On the mechanism of the hypolipidemic effect of sulfur-substituted hexadecanedioic acid (3-thiadicarboxylic acid) in normolipidemic rats

Jon Skorve,* Ayman Al-Shurbaji,† Daniel Asiedu,* Ingemar Björkhem,† Lars Berglund,† and Rolf K. Berge[1,*]

Laboratory of Clinical Biochemistry,* University of Bergen, Haukeland Sykehus, N-5021 Bergen, Norway, and Department of Clinical Chemistry,† Karolinska Institutet, Huddinge University Hospital, S-141 86 Huddinge, Sweden

Abstract The mechanism behind the hypolipidemic effect of the sulfur-substituted non-β-oxidizable fatty acid analogue 1,10 bis(carboxymethylthio)decane, also known as 3-thiadicarboxylic acid, was studied in normolipidemic rats. Treatment with 3-thiadicarboxylic acid markedly decreased plasma levels of free fatty acids, triglycerides, and cholesterol. This was accompanied by a corresponding reduction in very low density lipoprotein (VLDL)-triglyceride and low density lipoprotein (LDL)-cholesterol levels (by 46% and 42%, respectively), whereas the decrease in high density lipoprotein (HDL)-cholesterol levels was less pronounced (16%). However, the composition of the various plasma lipoprotein fractions was essentially unchanged. Fatty acid oxidation in both mitochondria and peroxisomes was stimulated in parallel; the activities of ATP:citrate lyase and fatty acid synthase, two key enzymes in fatty acid synthesis, were inhibited. Hepatic triglyceride biosynthesis was retarded, as indicated by a decrease in the liver triglyceride content along with a 30% reduction of hepatic VLDL-triglyceride secretion. This was accompanied by a 50% inhibition of phosphatidate phosphohydrolase. The activities of plasma lipoprotein lipase as well as hepatic lipase were somewhat higher (18%) in treated animals, suggesting a slight increase in the clearance potential of triglyceride-rich lipoproteins. The cholesterol-lowering effect was accompanied by a considerable reduction (75%) in HMG-CoA reductase activity and a less pronounced inhibition of cholesterol 7 α-hydroxylase (52%), and acyl-CoA:cholesterol acyltransferase (25%) activities. The present data suggest that the hypotriglyceridemic and hypocholesterolemic properties of sulfur-substituted fatty acid analogues are primarily due to effects on triglyceride and cholesterol synthesis.—**Skorve, J., A. Al-Shurbaji, D. Asiedu, I. Björkhem, L. Berglund, and R. K. Berge.** On the mechanism of the hypolipidemic effect of sulfur-substituted hexadecanedioic acid (3-thiadicarboxylic acid) in normolipidemic rats. *J. Lipid Res.* 1993. **34:** 1177–1185.

Supplementary key words plasma lipids • lipoprotein composition • hypolipidemic drug • triglyceride and cholesterol synthesis • lipoprotein lipase • hepatic lipase • lipogenic enzymes • HMG-CoA reductase • cholesterol 7 α-hydroxylase • ACAT

The hypolipidemic effect of sulfur-substituted long-chain fatty acids, where sulfur substitution prevents the β-oxidation of 3-thia fatty acids, is well documented in rats (1–3). The analogue 3-thiadicarboxylic acid has both hypotriglyceridemic and hypocholesterolemic properties (1). Recently, we found that the triglyceride-lowering effect was, at least in part, due to increased mitochondrial fatty acid oxidation along with an inhibition of phosphatidate phosphohydrolase activity in the liver (4). Data have accumulated underscoring the importance of the availability of triglycerides, cholesterol, and cholesteryl esters for the secretion of triglyceride-rich apolipoprotein B-containing lipoproteins (5–7). It seems, therefore, possible that 3-thiadicarboxylic acid could interfere with lipoprotein synthesis and/or secretion. However, so far very little information is available on the effect of these sulfur-substituted fatty acids on lipoprotein flux. Also, possible effects on plasma triglyceride clearance have not been previously investigated. In the present work a detailed characterization of the hypotriglyceridemic properties of 3-thiadicarboxylic acid and their underlying mechanisms was attempted. This included effects on fatty acid oxidation and synthesis, production and secretion of triglyceride-rich lipoprotein by the liver, effects on lipoprotein lipase and hepatic lipase activities, and the plasma lipoprotein pattern. 3-Thiadicarboxylic acid also has hypocholesterolemic properties. The mechanism by which this analogue reduces plasma cholesterol levels has not been addressed previously. Therefore, in the present study we have investigated whether 3-thiadicarboxylic acid influences key enzymes involved in cholesterol metabolism,

