	26 ± 8[a]
Total Cn	442 ± 75	125 ± 3[a]	153 ± 27[a]
SCA Cn/Free Cn	0.48 ± 0.13	0.51 ± 0.15	0.68 ± 0.15[a]
LCA Cn/Free Cn	0.19 ± 0.07	0.19 ± 0.12	0.35 ± 0.09[a,b]
Plasma (μmol/l)			
Free Cn	29.8 ± 9.3	5.6 ± 0.8[a]	4.6 ± 1.6[a]
SCA Cn	33.4 ± 12.9	5.4 ± 0.8[a]	4.9 ± 1.3[a]
LCA Cn	9.3 ± 1.5	1.6 ± 0.7[a]	1.6 ± 2.7[a]
Total Cn	72.6 ± 24.9	12.6 ± 0.7[a]	11.0 ± 4.2[a]

Cn, carnitine; LCA Cn, long-chain acylcarnitine; SCA Cn, short-chain acylcarnitine. Control and THP-treated rats (20 mg/100 g body weight) were studied after 3 or 6 weeks of treatment in the fasted state. The carnitine pools were determined by a radioenzymatic assay as described in Materials and Methods. Data are presented as mean ± SD.

[a] $P < 0.05$ versus control.

[b] $P < 0.05$ THP 6 weeks versus THP 3 weeks.

TABLE 2. State 3 oxidation rates by isolated rat liver mitochondria

	Control (n = 12)	THP 3 weeks (n = 6)	THP 6 weeks (n = 6)
L-Glutamate (20 mM)	82 ± 24	101 ± 17	80 ± 23
Succcinate (20 mM)	150 ± 40	207 ± 31[a]	123 ± 31[b]
Palmitoyl-L-carnitine (40 μM)	82 ± 26	116 ± 18[a]	62 ± 13[b]
Palmitoyl-CoA (20 μM)	83 ± 17	97 ± 28	68 ± 14[b]
Palmitate (80 μM)	46 ± 10	63 ± 14[a]	29 ± 10[a,b]

Control and THP-treated rats (20 mg/100 g body weight) were studied after 3 or after 6 weeks of treatment in the fasted state. Mitochondria were isolated by differential centrifugation and state 3 oxidation rates were determined in the presence of the substrates indicated using a Clark-type oxygen electrode as described in Materials and Methods. Units are natoms oxygen × minute^{-1} × milligram of mitochondrial protein^{-1}. Results are presented as mean ± SD.

[a] $P < 0.05$ versus control rats.

[b] $P < 0.05$ THP 6 weeks versus THP 3 weeks.

TABLE 3. Activities of enzyme complexes of the electron transport chain in isolated rat liver mitochondria

	Control (n = 12)	THP 3 weeks (n = 6)	THP 6 weeks (n = 6)
Complex I	17.5 ± 6.8	22.3 ± 12.6	15.5 ± 5.6
Complex II	12.0 ± 3.0	15.1 ± 5.9[a]	10.5 ± 4.1[b]
Complex III	1780 ± 440	1560 ± 340	1560 ± 420
Complex IV	419 ± 149	381 ± 195	367 ± 129

Control and THP treated rats (20 mg/100 g body weight) were studied after 3 or 6 weeks of treatment in the fasted state. Enzyme activities were determined using spectrophotometric methods as described in Materials and Methods. Units are mU × mg mitochondrial protein^{-1}. Data are presented as mean ± SD.

[a] $P < 0.05$ versus control rats.

[b] $P < 0.05$ THP 6 weeks versus THP 3 weeks.

TABLE 4. In vitro β-oxidation and ketogenesis

	Control (n = 12)	THP 3 weeks (n = 6)	THP 6 weeks (n = 6)
CPT I	347 ± 74	309 ± 53	263 ± 56[a]
β-Oxidation	0.86 ± 0.22	0.84 ± 0.2	0.45 ± 0.14[a,b]
Ketogenesis	15.0 ± 4.0	16.7 ± 6.3	16.4 ± 5.7
Ketone bodies			
Liver	1.96 ± 0.4	2.11 ± 0.5	1.94 ± 0.1
Plasma	903 ± 189	829 ± 172	805 ± 165

Control rats and THP treated rats (20 mg/100 g body weight) were studied after 3 or 6 weeks of treatment in the fasted state. Activities were determined using radioactive substrates as described in Materials and Methods. Units are mU × mg mitochondrial protein^{-1} for CPT I activity, β-oxidation and ketogenesis and nmol × mg liver wet weight^{-1} and μmol × l^{-1} for ketone bodies in liver and plasma, respectively. Data are presented as mean ± SD.

