Tetradecylthioacetic acid incorporated into very low density lipoprotein: changes in the fatty acid composition and reduced plasma lipids in cholesterol-fed hamsters

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Abstract The mechanism behind the hypolipidemic effect of tetradecylthioacetic acid (CMTTD, a non-β-oxidizable 3-thia fatty acid) was studied in hamsters fed a high cholesterol diet (2%), which resulted in hyperlipidemia. Treating hyperlipidemic hamsters with CMTTD resulted in a progressive hypocholesterolemic and hypotriacylglycerolemic effect. Decreased plasma cholesterol was followed by a 39% and 30% reduction in VLDL-cholesterol and LDL-cholesterol, respectively. In contrast, the HDL-cholesterol content was not affected, thus decreasing the VLDL-cholesterol/HDL-cholesterol and LDL-cholesterol/HDL-cholesterol ratios. 3-Hydroxy-3-methylglutaryl- (HMG) CoA reductase activity and its mRNA level were unchanged after CMTTD administration. Also, the LDL receptor and LDL receptor-related protein (LRP-4) mRNAs were unchanged. The decrease in plasma triacylglycerol was accompanied by a 45% and 56% reduction in VLDL-triacylglycerol and LDL-triacylglycerol, respectively. The hypolipidemic effect of CMTTD was followed by a 1.4-fold increase in mitochondrial fatty acid oxidation and a 2.3-fold increase in peroxisomal fatty acid oxidation. CMTTD treatment led to an accumulation of dihomo-γ-linolenic acid (20:3n-6) in liver, plasma, very low density lipoprotein, and heart. Noteworthy, CMTTD accumulated more in the heart, plasma, and VLDL particles compared to the liver, and in the VLDL particle α-linolenic acid (18:3n-3) decreased whereas eicosatetraenoic acid (20:4n-3) increased. In addition, linoleic acid (18:2n-6) and the total amount of polyunsaturated fatty acids decreased, the latter mainly due to a decrease in n-6 fatty acids. The present data show that CMTTD was detected in plasma and incorporated into VLDL, liver, and heart. The relative incorporation (mol %) of CMTTD was heart > VLDL > liver. In conclusion, CMTTD causes both a hypocholesterolemic and hypotriacylglycerolemic effect in hyperlipidemic hamsters.—**Frøyland, L., D. K. Asiedu, H. Vaagenes, A. Garras, Ø. Lie, G. K. Totland, and R. K. Berge.** Tetradecylthioacetic acid incorporated into very low density lipoprotein: changes in the fatty acid composition and reduced plasma lipids in cholesterol-fed hamsters. *J. Lipid Res.* 1995. **36:** 2529-2540.

Supplementary key words cholesterol • 3-thia fatty acid • fatty acid composition • hepatic fatty acid oxidation • plasma lipoproteins • peroxisome proliferator

Ample evidence exists to suggest that diet influences plasma lipoprotein lipid concentrations and may thus mediate an important role in the development of atherosclerotic vascular disease. Several studies have shown that high intake of dietary fat and cholesterol can cause hypercholesterolemia in several animal species including nonhuman species (1–3). Syrian hamsters are commonly used to model lipid metabolic studies because of similarities with human cholesterol and bile acid metabolism (4, 5). In general, saturated fats increase plasma cholesterol (6, 7) while polyunsaturated fats have the opposite effect (8). Historically, monounsaturated fatty acids have been regarded as having no influence on plasma cholesterol (9). In recent years, however, this has been challenged and it is suggested that monounsaturated fatty acids are potentially as effective as a diet rich in polyunsaturated fat in lowering plasma cholesterol (10, 11). The hamster carries a significant proportion of its serum cholesterol in the low density lipoprotein (LDL) fraction (12) and it has also

Abbreviations: CMTTD, tetradecylthioacetic acid (a non-b-oxidizable 3-thia fatty acid); VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL ,high density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26); FAO, fatty acyl-CoA oxidase (EC 1.3.3.6); HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.88); LRP- 4, LDL receptor-related protein.

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TABLE 1. Composition of diets

Content	Standard Diet	Cholesterol Diet
	g/100 g	
Cholesterol[a]	–	2.0
Fibre	2.0	2.0
Mineral mix[b]	3.0	3.0
Soya concentrate[c]	20.0	20.0
Soya oil	5.0	5.0
Starch	58.0	56.0
Sucrose	11.0	11.0
Vitamin mix[d]	1.0	1.0

[a]Sigma Chemical Company ash-free precipitated from alcohol 95–98%.

[b]Grams/1000 g: $CaCO_3$, 282.87; $CaHPO_4 \cdot 2H_2O$, 208.64; KH_2PO_4, 409.57; $MgCO_3$, 0.0065; $FeSO_4 \cdot 7H_2O$, 5.69; $MnSO_4 \cdot H_2O$, 5.01; Na_2SeO_3, 0.0066; $ZnSO_4 \cdot 7H_2O$, 1.73.

[c]Contains 62% protein.

[d]Milligrams/100 g: vitamin A, 80; vitamin D, 20; vitamin E (50%), 600; vitamin K (50%), 1; choline, 10,000; folic acid, 10; niacin, 200; pantothenic acid, 80; riboflavin, 30; thiamin, 40; pyridoxin, 60; vitamin B_{12} (1%), 50; dextrin, 88,829.

been shown that serum LDL-cholesterol increases in response to dietary cholesterol and saturated fat (13).

Tetradecylthioacetic acid (CMTTD), a saturated non-β-oxidizable fatty acid analogue, has been shown to act as an hypolipidemic agent in normolipidemic (14) and nephrotic hyperlipidemic rats (15). In contrast to rats, the capacity for liver cholesterol synthesis in humans and male hamsters is limited and may be further limited by exogenous cholesterol, thus allowing for liver homeostasis to be mediated by cholesterol influx/efflux rather than de novo cholesterol synthesis.

Based on our knowledge of the hypolipidemic action of CMTTD in the rat, the aim of the present study was to evaluate the hypolipidemic potential of CMTTD in an animal model comparable to human lipoproteins. With the above mentioned considerations in mind, we chose male golden Syrian hamsters for this study. We wanted to investigate the cholesterol content of lipoproteins, fatty acid composition, and the expression of 3-hydroxy-3-methylglutaryl-CoA reductase, LDL receptor, LDL receptor-related protein (LRP-4), and fatty acyl-CoA oxidase in liver of cholesterol-fed hamsters as well as in cholesterol-fed hamsters treated with CMTTD. In addition, it was of great interest to investigate whether CMTTD was incorporated into lipoproteins.