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; GPAT, glycerol-3-phosphate acyltransferase; PAP, phosphatidate phosphohydrolase; DGAT, diacylglycerol acyltransferase; ACAT, acyl-CoA:cholesterol acyltransferase.

[1]To whom correspondence should be addressed.

i.e., HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, cholesterol 7α-hydroxylase, the rate-limiting enzyme in cholesterol degradation, and acyl-CoA:cholesterol acyltransferase (ACAT), which is responsible for the esterification of hepatic cholesterol.

MATERIALS AND METHODS

Chemicals and drugs

[1-^{14}C]palmitic acid (56 mCi/mmol), L-[U-^{14}C]glycerol-3-phosphate (171 mCi/mmol), [^{14}C]acetyl-CoA (60 mCi/mmol), and NaH $^{14}CO_3$ (0.1 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. [4-^{14}C]cholesterol and [3-^{14}C]hydroxymethylglutaryl-CoA with specific activities of 55–60 mCi/mmol were obtained from New England Corp., Boston, MA. $^3H_2$-labeled 7α-hydroxycholesterol was synthesized as described previously (8). Tri [9,10-^{3}H]oleoylglycerol was synthesized and purified as described earlier (4). 3-Thiadicarboxylic acid was prepared as described earlier (9, 10). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and treatments

Male Sprague-Dawley rats from Möllegaard Breeding Laboratory, Ejby, Denmark, weighing about 200 g, were housed individually in metal wire cages maintained at 12 h light–dark cycles and a constant temperature of 20 ± 3°C. The animals were acclimatized for at least 1 week under these conditions before start of the experiment. 3-Thiadicarboxylic acid was suspended in 0.5% sodium carboxymethyl cellulose and administered by gastric intubation once a day for 5 days at a dose of 250 mg/day per kg body weight (2, 3). The animals were killed at 8 AM on the sixth day. All animals had free access to water and food. There was no significant difference in food consumption or weight gain between the two experimental groups.

At the end of the experiments, cardiac puncture was performed under neuroleptic anesthesia, and blood was collected in EDTA-containing tubes. Plasma was prepared by centrifugation at 3000 rpm for 20 min. For lipoprotein lipase and hepatic lipase assays, the rats were injected intravenously with heparin (1000 U/kg body weight) 8 min prior to blood sampling.

Preparation of subcellular fractions

The livers were removed and immediately chilled on ice and weighed. Portions of the livers from individual rats were homogenized in ice-cold sucrose–medium (0.25 M sucrose in 10 mM HEPES buffer, pH 7.4, and 1 mM EDTA) (3, 4). Mitochondrial and peroxisomal fractions were prepared as described earlier (3). Microsomal fractions were prepared by centrifugating the homogenate at 20,000 *g* for 15 min and recentrifugation of the supernatant at 100,000 *g* for 60 min. For analysis of microsomal and cytoplasmic enzyme activities, different buffers were used as detailed below.