[a] $P < 0.05$ versus control rats.

[b] $P < 0.05$ THP 6 weeks versus THP 3 weeks.

trol values. After 6 weeks of treatment, the activity of complex II was significantly decreased, whereas the activities of complex I, III, and IV were not altered in comparison to control rats. These findings agree well with the data obtained by the oxygen electrode.

Since reduced fatty acid oxidation could not be explained entirely by a reduced activity of the electron transport chain, we investigated the function of CPT I and of mitochondrial β-oxidation (**Table 4**). A direct toxic effect of THP on mitochondrial fatty acid metabolism was excluded, since THP did not impair CPT I activity or mitochondrial β-oxidation up to a concentration of 500 μmol/l and the addition of L-carnitine to THP prevented the development of liver steatosis in vivo (**Fig. 3**). CPT I activity, which can be rate-limiting for mitochondrial β-oxidation (41), was not different between mitochondria from THP-treated or control animals at 3 weeks, but showed a significant reduction in THP-treated rats at 6 weeks. Considering mitochondrial β-oxidation, no differences between THP-treated and control rats were observed after 3 weeks of treatment, whereas β-oxidation was reduced in rats treated with THP for 6 weeks. In contrast, ketogenesis was not affected at both time points. In agreement with ketogenesis, the β-hydroxybutyrate concentrations in liver and plasma were not different between control and THP-treated rats.

The results obtained so far show a decrease in the hepatic carnitine pool, impaired metabolism of palmitic acid, accumulation of hepatic fat at 3 weeks and 6 weeks of treatment with THP, and impaired hepatic mitochondrial β-oxidation and activity of CPT I at 6 weeks. These results therefore suggest that carnitine deficiency, which is present at both time points, is the major cause for liver steatosis in THP-treated rats, whereas decreased mitochondrial β-oxidation/CPT I activity may contribute at 6 weeks of treatment with THP. In order to investigate the consequences of carnitine deficiency in more detail, we determined the cytosolic and the mitochondrial CoA pools (**Table 5**). Expressed per gram of liver, the CoA pool was increased in THP treated rats at both time points. This increase could be explained by a rise in free and acetyl-CoA, whereas the short- and long-chain acyl-CoA contents were

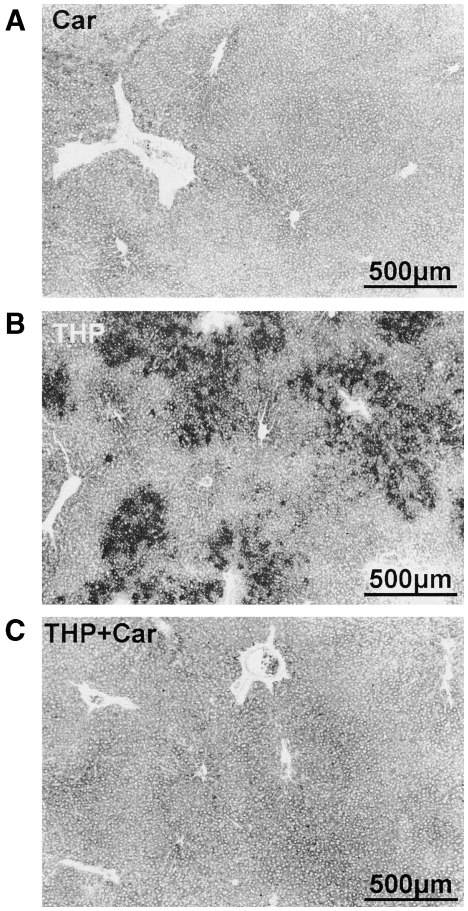

Fig. 3. Effect of L-carnitine on liver steatosis in rats treated with THP for 3 weeks. Cryosections stained with Sudan Black for specific labeling of lipids (enlargement 44×). Treatment of L-carnitine (50 mg/100 g/day for 3 weeks) is not associated with accumulation of fat (A). Similar to Fig. 2, treatment with THP (20 mg/100 g/day for 3 weeks) is associated with fat accumulation in the periportal region (B). The addition of L-carnitine (50 mg/100 g/day for 3 weeks) to THP (20 mg/100 g/day for 3 weeks) prevents the accumulation of fat almost completely (C).

not different from control rats. In contrast to total liver, the mitochondrial CoA content was decreased in THP-treated rats both when expressed per mitochondrial protein content or per mitochondria contained in 1 g of liver. This decrease could be explained by a reduced mitochondrial content in short- and long-chain CoAs. Consequently, the cytosolic CoA content (calculated as the difference between the total content and the content in the mitochondria per g liver) was increased in THP-treated rats and this increase was found for all CoA fractions determined.