MATERIALS AND METHODS

Chemicals

Tetradecylthioacetic acid (CMTTD, a 3-thia fatty acid) was synthesized as previously described (17). Isotopes used in this experiment were purchased from New England Nuclear (Boston, MA). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and diets

Pathogen-free male golden Syrian hamsters (90–100 g), obtained from Charles River Wiga GmbH, (Sulzfeld, Germany) were housed in individual cages and maintained on a 12-h cycle of light and dark, (the dark period ranging from 6:00 AM to 6:00 PM, rendering the animals easy to handle) at a temperature of 20 ± 3°C. The hamsters were acclimatized for 2 weeks before the start of the experiment and had free access to water and R34-EWOS-ALAB Grower rat maintenance feed (Ewos, Sweden) or standard maintenance chow coated with 2% cholesterol (Special Diets Services, UK). The composition of the diets is shown in **Table 1.** At the beginning of the experiment, the hamsters were randomly assigned to either a high-cholesterol group or an untreated group. At days 10, 20, 30, 35, and 40, one to three animals from each group were killed to monitor the plasma cholesterol concentration. At day 35 half of the high-cholesterol group received CMTTD and at the end of the experiment each group consisted of seven animals. Body weight was determined weekly.

TABLE 2. Effect of tetradecylthioacetic acid (CMTTD) on body and liver weights liver protein content and liver lipid levels in hypercholesterolemic hamsters

Type of Treatment	Body Weight		Liver Weight	Liver/Body Weight	Liver Protein	Liver Lipid Levels	
	Initial	Final				Triacylglycerol	Cholesterol
	g		*g*	*g/g × 100*	*mg/g liver*	*μmol/g liver*	
Untreated hamsters	92.4 ± 3.9[a]	114.1 ± 8.3[a]	3.7 ± 0.2[a]	3.2 ± 0.2[a]	148.5 ± 3.8[a]	14.5 ± 0.9[a]	6.4 ± 0.9[a]
Cholesterol-fed hamsters	94.4 ± 4.3[a]	91.6 ± 7.9[b]	6.7 ± 0.5[b]	7.3 ± 0.4[b]	110.6 ± 8.3[b]	10.2 ± 1.1[b]	13.2 ± 1.1[b]
Cholesterol-fed + CMTTD-treated hamsters	91.2 ± 3.0[a]	96.6 ± 5.1[b]	7.4 ± 0.4[b]	7.5 ± 0.6[b]	116.9 ± 4.8[b]	11.3 ± 1.6[b]	9.9 ± 1.7[c]

All values are means ± SD for seven hamsters. Means in a column with a different superscript are significantly different ($P < 0.05$).

TABLE 3. Morphometric analysis of hepatocytes in untreated, cholestrol-fed, and cholestrol-fed + CMTTD-treated hamsters

	Cells	Nuclei	Cytoplasm	Mitochondria		Fat Droplets			Peroxisomes	
Group	Area	Area	Area	Area	Volume Fraction	Area	Volume Fraction	No./ Cell	Area	Volume Fraction
	μm^2	μm^2	μm^2	μm^2	%	μm^2	%		μm^2	%
Untreated	455[a]	34[a]	421[a]	0.38a	8.1[a]	0.75[a]	5.6[a]	817[a]	0.21[a]	2.2[a]
Cholesterol-fed	581[b]	39[a]	542[b]	0.36[a]	5.3[b]	1.83[b]	26.1[b]	1463[b]	0.34[b]	1.9[a]
Cholesterol-fed + CMTTD-treated	635[b]	42[a]	593[b]	0.60[b]	11.3[c]	1.95[b]	19.4[c]	1111[c]	0.23[a]	1.9[a]

The analysis was performed on 100 hepatocytes from each animal (n = 3) per group. Values (means) within a column with a different superscript are significantly different ($P < 0.05$).

Three percent (w/v) CMTTD was prepared by suspending the fatty acid analogue in 0.5% CM-cellulose. A dose of 150 mg/kg body weight of the hypolipidemic 3-thia fatty acid was administered by oro-gastric intubation in a volume of 0.5 mL to the animals in the treatment group, each morning between 8:00 and 8:30 for 15 days. The untreated animals received only the vehicle (CM-cellulose).

At the end of the experimental period the overnight fasted hamsters were anesthetized with Hypnorm Dormicum® (fluanisone-fenatanylmidazolam, 0.8 mL • 100 g^{-1} body weight) subcutaneously; cardiac puncture was performed and blood was collected in EDTA vacutainers. The liver was removed and weighed and some portions were chilled on ice whereas other portions were freeze-clamped in liquid N_2 and stored at -80°C until analyzed. The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Preparation of total liver homogenates, subcellular fractions, and enzyme activities

Total homogenates of the liver were prepared by homogenizing weighed portions of the individual organs in ice-cold sucrose solution containing 0.25 mol/L sucrose in 10 mmol/L HEPES buffer and 1 mmol/L EDTA, pH 7.4. The resulting nuclear and post-nuclear fractions were used as the total homogenate. A mitochondrial-enriched fraction (M-fraction) was prepared from the post-nuclear fraction (E-fraction) at 12000 *g* for 10 min (SS 34 rotor). A peroxisome-enriched fraction (L-fraction) was prepared from the post-mitochondrial fraction by centrifugation at 14000 *g* for 30 min. A microsomal-enriched fraction (P-fraction) was isolated from the post-peroxisomal fraction at 100,000 *g* for 1 h (Ti 60 rotor). The remaining supernatant was collected as a cytosolic fraction (S-fraction). All procedures were performed at 0–4°C and fractions were washed twice. Aliquots of the whole homogenate and subcellular fractions were stored at -80°C until analyzed.

Mitochondrial β-oxidation was determined in the M-fraction as acid-soluble products using radiolabeled [1-^{14}C]palmitoyl-CoA or [1-^{14}C]palmitoyl-L-carnitine as substrates. The assay medium (0.25 mL) contained 13.2 mmol/L HEPES (pH 7.3), 16.5 mmol/L $MgCl_2$, 82.5 mmol/L KCl, 13.2 mmol/L dithiothreitol, 6.6 mmol/L ADP, 0.2 mmol/L NAD^+, 0.7 mmol/L EDTA, and 0.5–1.0 mg protein. Palmitoyl-L-carnitine oxidation was measured with 56 µmol/L [1-^{14}C]palmitoyl-L-carnitine and palmitoyl-CoA oxidation was measured with 115 µmol/L [1-^{14}C]palmitoyl-CoA supplemented with 1.2 mmol/L L-carnitine. All samples were preincubated for 2 min at 30°C. After incubation at 30°C for 4 min (palmitoyl-CoA) and 2 min (palmitoyl-L-carnitine), the rate of oxidation was stopped by addition of 150 µL 1.5