Enzyme assays

A fraction of the 100,000 *g* supernatant precipitated by 40% saturated ammonium sulfate was used as a source of cytosolic phosphatidate phosphohydrolase activity (11). Phosphatidate phosphohydrolase activity was assayed for 15 min at 37°C using ^{14}C-labeled phosphatidate as substrate as described earlier (11). The extraction procedure, thin-layer chromatography, and radioscanning of chromatography plates were performed as previously described (12). Phosphatidate phosphohydrolase was also determined by measuring the release of free phosphate in the presence of 5 mM $MgCl_2$ (13). Glycerol-3-phosphate acyltransferase (GPAT) was assayed for 3 min at 30°C, as essentially described by Bates and Saggerson (14). The reaction was started by the addition of mitochondria or microsomes (200 μg protein), and terminated by the addition of 2.0 ml of 1-n butanol. The incorporation of ^{14}C into butanol-soluble products was determined as described (14, 15). Diacylglycerol acyltransferase (DGAT) was assayed for 5 min at 37°C. The reaction was initiated by the addition of microsomes (30–40 μg protein), and terminated by the addition of 7.5 ml of 2-propanol–hexane–water 89:20:16. The extraction of triglycerides into hexane was performed as described by Bell and Coleman (16). The microsomal fractions used for the GPAT and DGAT assays (see below) were isolated as described previously (17).

ATP:citrate lyase activity (18), peroxisomal β-oxidation (19), fatty acyl-CoA oxidase activity (20), and mitochondrial β-oxidation (21) were determined as described earlier. Acetyl-CoA carboxylase activity was measured in the cytosolic fraction as fixation of $^{14}CO_2$ from NaH$^{14}CO_3$ into malonyl-CoA essentially as described by Tanabe et al. (22). Fatty acid synthase activity was measured in the cytosolic fraction as described by Roncari (23), except that 300 μM NADPH was used instead of an NADPH-generating system, and [^{14}C]acetyl-CoA was used as the radioactive substrate. The assay was started by the addition of preincubated enzyme fraction (100 μg protein) and run for 6 min at 37°C.

For assay of HMG-CoA reductase and cholesterol 7α-hydroxylase, a 10% (w/v) liver homogenate was prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3 M sucrose, 50 mM NaCl, 10 mM EDTA, and 10 mM dithiothreitol (DTT). The microsomal fraction was prepared by centrifugation of the homogenate at 20,000 *g* for 15 min and recentrifugation of the supernatant at 100,000 *g* for 60 min. Half of the microsomal fraction was resuspended in the homogenizing buffer and recentrifuged at 100,000 *g* for 60 min. The resulting fraction was

suspended in 20 mM imidazol-HCl buffer (pH 7.4) containing 10 mM DTT, and used for assay of HMG-CoA reductase. The other half of the original microsomal fraction was suspended in a homogenizing buffer lacking DTT and recentrifuged at 100,000 *g* for 60 min. This fraction was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, and used for assay of cholesterol 7α-hydroxylase.

HMG-CoA reductase activity was assayed essentially as described by Brown, Goldstein, and Dietschy (24). The incubation was run for 15 min and terminated by addition of 25 μl 6 M HCl. Tritium-labeled mevalonic acid (0.01 μCi) together with 3 mg unlabeled mevalonic acid lactone was added as internal standard. The incubation mixture was further lactonized, subjected to thin-layer chromatography, and analyzed for radioactivity (24).

For assay of cholesterol 7α-hydroxylase, the microsomal fraction was incubated with [4-^{14}C]cholesterol as described previously (8). In this procedure the conversion of [4-^{14}C]cholesterol into 7α-hydroxycholesterol is measured by radio-scanning after separation of the product from the substrate by thin-layer chromatography. Also, the conversion of the endogenous microsomal cholesterol into 7α-hydroxycholesterol was measured by isotope dilution mass spectrometry.

Acyl-CoA:cholesterol acyltransferase activity was measured in the microsomal fraction essentially as described by Field, Albright, and Mathur (25). The assay was performed for 3 min at 37°C.

Lipoprotein lipase and hepatic lipase activities were assayed essentially as previously described (26).