Mitochondrial dysfunction can be associated with secondary hepatic changes such as mitochondrial proliferation (21, 23). Mitochondrial proliferation was assessed by determining the mitochondrial protein content per gram of liver and by performing Western blots of subunit IV of cytochrome c oxidase. Mitochondrial protein content was not significantly different between control and THP treated rats (111 ± 25 mg /g in control rats, and 89 ± 16 and 103 ± 23 mg/g in rats treated with THP for 3 and 6 weeks, respectively). In addition, the protein content of

TABLE 5. Liver CoA pool

	Control (n = 12)	THP 3 weeks (n = 6)	THP 6 weeks (n = 6)
Total liver			
CoASH + acetyl-CoA	223 ± 73	301 ± 89[a]	365 ± 64[a]
Short-chain acyl-CoA	68 ± 57	63 ± 27	68 ± 47
Long-chain acyl-CoA	90 ± 31	102 ± 32	97 ± 13
Total CoA	380 ± 84	466 ± 61[a]	528 ± 90[a]
Liver cytosol			
CoASH + acetyl-CoA	172 ± 63	nd	323 ± 62[a]
Short-chain acyl-CoA	26 ± 15	nd	52 ± 23[a]
Long-chain acyl-CoA	55 ± 16	nd	75 ± 10[a]
Total CoA	251 ± 46	nd	446 ± 43[a]
Liver mitochondria			
CoASH	381 ± 156	nd	365 ± 64
Acetyl-CoA	76 ± 15	nd	51 ± 22[a]
Short-chain acyl-CoA	379 ± 129	nd	159 ± 91[a]
Long-chain acyl-CoA	315 ± 92	nd	212 ± 55[a]
Total CoA	1160 ± 160	nd	793 ± 108[a]

Control and THP treated rats (20 mg/100 g body weight) were studied after 3 or 6 weeks of treatment in the fasted state. CoASH and acyl-CoAs (except acetyl-CoA) were determined fluorimetrically, and acetyl-CoA by a radioenzymatic method as described in Materials and Methods. Units are nmol × g liver wet weigh^{-1} (total liver and liver cytosol) or nmol × g mitochondrial protein^{-1}. The cytosolic CoA content was calculated by subtracting the mitochondrial from the total liver content as described in Materials and Methods. Data are presented as mean ± SD.

[a] $P < 0.05$ versus control rats.

COX subunit IV, as assessed by Western blotting, was not different between THP and control rats (results not shown), excluding mitochondrial proliferation.

When mitochondrial fatty acid metabolism is impaired, peroxisomal proliferation could be a consequence due to hepatic accumulation of long-chain fatty acids and fatty acid metabolites that may stimulate peroxisome proliferator-activated receptor (PPAR)α (42). Indeed, a significant panlobular peroxisomal proliferation occurred in livers of THP-treated rats (**Fig. 4**). In agreement with these results, peroxisomal fatty acyl-CoA oxidase activity and protein content were both increased in THP-treated rats (**Fig. 5**).

A third consequence of decreased mitochondrial fatty acid oxidation could be an increase in the hepatic VLDL production. This was assessed indirectly by measuring the plasma lipid composition. As shown in **Table 6**, VLDL triglycerides and phospholipids were all increased in THP-treated versus control rats, which may reflect increased hepatic availability of activated fatty acids (as shown above) with increased hepatic VLDL synthesis. On the other hand, Western blot analysis revealed that the hepatic content of apoB was not different between THP-treated and control rats (results not shown).

DISCUSSION

Our study demonstrates that THP-treated rats have an ~70% reduction in the hepatic carnitine content, reduced in vivo metabolism of palmitate, liver steatosis, accumulation of long-chain acyl-CoAs in the cytosol of the hepatocytes, hepatic proliferation of peroxisomes, and increased plasma VLDL.