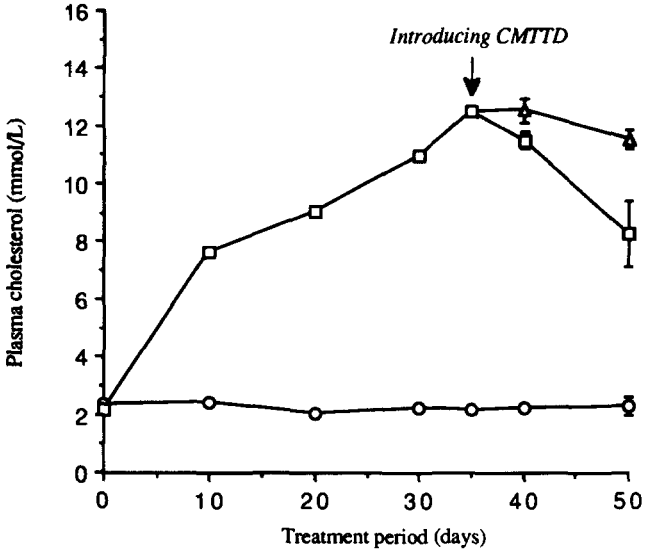

Fig. 1. Time course of the hypocholesterolemic effect of tetradecylthioacetic acid (CMTTD). Male hamsters were kept on a high-cholesterol diet (2%) for 34 days. From day 35, half of the animals received CMTTD (150 mg/kg body weight/day) (□) in addition to the high-cholesterol diet, whereas the others only received a high-cholesterol diet (△). In addition, one group was given a standard maintenance diet (○). Values up to day 40 denote means ± SD for 1–3 animals, and at day 50 means ± SD (n = 7).

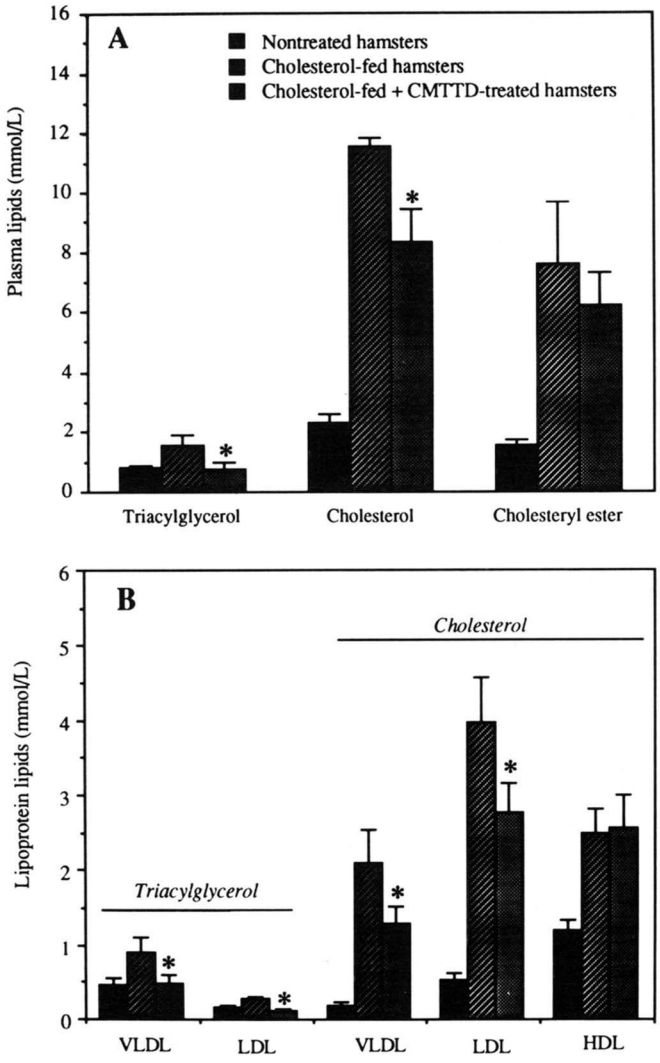

Fig. 2. Effect of tetradecylthioacetic acid (CMTTD) on plasma (A) and lipoprotein (B) lipids in hypercholesterolemic (cholesterol-fed) hamsters. *Significantly different from cholesterol-fed animals ($P<0.05$).

mol/L KOH. Twenty-five µL fatty acid-free BSA (100 mg/mL) was then added to the suspension in order to bind non-oxidized substrates. Five hundred µL of 4 mol/L $HClO_4$ was added to precipitate non-oxidized substrates bound to BSA. The total solution was then centrifuged at 2010 *g* for 10 min. Aliquots of 500 µL were assayed for radioactivity. Fatty acyl-CoA oxidase (21) was determined in the L-fraction as described elsewhere. 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (22) and acyl-CoA:cholesterol acyltransferase (23) were determined in the P-fraction as described elsewhere. All enzyme assays were run in duplicate and performed under conditions where product formation was linear with respect to both the time of incubation and the amount of protein. Protein was determined using the Bio-Rad protein kit (Bio-Rad, Richmond, CA.).

Lipid analysis

Plasma was prepared by centrifugation of whole blood at 1000 *g* for 10 min. Triacylglycerol and cholesterol were determined according to Technicon Method no. SA4-0324L90, USA and Technicon Method no. SA4-0305L90, USA, respectively.

Lipoprotein isolation

Lipoprotein quantitation was performed by a combination of ultracentrifugation and precipitation as described in detail elsewhere (24, 25). Briefly, plasma samples were centrifuged at a density of 1.006 g/mL for 18 h at 40,000 rpm in a Centrikon T-2060 ultracentrifuge (Contron Roche, Zurich, Switzerland) equipped with a 45.6 Ti rotor. The tubes were sliced, and the floating fractions, very low density lipoprotein (VLDL), were analyzed for cholesterol and triacylglycerol. The infranatant fractions obtained after the initial ultracentrifugation procedure were subjected to sequential ultracentrifugation for 20 h at 40,000 rpm and 40 h at 40,000 rpm to obtain low density lipoprotein (LDL) ($1.025 < d < 1.063$ g/mL) and high density lipoprotein (HDL) ($1.063 < d < 1.21$ g/mL) fractions, respectively. The resulting fractions were analyzed for cholesterol and triacylglycerol.

Determination of fatty acid composition

Total lipids were extracted from plasma and liver as described by Lie, Lied, and Lambertsen (26). The lipid fractions were evaporated, saponified, and nondecanoic acid (19:0) was added as internal standard and the fatty acids were esterified in 12% BF_3 in methanol. The methyl esters were separated using a Carlo Erba 2900 gas-chromatograph ("cold on column" injection, $60^{49°C/min}$ $160^{1°C/min}$ $190^{4°C/min}$ 220°C), equipped with a 50 m CP-sil 88 (Chrompack) fused silica capillary column (id: 0.32 mm). The relative fatty acid distribution (% w/w of total fatty acid) was calculated from the peak areas obtained using a Maxima 820 Chromatography Workstation software, installed in an IBM-AT, connected to the gas chromatograph and identification was ascertained by standard mixtures of methyl esters.