Analytical methods

Lipoprotein quantitation was performed by a combination of ultracentrifugation and precipitation (27, 28). Briefly, plasma samples were centrifuged at 1.006 g/ml for 18 h at 45000 rpm in a Beckman 2-TL centrifuge equipped with a 70 Ti-rotor. The tubes were sliced, and the floating fractions as well as the infranatants were analyzed for cholesterol and triglycerides. One portion of each infranatant was treated with phosphotungstic acid to precipitate apoB-containing lipoproteins, and the resulting supernatant fraction was analyzed for triglyceride and cholesterol. By this procedure, it was possible to calculate triglyceride and cholesterol content in the VLDL, LDL, and HDL fractions (27). Furthermore, the infranatant fractions obtained after the initial ultracentrifugation procedure were subjected to sequential ultracentrifugation to obtain LDL ($1.025 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml) fractions. In order to optimize recovery, the respective fractions from all animals in each group were pooled. Each lipoprotein fraction, including the VLDL ($d < 1.006$ g/ml) fraction, was then washed by ultracentrifugation at the upper density prior to use. SDS polyacrylamide gel electrophoresis was performed using 4–20% gradient gels and the gels were stained for protein with Coomassie Brilliant Blue. VLDL samples were delipidated with ethanol–ether 3:1 (v/v) prior to electrophoresis. Protein was determined by the procedure of Lowry et al. (29). Phospholipids were analyzed by a modification of the procedure described by Bartlett (30). Unesterified cholesterol was measured enzymatically (Boehringer Mannheim, Mannheim, Germany).

Analysis of hepatic lipids was carried out by the Monotest cholesterol and phospholipid enzymatic kits (Boehringer Mannheim, Germany) and the Biopak triglyceride enzymatic kit (Biotrol, Paris, France).

VLDL-triglyceride secretion rate was determined by measuring the increase in plasma triglyceride concentration after an intravenous injection of Triton WR 1339 as described before (31, 32).

Plasma free fatty acids were determined by an enzymatic colorimetric method (NEFAC, Wako Chemicals GmbH) (33, 34).

Statistical analysis

Results were evaluated for statistical significance by Student's *t*-test. The level of significance was taken as $P < 0.05$.

RESULTS

Plasma lipids and lipoproteins

As shown in **Table 1,** administration of 3-thiadicarboxylic acid to rats maintained on a balanced pelleted control diet resulted in a reduction of plasma triglycerides (38%), cholesterol (34%), and free fatty acids (49%), in accordance with our previous results (1–3). The observed decrease in plasma triglyceride and cholesterol levels was

TABLE 1. Changes of plasma lipids in 3-thiadicarboxylic acid-treated rats

Group	Triglycerides	Cholesterol	Free Fatty Acid
		mmol/l	
Control	1.21 ± 0.14	2.55 ± 0.31	0.84 ± 0.08
3-Thiadicarboxylic acid	0.75 ± 0.09[a]	1.69 ± 0.11[a]	0.43 ± 0.07[a]

The plasma lipid values represent means ± SEM for six animals in each experimental group.
[a] $P < 0.02$ for differences between control and treated rats.

TABLE 2. Plasma lipoproteins levels in 3-thiadicarboxylic acid-treated and control rats

Composition	Nontreated	3-Thiadicarboxylic Acid-Treated	Decrease during Treatment
		mmol/l	
Triglycerides			
Total	1.07 ± 0.10	0.67 ± 0.06[a]	−38%
VLDL	0.62 ± 0.12	0.34 ± 0.04[b]	−46%
LDL	<0.05	<0.05	
HDL	0.44 ± 0.02	0.33 ± 0.04[a]	−25%
Cholesterol			
Total	2.32 ± 0.12	1.53 ± 0.15[a]	−34%
VLDL	0.44 ± 0.06	0.27 ± 0.02[a]	−39%
LDL	1.22 ± 0.20	0.71 ± 0.11[a]	−42%
HDL	0.64 ± 0.08	0.54 ± 0.02[a]	−16%

The values are given as mean ± SEM for six animals in each group. To convert mmol/l to mg/dl, multiply cholesterol values by 38.7 and triglyceride values by 88.5.
[a] $P < 0.05$.
[b] $P = 0.055$.

accompanied by a corresponding reduction in VLDL-triglyceride and LDL-cholesterol, whereas the decrease in HDL-cholesterol was less pronounced (**Table 2**). The composition of the VLDL, LDL, and HDL fractions was not significantly affected by 3-thiadicarboxylic acid treatment (**Table 3**). Further, the apolipoprotein pattern of VLDL as judged by SDS polyacrylamide gel electrophoresis was essentially unchanged (data not shown).