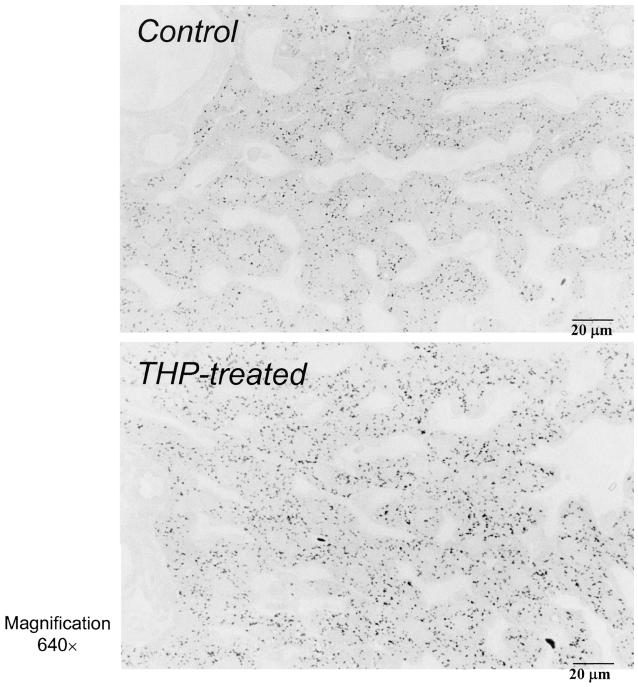

Fig. 4. Hepatic peroxisomal proliferation in rats treated with THP for 3 weeks. Peroxisomes were stained with the alkaline DAB method for cytochemical localization of catalase. Hepatocytes of rats treated with THP contain an increased number of peroxisomes. In comparison to lipid accumulation (Fig. 2), the increase in the number of peroxisomes shows no significant lobular gradient (enlargement 640×).

The study was designed to find out the mechanisms leading to micro- and macrovesicular steatosis THP-treated rats. Liver steatosis was present already at 3 weeks of treatment, a time point when THP-treated rats had developed systemic carnitine deficiency, and had reduced in vivo metabolism of palmitate, whereas oxidative metabolism and β-oxidation of isolated liver mitochondria was normal or even higher in mitochondria from THP-treated as compared with control rats. Since THP has no apparent toxicity on liver mitochondria, liver steatosis is explained, at least at this early time point, entirely by a reduction in the hepatic carnitine pool.

After 6 weeks of treatment with THP, oxidative metabolism of different substrates (including fatty acids) as well as β-oxidation and activity of CPT I were reduced in THP-treated rats, suggesting that impaired mitochondrial function could contribute to the development of liver steatosis. Although the study was not designed to find out the mechanisms leading to mitochondrial dysfunction at this time point, it can be speculated that it may reflect changes in the lipid composition of mitochondrial membranes and/or oxidative damage to mitochondria, as observed in other animal models with alterations in hepatic fatty acid metabolism (43) or liver steatosis (44, 45).

In THP-treated rats, the hepatic content of long-chain acyl-CoAs was increased at 3 and 6 weeks, compatible with a decreased cytosolic conversion of long-chain acyl-CoAs to the respective carnitine derivatives due to carnitine deficiency and/or reduced activity of CPT I (see Fig. 1 for