RNA isolation and hybridization analysis

Total cellular RNA was purified using the acid guanidinium thiocyanate–phenol method (27). The RNA concentrations were determined by measuring the UV absorbance at 260 nm. Slot blotting of RNA on to nylon membranes was carried out as described by

TABLE 4. Effect of tetradecylthioacetic acid (CMTTD) on liver, plasma, and very low density lipoprotein (VLDL) fatty acid composition (mol % of total lipid) in hypercholesterolemic hamsters

Fatty Acid	Liver			Plasma			VLDL		
	Untreated Hamsters	Cholesterol-Fed Hamsters	Cholesterol-Fed + CMTTD -Treated Hamsters	Untreated Hamsters	Cholesterol-Fed Hamsters	Cholesterol-Fed + CMTTD -Treated Hamsters	Untreated Hamsters	Cholesterol-Fed Hamsters	Cholesterol-Fed + CMTTD -Treated Hamsters
14:0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.0	0.7 ± 0.2	0.6 ± 0.1	1.0 ± 0.6
16:0	18.4 ± 0.9	13.1 ± 1.3[a]	12.6 ± 0.5[a]	18.0 ± 1.5	15.5 ± 1.5	15.6 ± 1.0	19.9 ± 1.0	13.5 ± 0.5[a]	14.8 ± 1.4[a]
16:1n-7	1.2 ± 0.2	2.6 ± 0.3[a]	2.4 ± 0.4[a]	1.3 ± 0.3	1.8 ± 0.3	1.4 ± 0.3	1.6 ± 0.1	2.0 ± 0.2	1.7 ± 0.3
16:1n-9	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	1.0 ± 0.2	0.7 ± 0.1	0.9 ± 0.1
CMTTD	ND	ND	0.3 ± 0.1[a,b]	ND	ND	1.2 ± 0.4[a,b]	ND	ND	1.7 ± 0.7[a,b]
18:0	15.8 ± 1.2	7.1 ± 1.3[a]	8.8 ± 1.7[a]	6.6 ± 1.3	5.4 ± 0.8	6.8 ± 0.7	4.9 ± 0.3	4.4 ± 0.5	5.4 ± 0.2
18:1n-7	1.4 ± 0.1	2.4 ± 0.3[a]	2.2 ± 0.2[a]	1.4 ± 0.1	2.4 ± 0.5[a]	1.7 ± 0.1	2.0 ± 0.1	2.4 ± 0.1	2.1 ± 0.2
18:1n-9	15.6 ± 3.1	37.0 ± 4.9[a]	38.1 ± 4.2[a]	18.1 ± 1.7	25.2 ± 2.9[a]	26.0 ± 1.6[a]	30.1 ± 1.6	30.6 ± 1.9	33.9 ± 4.1
18:2n-6	20.5 ± 0.3	23.2 ± 1.7	20.3 ± 1.1	37.9 ± 2.0	36.4 ± 1.6	32.7 ± 2.2[a]	26.0 ± 0.7	27.9 ± 1.6	21.9 ± 0.4[a,b]
18:3n-3	0.4 ± 0.1	1.4 ± 0.2[a]	1.0 ± 0.1[a]	0.5 ± 0.1	1.2 ± 0.3[a]	0.7 ± 0.1	1.4 ± 0.1	2.0 ± 0.3[a]	1.3 ± 0.2[b]
20:3n-6	0.8 ± 0.0	0.6 ± 0.2	1.5 ± 0.4[a,b]	0.3 ± 0.1	0.4 ± 0.0	1.4 ± 0.2[a,b]	0.1 ± 0.1	0.3 ± 0.1	0.6 ± 0.1[a,b]
20:4n-3	0.1 ± 0.1	Tr.	Tr.	0.1 ± 0.0	Tr.	Tr.	Tr.	Tr.	0.9 ± 0.2[a,b]
20:4n-6	7.9 ± 0.9	3.5 ± 1.2[a]	4.7 ± 1.2[a]	3.9 ± 0.9	2.7 ± 0.7	3.9 ± 0.6	0.7 ± 0.1	1.1 ± 0.2	1.4 ± 0.1
20:5n-3	1.2 ± 0.3	0.2 ± 0.1[a]	0.3 ± 0.1[a]	1.0 ± 0.2	0.3 ± 0.0[a]	0.4 ± 0.1[a]	0.7 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
NN	ND	ND	ND	ND	ND	0.6 ± 0.2[a,b]	ND	ND	ND
22:1n-9	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	ND	ND	ND
22:5n-3	1.6 ± 0.4	0.4 ± 0.1[a]	0.4 ± 0.2[a]	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
22:6n-3	12.3 ± 0.9	4.1 ± 1.1[a]	3.4 ± 0.8[a]	4.0 ± 0.8	2.3 ± 0.7	1.8 ± 0.2[a]	1.9 ± 0.2	1.4 ± 0.2	1.0 ± 0.1[a]
Σ Saturated	35.1 ± 1.8	21.2 ± 2.3[a]	22.2 ± 1.9[a]	26.0 ± 2.4	22.0 ± 2.3	23.4 ± 1.3	27.5 ± 1.1	19.6 ± 0.9[a]	22.3 ± 1.9[a]
Σ Monounsat.	18.8 ± 3.5	44.2 ± 4.7[a]	43.9 ± 4.8[a]	21.8 ± 2.0	30.4 ± 3.7[a]	29.9 ± 1.7[a]	36.7 ± 0.9	36.9 ± 2.1	39.4 ± 4.5
Σ Polyunsat.	45.2 ± 2.2	33.8 ± 3.8[a]	32.2 ± 3.1[a]	48.8 ± 2.1	44.5 ± 2.1	42.0 ± 2.1[a]	32.4 ± 0.6	35.0 ± 2.2	28.5 ± 0.7[a,b]
Σ n-3	15.6 ± 1.5	6.0 ± 1.1[a]	5.3 ± 0.9[a]	6.1 ± 1.1	4.1 ± 0.5[a]	3.3 ± 0.2[a]	4.9 ± 0.5	4.2 ± 0.3	4.0 ± 0.2
Σ n-6	29.6 ± 0.8	27.8 ± 2.8	27.0 ± 2.3	42.3 ± 1.9	39.9 ± 1.9	38.3 ± 2.1	27.0 ± 0.8	29.8 ± 1.7	24.1 ± 0.5[a,b]
n-3/n-6	0.53 ± 0.04	0.21 ± 0.02[a]	0.19 ± 0.02[a]	0.14 ± 0.03	0.09 ± 0.01[a]	0.10 ± 0.01	0.20 ± 0.05	0.10 ± 0.05	0.20 ± 0.05

Liver and plasma values are expressed as means ± SD (n = 7). Very low density lipoprotein (VLDL) values represent means ± SD for four preparations of pooled plasma. Tr., trace (< 0.01%); ND, not detected; NN, unknown.

[a]Significantly different from untreated hamsters ($P < 0.05$).

[b]Significantly different from cholesterol-fed hamsters ($P < 0.05$).