Lipogenesis and fatty acid oxidation

As the availability of fatty acids is a major determinant of the hepatic triglyceride synthetic rate, it was important to investigate the effect of 3-thiadicarboxylic acid on lipogenesis and fatty acid oxidation. As shown in **Table 4,** treatment with 3-thiadicarboxylic acid resulted in inhibition of ATP:citrate lyase (21%) and fatty acid synthase (33%), two key enzymes in fatty acid synthesis. However, acetyl-CoA carboxylase, which catalyzes the rate-limiting step in fatty acid biosynthesis, was not significantly affected.

In accordance with our previous results (5), fatty acyl-CoA oxidase activity and peroxisomal β-oxidation were stimulated by drug treatment (**Table 5**). Also, mitochondrial fatty acid oxidation increased, as measured with palmitoyl-CoA or palmitoyl-L-carnitine as substrates (Table 5).

Hepatic triglyceride synthesis and secretion

The activities of several enzymes involved in triglyceride biosynthesis were affected by treatment with 3-thiadicarboxylic acid (**Table 6**). The activities of glycerol-3-phosphate acyltransferase (GPAT), both in mitochondria and microsomes, and diacylglycerol acyltransferase (DGAT) were stimulated, whereas phosphatidate phosphohydrolase (PAP) activity was inhibited, confirming our earlier observations (5). Because the drug substantially increased the β-oxidation of fatty acids (Table 5), thereby limiting their availability for triglyceride biosynthesis, it was important to determine the overall effect of drug treatment on VLDL-triglyceride production. This was accomplished by measuring the rate of plasma triglyceride entry after blocking the clearance of triglyceride-rich lipoproteins by Triton WR 1339. During the next 3 h, the rate of plasma triglyceride entry was

TABLE 3. The effect of 3-thiadicarboxylic acid on plasma lipoprotein composition

Composition	VLDL		LDL		HDL	
	Nontreated	TD-Treated	Nontreated	TD-Treated	Nontreated	TD-Treated
			% of total weight			
Triglycerides	64.0	67.0	22.5	31.5	2.3	5.1
Free cholesterol	3.0	1.5	2.0	1.0	2.7	1.9
Cholesteryl ester	5.5	8.5	26.5	23.0	25.3	25.3
Phospholipid	13.0	11.5	18.0	15.0	22.2	17.2
Protein	14.5	11.5	31.0	29.5	47.5	50.4

The results are presented as mean values from two different experiments. In each experiment, the lipoprotein fractions were prepared by ultracentrifugation of pooled plasma from six control and six 3-thiadicarboxylic acid-treated rats. TD, 3-thiadicarboxylic acid.

TABLE 4. The effect of 3-thiadicarboxylic acid on lipogenic enzymes

Enzymes	Nontreated Rats	3-Thiadicarboxylic Acid-Treated Rats
	nmol/min/mg protein	
ATP-citrate lyase	12.50 ± 0.18	9.84 ± 0.64[a]
Acetyl-CoA carboxylase	4.71 ± 0.58	4.66 ± 0.49
Fatty acid synthase	0.15 ± 0.02	0.10 ± 0.02[a]

Values are expressed as means ± SEM for six rats in each experimental group.
[a] $P < 0.05$.