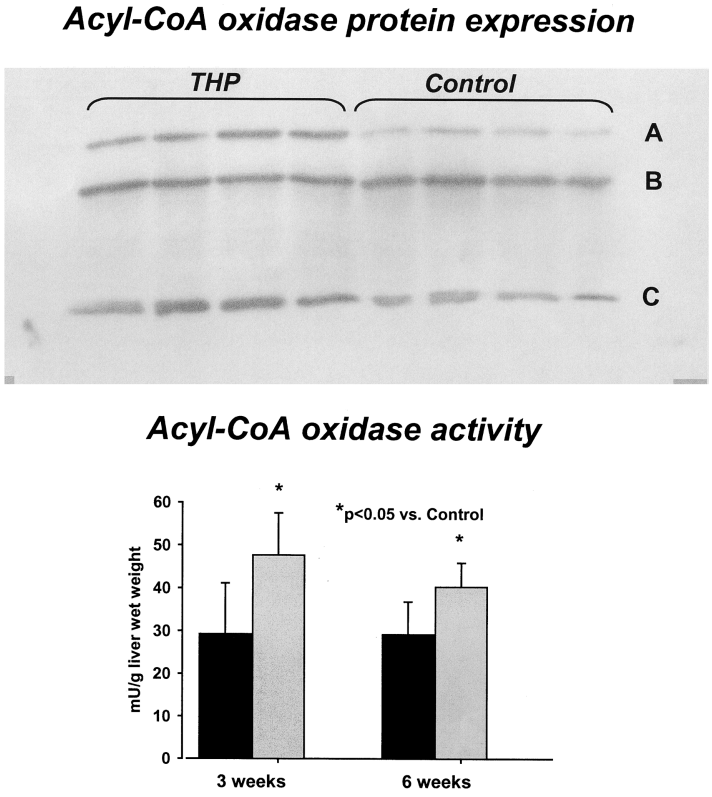

Fig. 5. Protein expression and activity of acyl-CoA oxidase. Similar to earlier observations, the Western blot of liver homogenate with polyclonal antibodies against acyl-CoA oxidase (AOX) reveals three bands (58, 59). These three bands correspond to AOX subunits A (71.9 kDa), B (51.7 kDa), and C (20.5 kDa). All three bands exhibited a significant ($P < 0.05$) augmentation in livers from THP-treated rats. The densitometric analysis yielded 3.52 ± 1.29 versus 1.00 ± 0.43 for band A (THP-treated vs. control), 1.55 ± 0.12 versus 1.00 ± 0.27 for band B, and 1.70 ± 0.39 versus 1.00 ± 0.17 for band C. The activity for AOX was increased ~1.5-fold, corresponding well with the densitometric results for bands B and C.

explanation). Peroxisomal proliferation may result from stimulation of PPARα by accumulated fatty acids and fatty acid derivatives in THP-treated rats (42), and may represent a mechanism to compensate for decreased mitochondrial metabolism of fatty acids. In support of this interpretation, a significant increase in the surface density of peroxisomes has been described in humans with liver steatosis (46). Since peroxisomes produce also medium chain acyl-CoAs during β-oxidation, the observed increase in the cytosolic content of short-chain acyl-CoAs and of the short-chain acylcarnitine to carnitine ratio may result from increased peroxisomal metabolism of long-chain fatty acids. Medium-chain acylcarnitines may serve as additional substrates for mitochondrial β-oxidation in THP-treated rats since they can enter the mitochondria without the action of CPT I.

TABLE 6. Plasma lipid and lipoprotein analysis

	CON (n = 6)	THP (n = 6)
Lipids and glycerol		
Triglycerides	0.47 ± 0.12	0.58 ± 0.12
Phospholipids	1.17 ± 0.17	1.51 ± 0.35[a]
Cholesterol	0.12 ± 0.03	0.13 ± 0.02
Free fatty acids	0.72 ± 0.10	0.71 ± 0.20
Glycerol	0.023 ± 0.004	0.023 ± 0.005
VLDL		
Triglycerides	0.49 ± 0.15	0.72 ± 0.15[a]
Phospholipids	0.10 ± 0.03	0.16 ± 0.03[a]
Cholesterol	0.11 ± 0.02	0.08 ± 0.03
LDL		
Phospholipids	0.23 ± 0.03	0.31 ± 0.14
Cholesterol	0.25 ± 0.05	0.28 ± 0.12
HDL		
Triglycerides	0.15 ± 0.01	0.15 ± 0.02
Phospholipids	0.90 ± 0.09	1.09 ± 0.31
Cholesterol	0.52 ± 0.07	0.55 ± 0.18

Control and THP treated rats (20 mg/100 g body weight) were studied after weeks of treatment in the fasted state. Plasma lipoproteins were isolated using a combined centrifugation/precipitation technique and lipids were analyzed enzymatically as described in Materials and Methods. Data are presented as mean ± SD. Units are mmol/l for free fatty acids and g × l^{-1} for all other lipids or metabolites.

[a] $P < 0.05$ THP versus CON.

In THP-treated rats, carnitine deficiency was associated with a significant increase in the cytosolic CoA content of the hepatocytes, whereas the mitochondrial content decreased. CoA is synthesized from pantothenate, cysteine, and ATP, with the rate-limiting step being phosphorylation of pantothenate located in the cytosol (47). Mitochondria obtain CoA either by endogenous biosynthesis [the final steps in CoA synthesis are also found in mitochondria (48)] or by transport across the inner mitochondrial membrane by a specific transport system (49). In comparison, degradation of CoA appears to be a cytosolic process (50). Interestingly, rats treated with clofibrate, a ligand for PPARα leading to peroxisomal proliferation (51), also reveal an increase in the hepatic CoA pool (52–54), suggesting that an increase in the hepatic CoA pool and peroxisomal proliferation may somehow be connected. The rise in the hepatic CoA pool by clofibrate has clearly been shown to result from increased biosynthesis (54), and the distribution of CoA between the mitochondria and the cytosol was not affected in clofibrate-treated rats (53). In contrast, in THP treated rats, the total hepatic CoA pool is increased due to a rise in the cytosolic content of CoA, whereas the mitochondrial content is decreased. Since the changes in the hepatic CoA pool could already be observed at 3 weeks of treatment with THP, a