TABLE 5. Effect of tetradecylthioacetic acid (CMTTD) on heart and brain fatty acid composition (% of total lipid) in hypercholesterolemic hamsters

Fatty Acid	Heart			Brain		
	Untreated Hamsters	Cholesterol-Fed Hamsters	Cholesterol-Fed + CMTTD-Treated Hamsters	Untreated Hamsters	Cholesterol-Fed Hamsters	Cholesterol-Fed + CMTTD-Treated Hamsters
14:0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	Tr.	Tr.	Tr.
16:0	15.0 ± 0.5	13.8 ± 0.8	11.8 ± 1.1[a]	20.2 ± 0.2	20.2 ± 0.5	20.9 ± 0.3
16:1n-7	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
16:1n-9	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
CMTTD	ND	ND	2.0 ± 0.6[a,b]	ND	ND	ND
18:0	14.2 ± 0.6	13.1 ± 1.0	14.2 ± 0.3	13.7 ± 0.5	13.9 ± 0.5	14.5 ± 0.3
18:1n-7	1.3 ± 0.1	1.4 ± 0.1	0.9 ± 0.1[a,b]	5.3 ± 0.1	5.3 ± 0.1	5.4 ± 0.1
18:1n-9	12.8 ± 1.5	13.9 ± 2.2	11.1 ± 0.7	15.3 ± 0.5	15.4 ± 0.7	15.5 ± 0.4
18:2n-6	28.4 ± 1.5	31.2 ± 2.7	29.1 ± 1.5	0.9 ± 0.1	1.5 ± 0.2[a]	1.5 ± 0.4
18:3n-3	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	ND	ND	ND
20:3n-6	0.3 ± 0.1	0.4 ± 0.1	0.9 ± 0.1[a,b]	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
20:4n-3	Tr.	Tr.	0.1 ± 0.1	Tr.	Tr.	Tr.
20:4n-6	6.6 ± 0.7	6.9 ± 0.7	7.6 ± 0.6	10.1 ± 0.5	10.4 ± 0.5	11.4 ± 0.4[a]
20:5n-3	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	Tr.	Tr.	Tr.
NN	ND	ND	ND	ND	ND	ND
22:1n-9	2.4 ± 0.5	2.4 ± 0.7	2.3 ± 0.6	1.3 ± 1.0	2.1 ± 0.4	0.3 ± 0.1[b]
22:5n-3	1.1 ± 0.1	0.9 ± 0.2	1.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
22:6n-3	11.5 ± 1.7	9.3 ± 1.1	12.4 ± 0.7[b]	13.4 ± 0.6	13.8 ± 0.6	14.1 ± 0.4
Σ Saturated	30.1 ± 0.5	27.8 ± 1.4	26.8 ± 1.0[a]	34.8 ± 0.5	34.8 ± 0.8	36.2 ± 0.6
Σ Monounsaturated	17.5 ± 1.6	19.0 ± 2.2	15.1 ± 0.9[b]	25.6 ± 1.6	26.3 ± 1.0	24.7 ± 0.8
Σ Polyunsaturated	48.8 ± 1.6	49.7 ± 2.0	52.5 ± 1.4[a]	25.0 ± 0.9	26.3 ± 0.9	27.6 ± 0.7
Σ n-3	13.4 ± 1.8	11.0 ± 1.1	14.6 ± 0.7[b]	13.7 ± 0.6	14.1 ± 0.6	14.3 ± 0.4
Σ n-6	35.4 ± 2.0	38.7 ± 2.7	37.8 ± 1.4	11.4 ± 0.4	12.3 ± 0.3[a]	13.3 ± 0.7[a]
n-3/n-6	0.38 ± 0.06	0.29 ± 0.05	0.39 ± 0.03[b]	1.20 ± 0.05	1.15 ± 1.03	1.08 ± 0.06[a]

Heart and brain values are expressed as means ± SD (n = 7). ND, not detected; Tr, trace (< 0.01 %); NN, unknown.
[a]Significantly different from untreated hamsters ($P < 0.05$).
[b]Significantly different from cholesterol-fed hamsters ($P < 0.05$).

Aasland et al. (28). Hybridization to immobilized RNA was carried out according to Sambrook, Fritsch, and Maniatis (29) in the presence of 50% formamide, 5 × SSC, 200 μg/mL heat-denaturated herring sperm DNA, 0.1% SDS, 25 μmol/L sodium phosphate, pH 6.4, 8.25% dextran sulfate at 42°C for 24–48 h. Filters were stringently washed twice in 0.2 × SSC, 0.1% SDS, 0.1% $Na_4P_2O_7$ at 65°C (or at 60°C for DNA probes not obtained from rats). When the filters were to be rehybridized, the bound probe was first stripped off by incubation of the filters in 0.1% SDS at 95–100°C for 7 min.

Kodak XAR-5 X-ray films were exposed to the membranes at -80°C in the presence of intensifying screens. Autoradiograms were analyzed by densiometric scanning using an LKB Ultrogel laser densiometer (Bromma, Sweden). The relative level of mRNA expression was estimated as the amount of radioactive probe hybridized to each sample of RNA relative to the level of 28S rRNA in each sample.

Preparation of hybridization probes

The appropriate DNA fragments were excised from plasmids by restriction enzymes and the purified fragments were ^{32}P-labeled using the oligo-labeling technique (30). The resulting specific activities ranged from 1 to 5 × 10^9 cpm/μg. The probes were purified fragments of cloned genes: rat LDL-receptor; 2.2 bp *Eco* RI inserted into pGEM7 (31), HMG-CoA reductase cDNA from baby hamster kidney cells; full length *Eco* RI fragment of pDGS2, rat LRP-4; *Bam* HI fragment, 3.6 kb cDNA inserted into pGEM4 (32), rat fatty acyl-CoA oxidase (FAO); *PST1* fragment, 1.4 kb inserted into pMJ125 and 28S rRNA; 1.4 kb *Bam* HI fragment of pA (33).