3.29 ± 0.43 mmol/l per h in thiadicarboxylate-treated rats and 4.43 ± 0.23 mmol/l per h in controls (**Fig. 1**). To rule out the possibility that drug treatment might block VLDL secretion without affecting triglyceride biosynthesis, the liver triglyceride content was measured. This was reduced by 52% (6.77 ± 0.40 μmol/g tissue in control and 3.23 ± 0.22 μmol/g tissue in treated rats), excluding a retention of triglyceride in the liver as an explanation to the observed decrease in VLDL-triglyceride secretion. No difference was found in liver cholesterol content. In contrast, hepatic phospholipid levels and the liver/body weight ratio increased during 3-thiadicarboxylic acid administration (**Table 7**).

Plasma triglyceride clearance

The hypotriglyceridemic effect of 3-thiadicarboxylic acid might not be exclusively due to retarded VLDL-triglyceride secretion rate. As changes in the clearance rate might contribute to the triglyceride-lowering effect, we examined whether the activities of lipoprotein lipase and hepatic lipase were changed after feeding 3-thiadicarboxylic acid. Table 6 shows that administration of the fatty acid analogue stimulated both lipase activities by 18–20%.

Cholesterol synthesis, degradation, and esterification

In view of the cholesterol-lowering effect observed, the possibility that 3-thiadicarboxylic acid treatment might affect the rate-limiting enzymes in cholesterol synthesis, degradation, and esterification was studied. The activities of HMG-CoA reductase and cholesterol 7α-hydroxylase were inhibited by 75% and 52%, respectively. Also, ACAT activity decreased by 24% (**Table 8**).

DISCUSSION

Plasma triglyceride levels are determined by a delicate balance between hepatic triglyceride synthesis and secretion on one hand and plasma triglyceride clearance on the other. Thus, the observed reduction in plasma triglyceride levels during 3-thiadicarboxylic acid administration could be accomplished by retarded synthesis, reduced hepatic output, enhanced clearance, or a combination of these factors. In the present study 3-thiadicarboxylic acid was found to decrease triglyceride biosynthesis in the liver by several mechanisms. As shown above, and in agreement with previous studies, 3-thiadicarboxylic acid treatment stimulated both mitochondrial and peroxisomal β-oxidation of fatty acids (75% and 450%, respectively) (1, 21). The relative importance of the induced β-oxidation in peroxisomes versus mitochondria for the overall fatty acid oxidation in the liver during 3-thiadicarboxylic acid treatment remains, however, to be established. The previous finding that the effects of 3-thiadicarboxylic acid on peroxisomal β-oxidation and plasma triglyceride levels were dissociable depending on dose and time of treatment indicates that the role of 3-thiadicarboxylic acid as a peroxisomal proliferator might not be crucial for the hypotriglyceridemic effect observed (1). Moreover, it should be emphasized that mitochondria are by far the quantitively dominating organelle in liver cells, suggesting that a twofold increase in mitochondrial oxidation

TABLE 5. The effect of 3-thiadicarboxylic acid on fatty acid oxidation

Oxidation	Nontreated Rats	3-Thiadicarboxylic Acid-Treated Rats
	nmol/min/mg protein	
Mitochondrial β-oxidation		
Palmitoyl-L-carnitine as substrate	4.27 ± 0.33	6.83 ± 0.28[a]
Palmitoyl-CoA as substrate	2.56 ± 0.68	4.70 ± 0.16[a]
Peroxisomal β-oxidation	1.56 ± 0.18	7.84 ± 0.66[a]
	nmol H_2O_2/min/mg protein	
Fatty acyl-CoA oxidase	9.33 ± 0.30	46.89 ± 1.44[a]

Values are expressed as means ± SEM for six animals in each experimental group.
[a] $P < 0.05$.