time point when β-oxidation was not impaired, these alterations in the CoA pool did probably not affect significantly hepatic fatty acid metabolism in THP-treated rats.

In THP-treated rats, the phospholipid and triglyceride contents were increased in the VLDL fraction in plasma and also per gram of liver, whereas the hepatic apoB content was unchanged. These findings are compatible with increased loading of VLDL with triglycerides and phospholipids in the liver, which is most probably a consequence of increased cytosolic availability of fatty acids and acyl-CoAs due to impaired mitochondrial fatty acid metabolism. Hepatic accumulation of triglycerides has been associated with the development of macrovesicular steatosis of the liver (9, 11). Since isolated inhibition of mitochondrial fatty acid metabolism is considered to result in microvesicular steatosis (11), secondary accumulation of cytosolic triglycerides and phospholipids in the presence of initial mitochondrial damage may explain the development of a mixed type of liver steatosis over time.

Despite reduced in vivo metabolism of palmitate and decreased activity of CPT I, as well as mitochondrial β-oxidation in THP-treated rats, the plasma and liver β-hydroxybutyrate concentrations were not different from control rats. Hepatic long-chain fatty acid metabolism is controlled at two sites, namely at CPT I and mitochondrial HMG-CoA synthase (55, 56), both of them regulated enzymes. Since ketogenesis was assessed specifically and was not different between mitochondria from THP-treated and control rats, the activity of the HMG-CoA synthase can be assumed to be identical in THP-treated and control rats. The observed decrease of palmitate metabolism in THP rats in vivo, which is explained primarily by hepatic carnitine deficiency and, at 6 weeks, also reduced activity of CPTI/mitochondrial β-oxidation, could therefore be expected to result in decreased hepatic production of ketone bodies, e.g., β-hydroxybutyrate. Indeed, in agreement with reduced CPT I activity/mitochondrial β-oxidation, liver mitochondria from THP-treated rats contained less acetyl-CoA than control mitochondria. On the other hand, the plasma and liver β-hydroxybutyrate concentrations were not different between THP-treated and control rats. Among the possibilities to explain this discrepancy are decreased consumption of ketone bodies by peripheral tissues such as skeletal muscle, or increased production of ketone bodies by extrahepatic tissues such as kidneys in THP-treated rats. These two possibilities are unlikely, however, since not only plasma, but also the hepatic β-hydroxybutyrate concentration was unaffected by treatment with THP. The most likely possibility is therefore that during starvation, the HMG-CoA cycle becomes rate-limiting for ketogenesis. During starvation, the activity of CPT I increased due to a decrease in the hepatic concentration of malonyl-CoA, an endogenous inhibitor of CPT I (55). This increase in CPT I activity during starvation may be sufficient so that ketogenesis is controlled solely by the HMG-CoA cycle, as suggested by our findings. In support of this interpretation, starved rats with acute cholestasis have reduced production of ketone bodies and a reduced activity of HMG-CoA synthase, the rate-limiting enzyme of the HMG-CoA cycle, while the function of CPT I is normal (25). On the other hand, ketogenesis is reduced in JVS mice, an animal model with severe systemic carnitine deficiency (57). The hepatic carnitine content in JVS mice is less than 5% of normal, limiting the activity of CPT I to a higher extent than in THP-treated rats. In JVS mice, ketogenesis was maximal at carnitine concentrations above 100 nmol/g liver wet weight (57), a value which is just reached in THP-treated rats.

We conclude that a reduction in the hepatic carnitine pool is the principle mechanism leading to impaired hepatic fatty acid metabolism and liver steatosis after 3 weeks of treatment with THP. After 6 weeks of treatment, impaired activity of CPT I and mitochondrial β-oxidation may contribute. Cytosolic accumulation of fatty acids and long-chain acyl-CoAs is associated with increased plasma VLDL triglyceride and phospholipid concentrations and peroxisomal proliferation.

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