TABLE 6. Effect of tetradecylthioacetic acid (CMTTD) on hepatic fatty acid oxidation and cholesterol metabolism in hypercholesterolemic hamsters

Enzyme Activities	Untreated Hamsters	Cholesterol-Fed Hamsters	Cholesterol-Fed + CMTTD-Treated Hamsters
Fatty acid oxidation			
Mitochondrial			
Palmitoyl-L-carnitine as substrate			
(nmol/min/mg protein)	0.41 ± 0.03[a]	0.29 ± 0.02[b]	0.41 ± 0.07[a]
(nmol/min)	80.07 ± 4.03[a]	58.55 ± 5.87[b]	117.34 ± 24.03[c]
Palmitoyl-CoA as substrate			
(nmol/min/mg protein)	0.24 ± 0.01[a]	0.18 ± 0.02[b]	0.24 ± 0.02[a]
(nmol/min)	48.70 ± 2.43[a]	37.68 ± 4.70[b]	67.80 ± 11.08[c]
Peroxisomal			
Fatty acyl-CoA oxidase			
(nmol/min/mg protein)	15.7 ± 1.5[a]	16.9 ± 3.4[a]	38.7 ± 10.4[b]
(μmol/min)	3.0 ± 0.4[a]	3.6 ± 0.8[a]	10.8 ± 2.1[b]
Cholesterol metabolism			
HMG-CoA reductase			
(pmol/min/mg protein)	20 ± 4[a]	12 ± 6[a]	16 ± 3[a]
(nmol/min)	2.2 ± 0.6[a]	1.0 ± 0.2[b]	2.6 ± 0.6[a]
ACAT			
(pmol/min/mg protein)	34 ± 5[a]	107 ± 31[b]	113 ± 22[b]
(nmol/min)	3.6 ± 0.7[a]	13.6 ± 2.5[b]	18.2 ± 3.5[c]

HMG-CoA reductase indicates 3-hydroxy-3-methylglutaryl-coenzyme A and ACAT, acyl-CoA:cholesterol acyltransferase. The values represent mean ± SD for seven hamsters in each group. Means in a row with a different superscript are significantly different ($P < 0.05$).

Morphological methods

Parts of the ventral lobe of the liver were dissected into small cubes (< 1 μm^3) and fixed by immersion in a 0.2% phosphate buffer (pH 7.35) containing 1% formaldehyde and 2.5% glutaraldehyde. They were then rinsed in buffer, postfixed in 1% osmium tetroxide, dehydrated in graded series of alcohol, and embedded in Epon 812. Semi-thin sections were stained in toluidine blue and studied in the light microscope, while ultra-thin sections, stained in uranyl acetate and lead citrate, were studied in a JEOL 100 CX transmission electron microscope. Morphometric analysis was carried out on random selected hepatocytes from all areas of the liver lobule using a Leitz ASM 64 K morphometrical analyzer.

Statistical analysis and presentation of results

The data are presented as mean ± standard deviation (SD) from 7 animals. Treatment means were compared by one-way analysis of variance, single factorial model with multiple comparison, Fisher's PLSD using a statistical software StatView SE + Graphics™on an Apple Macintosh.

RESULTS

Cholesterol feeding significantly decreased the weight gain and liver protein and triacylglycerol content compared to nontreated hamsters (**Table 2**). However, the liver weight, liver-to-body weight, and hepatic cholesterol level were significantly higher in cholesterol-fed hamsters compared to untreated hamsters. Administration of tetradecylthioacetic acid (CMTTD) to hamsters fed a high cholesterol diet (2%) significantly decreased hepatic cholesterol concentration compared to hamsters receiving cholesterol.

Morphometric analysis of hepatocytes revealed marked morphological alterations in the presence of a high cholesterol diet and treatment with CMTTD (**Table 3**). Both groups that received a high cholesterol diet had significantly increased cell size and cytoplasmic volume. Cholesterol feeding decreased the volume fraction of mitochondria as compared to controls, whereas CMTTD treatment increased the mitochondrial volume fraction. Cholesterol feeding increased the volume fraction of fat droplets by 4.7-fold as compared to controls. Treating hypocholesterolemic hamsters with CMTTD decreased the volume fraction of fat droplets by 25% as compared to cholesterol-fed animals. The peroxisomal

volume fraction was not significantly changed by either of the two feeding regimes.

The time course of cholesterol feeding and the hypocholesterolemic effect of CMTTD is shown in **Fig. 1.** The plasma cholesterol content in cholesterol-fed animals progressively increased up to day 35, reaching a steady-state level of approximately 12 mmol/L. At day 35 we introduced CMTTD (150 mg/kg body weight) to hypercholesterolemic hamsters (n = 7) and the plasma cholesterol concentration of CMTTD-treated cholesterol-fed animals decreased with time up to 15 days of administration (day 50).

Hamsters fed a high cholesterol diet had a significantly increased amount of plasma triacylglycerol compared to nontreated hamsters (**Fig. 2A**). In contrast, addition of CMTTD to hypercholesterolemic animals normalized the plasma triacylglycerol concentration, and this was accompanied by a 45% and 56% reduction in VLDL-triacylglycerol and LDL-triacylglycerol, respectively (Fig. 2B). The amount of cholesterol and cholesteryl ester in plasma of cholesterol-fed animals was increased compared to that in untreated animals. The plasma cholesterol content in CMTTD-treated cholesterol-fed animals was reduced, ascribed to a reduction in VLDL-cholesterol and LDL-cholesterol but not HDL-cholesterol (Fig. 2B).

Hamsters receiving cholesterol had an increased total amount of monoenes in liver and plasma (**Table 4**) and this increase was attributed to increased amounts of palmitoleic acid (16:1n–7), vaccenic acid (18:1n–7), and especially oleic acid (18:1n–9). No such changes were observed in the heart and brain (**Table 5**). The total amount of saturated fatty acids was decreased in liver and VLDL. This was mainly due to decreased amounts of palmitic acid (16:0) and especially stearic acid (18:0). Cholesterol feeding decreased the total amount of polyenes and n–3/n–6 ratio in liver and plasma due to reduced amounts of eicosapentaenoic acid (20:5n–3), docosapentaenoic acid (22:5n–3), and docosahexaenoic acid (22:6n–3). A similar tendency was observed in VLDL (Table 4). The amount of α-linolenic acid (18:3n–3) was significantly higher in liver, plasma, and VLDL, whereas arachidonic acid (20:4n–6) was decreased in liver of cholesterol-fed animals compared to untreated animals. Noteworthy, no changes in the fatty acid composition of the heart were observed in cholesterol-fed animals, whereas the amount of linolenic acid (18:2n–6) was higher in the brain compared to untreated animals, leading to an increased amount of n–6 fatty acids (Table 5).

Interestingly, CMTTD was found in liver, plasma, VLDL, and heart, but could not be detected in the brain. Moreover, a new, yet unidentified fatty acid was detected in plasma (Table 4). Administration of CMTTD to cholesterol-fed hamsters significantly increased the amount of dihomo-γ-linolenic acid (20:3n–6) in the liver and plasma. The amounts of 18:2n–6, 18:3n–3, and total amount of polyenes decreased, whereas the amount of 20:3n–6 and eicosatetraenoic acid (20:4n–3) increased in the VLDL-fraction after CMTTD treatment compared to cholesterol-fed animals. In addition, in the heart, CMTTD administration decreased 18:1n–7 and the total amount of monoenes, and increased the amount of 22:6n–3 and total amount of n–3 fatty acids, thereby increasing the ratio between n–3 and n–6 fatty acids compared to cholesterol-fed hamsters. In the brain a decrease in erucic acid (22:1n–9) was observed after CMTTD treatment compared to cholesterol-fed animals (Table 5).