TABLE 6. The effect of 3-thiadicarboxylic acid on key enzymes in triglyceride biosynthesis and degradation

Enzymes	Nontreated Rats	3-Thiadicarboxylic Acid-Treated Rats
	nmol/min/mg protein	
Glycerophosphate acyltransferase		
Microsomal fraction	2.08 ± 15	9.56 ± 0.25[a]
Mitochondrial fraction	0.40 ± 0.09	0.85 ± 0.09[a]
Diacylglycerol acyltransferase	2.83 ± 0.17	4.15 ± 0.40[a]
Phosphatidate phosphohydrolase		
Release of phosphate	12.23 ± 1.45	6.37 ± 0.70[a]
Production of [^{14}C]diacylglycerol	1.68 ± 0.09	0.82 ± 0.05[a]
	mU/ml	
Lipoprotein lipase	9.9 ± 0.2	11.7 ± 0.2[a]
Hepatic lipase	9.3 ± 0.3	11.0 ± 0.2[a]

The activities reported represent means ± SEM for seven animals in each experimental group. One mU of enzyme activity is defined as the release of 1 nm of fatty acid from triolein per min under the conditions of the assay.
[a] $P < 0.05$.

might have a greater impact on the total β-oxidation of fatty acids than the fivefold increase in peroxisomal oxidation (Table 5).

3-Thiadicarboxylic acid treatment resulted in a slight inhibition in the activities of ATP:citrate lyase and fatty acid synthase (Table 4). However, the impact of these effects on lipogenesis, and consequently triglyceride biosynthesis, is not self-evident. On the one hand an inhibition of two of the enzymes involved in fatty acid synthesis is consistent with a retarded lipogenesis. On the other hand, the finding that the activity of the enzyme considered to be rate-limiting in fatty acid synthesis, i.e., acetyl-CoA carboxylase, was unaffected by drug treatment argues against the contention that decreased fatty acid synthesis is of major importance for the triglyceride-lowering effect observed. During administration of the drug, plasma free fatty acid levels decreased (Table 1). Administration of nicotinic acid or acipimox has previously been shown to affect plasma triglyceride levels primarily via this mechanism (32, 35). Thus, 3-thiadicarboxylic acid treatment might interfere with both the exogenous and the endogenous supply of hepatic fatty acids, affecting their availability for esterification and triglyceride biosynthesis. In addition, the observed inhibition of phosphatidate phosphohydrolase activity during 3-thiadicarboxylic acid treatment (Table 6) would further contribute to a lower triglyceride synthetic rate. The parallel decrease in phosphatidate phosphohydrolase activity and the hepatic and plasma triglyceride concentrations suggests that the hypotriglyceridemic effect of 3-thiadicarboxylic level may be largely due to its effect on the synthetic level. Also, this underscores the regulatory importance of phosphatidate phosphohydrolase in triglyceride biosynthesis. In contrast, both glycerol-3-phosphate and diacylglycerol acyltransferase activities (Table 6) increased when hepatic triglyceride synthesis and secretion were retarded. This

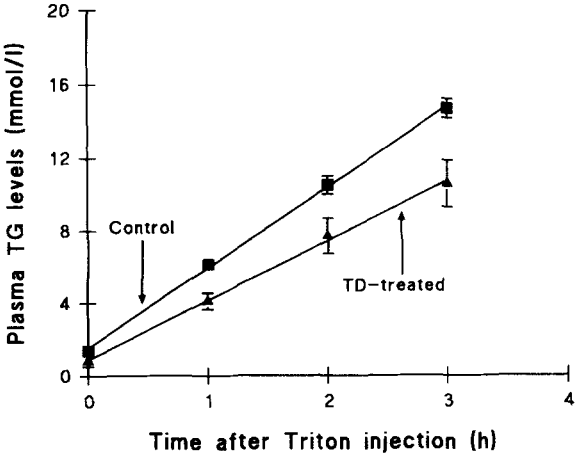

Fig. 1. Effect of 3-thiadicarboxylic acid (TD) on plasma triglyceride entry rate after a single intravenous injection of Triton WR 1339. The values are expressed as means ± SD for six rats in each experimental group; (■) control rats; (▲) TD-treated rats. To convert mmol/l to mg/dl, multiply by 88.5.