The mitochondrial fatty acid oxidation was stimulated 1.4- and 1.3-fold with palmitoyl-L-carnitine and palmitoyl-CoA as substrates, respectively, after CMTTD treatment (**Table 6**). Also, the activity of fatty acyl-CoA oxidase (FAO), the rate-limiting enzyme in peroxisomal fatty acid oxidation, was markedly stimulated (2.3-fold). The FAO activity was unchanged after cholesterol feeding, whereas the acyl-CoA:cholesterol acyltransferase (ACAT) activity was increased after cholesterol feeding and further increased with CMTTD treatment (Table 6). In addition, the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase was found to respond to the cholesterol status of the liver, being down-

TABLE 7. Expression of HMG-CoA reductase, fatty acyl-CoA oxidase (FAO), LRP-4 and LDL-receptor mRNAs in liver of hypercholesterolemic hamsters treated with tetradecylthioacetic acid (CMTTD) for 2 weeks

Type of Treatment	HMG-CoA Reductase	FAO	LRP-4	LDL-Receptor	Δ^9 Desaturase
Untreated hamsters	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.1
Cholesterol-fed hamsters	1.1 ± 0.3	1.5 ± 0.6	1.4 ± 0.4	1.1 ± 0.2	1.7 ± 0.4[a]
Cholesterol-fed + CMTTD-treated hamsters	1.3 ± 0.4	1.9 ± 0.8	1.7 ± 0.7	1.0 ± 0.2	1.4 ± 0.3

The relative mRNA levels were determined by densiometric scanning of the autoradiograms (Materials and Methods). The expression of mRNA was normalized to the corresponding 28S rRNA levels (i.e., the ratio of mRNA:28S rRNA), mean of controls are set to 1.0. Values are presented as mean ± SD.

[a]Significantly different from controls ($P < 0.05$).

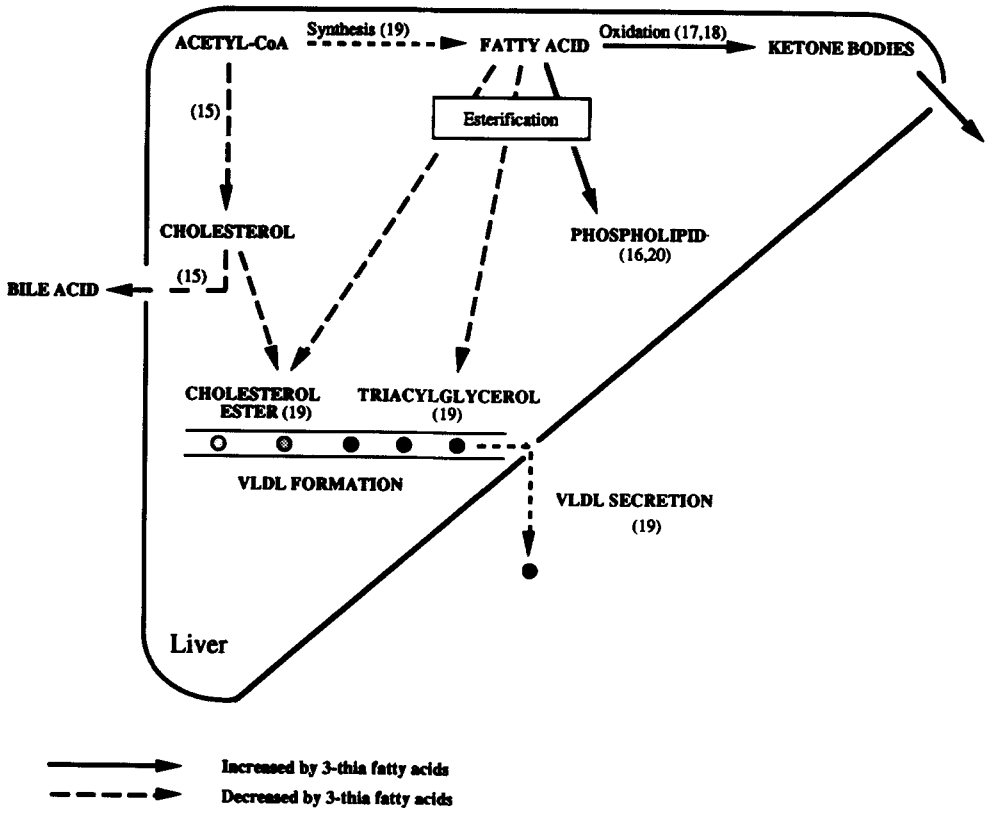

Fig. 3. Schematic presentation of the possible mechanisms of 3-thia fatty acids on hepatic lipid and lipoprotein metabolism. 3-Thia fatty acyl-CoA inhibits acetyl-CoA carboxylase, leading to decreased synthesis of malonyl-CoA and fatty acids. Decreased malonyl-CoA levels lead to an increased fatty acid oxidation. As the 3-thia fatty acids are non-β-oxidizable, they are incorporated into hepatic lipids, mainly phospholipids instead of endogenous fatty acyl-CoA, which are then β-oxidized. The 3-thia fatty acyl-CoA inhibits HMG-CoA reductase and cholesterol 7α-hydroxylase activities, leading to decreased production of cholesterol and bile acids, respectively. Decreased production of cholesteryl esters and triacylglycerol decreases the VLDL secretion; VLDL, very low density lipoprotein.

regulated by cholesterol treatment (Table 6). The latter enzyme activity was not affected by administration of CMTTD as compared to untreated hamsters (Table 6). The mRNA levels of HMG-CoA reductase, FAO, LDL receptor-related protein (LRP-4), and LDL receptor were not significantly changed by feeding exogenous cholesterol with or without addition of CMTTD. However, hamsters receiving a high cholesterol diet had a significantly increased amount of Δ^9 desaturase mRNA level as compared to untreated hamsters (**Table 7**).

DISCUSSION

In the present study liver weight was increased considerably by a high cholesterol diet (Table 2). Morphometric analysis revealed a considerable increase in cell size, cytoplasmic volume, and volume fraction of lipid droplets (Table 3). Treating hypocholesterolemic hamsters with tetradecylthioacetic acid (CMTTD) induced an increase in size and volume fraction of mitochondria but not of peroxisomes as compared to cholesterol-fed animals. Furthermore, CMTTD treatment significantly increased the hepatic capacity of mitochondrial fatty acid oxidation (Table 6) and decreased the volume fraction and number of lipid droplets as compared to cholesterol-fed hamsters (Table 3).

After oral administration for 2 weeks, tetradecylthioacetic acid (CMTTD) was absorbed and detectable in plasma, VLDL, liver, and heart. Moreover, the mol % of CMTTD was 6.7-fold higher in the heart than in the liver, indicating that VLDL particles may be the primary mode of delivery of CMTTD to the heart. How CMTTD is absorbed and carried needs to be further explored.

The detection of CMTTD in plasma is in agreement with previous findings (34, 35). An important finding was that CMTTD is incorporated into VLDL. As CMTTD is mainly incorporated into phospholipids (16, 20), this finding may be explained in the following way. 3-Thia fatty acids are activated to acyl-CoAs (14, 36) but

as they cannot be β-oxidized due to a sulfur atom in the β-position, they accumulate within the cell and are subsequently incorporated into phospholipids. The relative incorporation of CMTTD (mol %) was 5.7-fold higher in VLDL compared to the liver (Table 4), and it is possible that newly synthesized CMTTD-enriched phospholipids are incorporated into new VLDL particles. Thus, CMTTD carried in VLDL may be found mainly in the phospholipid layer as the flux of CMTTD acyl units into hepatic triacylglycerols and/or into cholesteryl esters appears less efficient (19).

Accumulation of CMTTD into phospholipids may affect the membrane fluidity and membrane signalling system. It has been shown in rats treated with peroxisome-proliferating drugs that the expression of fatty acyl-CoA oxidase (FAO), the rate-limiting enzyme in peroxisomal fatty acid oxidation, is regulated at the level of transcription (33). In the present study using cholesterol-fed hamsters, the peroxisome-proliferating drug CMTTD increased the enzyme activity without a concomitant increase in mRNA expression (Tables 6 and 7).

The current findings demonstrate changes in fatty acid composition in plasma, VLDL, and liver of hamster after treatment with cholesterol compared to untreated normolipidemic hamsters. Only marginal effects were observed in heart and brain after cholesterol feeding. Changes in the fatty acid composition caused by the cholesterol diet in liver, plasma, and VLDL included an increased content of α-linolenic acid (18:3n–3), whereas the desaturated and elongated products, eicosapentaenoic acid (20:3n–3), docosapentaenoic acid (22:5n–3), and docosahexaenoic acid (22:6n–3) were reduced (Table 4). This suggests that cholesterol treatment might decrease the hepatic desaturases (Δ^6, Δ^5) and elongase system (37), thereby contributing to a reduced amount of n–3 fatty acids. Nakamura et al. (38) suggested that a selective reduction of Δ^6 and Δ^5 desaturase activities was responsible for an altered liver phospholipid profile, including a decrease in n–3 fatty acids in micropigs chronically fed ethanol. In addition, similar effects were observed in hamsters treated with cholesterol and ethanol (39).

Administration of cholesterol decreased the hepatic content of palmitic acid (16:0) and stearic acid (18:0) and increased the amount of palmitoleic acid (16:1n–7), vaccenic acid (18:1n–7), and especially oleic acid (18:1n–9). These unsaturated products all need to go through a Δ^9 desaturation. Whether cholesterol upregulates the Δ^9 desaturase activity in the liver should be considered. As shown in Table 7, the expression of Δ^9 desaturase mRNA was increased by cholesterol. Interestingly, the changes in fatty acid composition observed in the cholesterol-fed hamster were similar to results obtained in normolipidemic rats treated with CMTTD for 3 months (D. K. Asiedu, L. Frøyland, H. Vaagenes, Ø. Lie, A. Demoz, and R. K. Berge, unpublished results).

Recently, in a study by Sánches et al. (40), it was shown that fibrates, peroxisome-proliferating hypolipidemic compounds, inhibited Δ^5 and Δ^6 desaturates as well as the elongation system in rats. However, the Δ^9 desaturase activity was not affected by these drugs. Importantly, prolonged administration of CMTTD to normolipidemic rats increased the mRNA expression of Δ^9 desaturase in liver (L. Madsen, L. Frøyland, H. J. Grav, and R. K. Berge, unpublished results). CMTTD acts as an hypolipidemic agent (14), and in this study the amount of dihomo-γ-linolenic acid (20:3n–6) was significantly increased in liver, plasma, VLDL, as well as in the heart (Tables 4 and 5). The accumulation of 20:3n–6 fatty acid is noteworthy because it is a precursor of PGE_1, an antiaggregatory factor, and also because 12- and 15-hydroxyeicosaenoic acids formed from 20:3n–6 are inhibitors of platelet aggregation (41, 42). This finding indicates that Δ^6 desaturase activity may be stimulated or at least not inhibited by CMTTD-treatment. How CMTTD affects the different desaturates as well as the elongation system needs to be further explored.

Cholesterol feeding increased the plasma concentration of triacylglycerol 2-fold, and this increase was reflected by 1.9- and 1.7-fold increases in VLDL-triacylglycerol and LDL-triacylglycerol, respectively (Fig. 2A). Feeding cholesterol increased the amount of cholesterol and cholesteryl ester in plasma by 5- and 4.8-fold, respectively, with concomitant increases in VLDL-cholesterol, LDL-cholesterol, and HDL-cholesterol by 11.7-, 7.6-, and 2.1-fold, respectively. Treating hypercholesterolemic hamsters with CMTTD resulted in both a hypotriacylglycerolemic and a hypocholesterolemic effect, and this was reflected by: *1)* 45% and 56% reduction in VLDL-triacylglycerol and LDL-triacylglycerol, respectively, and *2)* 39% and 30% reduction in VLDL-cholesterol and LDL-cholesterol, respectively, but no reduction in HDL-cholesterol. The complete normalization of plasma triacylglycerol content after CMTTD treatment was followed by an increased capacity of mitochondrial fatty acid oxidation as measured by palmitoyl-L-carnitine and palmitoyl-CoA as substrates by 2.0- and 1.8-fold, respectively, as compared to cholesterol-fed animals (Table 6). Moreover, CMTTD treatment increased the volume fraction of mitochondria by 2.1-fold and decreased the amount of fat droplets in the liver by 25% (Table 3). Increased fatty acid oxidation may lead to a decrease in the availability of fatty acids for triacylglycerol synthesis (19) with a concomitant decreased VLDL (19, 43). Increased capacity of fatty acid oxidation may explain the hypotriacylglycerolemic effect of CMTTD.

The hypocholesterolemic effect of CMTTD was accompanied by a 2.3-fold increase in FAO activity (Table 6). The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase was not affected in this study (Table 6). Also, an increased synthesis of bile acids could contribute to a loss of cholesterol. However, CMTTD has been shown to inhibit cholesterol 7α-hydroxylase (15), the rate-limiting enzyme in bile acid formation. As VLDL is a precursor to LDL, it is conceivable that a reduction of VLDL secretion and of plasma VLDL levels will affect LDL formation, which might contribute to the lowering of VLDL-cholesterol and LDL-cholesterol (**Fig. 3**).

In conclusion, cholesterol feeding changed the fatty acid composition of various organs in hamsters and produced hyperlipidemia. Treating cholesterol-fed hamsters with CMTTD (a 3-thia fatty acid) resulted in both a hypotriacylglycerolemic and a hypocholesterolemic effect.

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