TABLE 7. Liver mass and hepatic lipids after 3-thiadicarboxylic acid treatment

Variable	Nontreated Rats	3-Thiadicarboxylic Acid-Treated Rats
Liver weight/body weight (%)	3.27 ± 0.09	5.57 ± 0.33[a]
Hepatic triglycerides (μmol/g)	6.77 ± 0.40	3.23 ± 0.22[a]
Hepatic cholesterol (μmol/g)	5.32 ± 0.12	5.24 ± 0.09
Hepatic phospholipids (μmol/g)	18.12 ± 0.41	21.10 ± 0.27[a]

Values are expressed as means ± SEM for six animals in each experimental group.
[a] $P < 0.05$.

TABLE 8. The effect of 3-thiadicarboxylic acid on the rate-limiting enzymes in cholesterol synthesis, degradation, and esterification

Enzymes	Nontreated Rats	3-Thiadicarboxylic Acid-Treated Rats
HMG-CoA reductase (pmol/min/mg/protein	1039 ± 33	256 ± 41[a]
Cholesterol 7α-hydroxylase (nmol/min/mg protein)	36.2 ± 2.3	17.3 ± 3.3[a]
ACAT (pmol/min/mg protein)	364 ± 8	277 ± 2[a]

Values represent means ± SEM for seven animals in each experimental group.
[a] $P < 0.05$.

argues against the initial and the last esterification steps in triglyceride biosynthesis being potential sites at which 3-thiadicarboxylic acid might modulate triglyceride synthesis.

A major new finding in the present study was the effect of 3-thiadicarboxylic acid treatment on the lipoprotein fractions, with a decrease in both VLDL-triglyceride and LDL-cholesterol levels. The decrease in VLDL-triglyceride was associated with a reduction in the secretion of newly synthesized triglycerides (Fig. 1). As VLDL is a precursor to LDL, it is conceivable that the observed reduction in VLDL secretion and plasma VLDL levels will affect LDL formation, which might contribute to the lowering of LDL cholesterol levels. It is of interest that the reduction in plasma triglyceride levels was slightly more pronounced (38% decrease) than the decrease in the VLDL-triglyceride secretion (25%), implying that alterations in the clearance of VLDL-triglycerides might contribute to the decrease in plasma triglycerides. In agreement with this, the activities of plasma lipoprotein lipase and hepatic lipase were somewhat higher in 3-thiadicarboxylic acid-treated animals, indicating a possible increase in the clearance potential of triglyceride-rich lipoproteins. However, no difference was found in either the chemical composition or the apolipoprotein pattern in isolated VLDL from control or 3-thiadicarboxylic acid-treated rats, arguing against any larger conformational changes that could affect their catabolism. However, at present we cannot exclude that minor conformational changes in VLDL could be induced by 3-thiadicarboxylic acid administration, and further studies are needed to establish whether treatment with this drug affects the metabolic properties of VLDL.

The effects of 3-thiadicarboxylic acid on plasma cholesterol levels could be due to a number of factors. Of prime significance is the possibility that this effect could be obtained through retarded cholesterol synthesis and/or increased degradation. It is well documented that HMG-CoA reductase is rate-limiting in the synthesis of cholesterol under almost all experimental conditions, and inhibition of this enzyme has been shown to reduce plasma cholesterol levels (36–38). Repeated administration of the 3-thiadicarboxylic fatty acid led to a 75% reduction of the HMG-CoA reductase activity. It is therefore tempting to suggest that the decrease in plasma cholesterol was mediated largely by an inhibition of HMG-CoA reductase. In addition to the effect on HMG-CoA reductase, the activity of the rate-limiting enzyme in the degradation of cholesterol into bile acids, i.e., cholesterol 7α-hydroxylase, was also depressed (52%). Furthermore, ACAT activity was inhibited (24%) (Table 8), compatible with a decrease in cholesteryl ester formation. Recently, a possible role of cholesteryl ester for VLDL secretion has been proposed (7). The impact of the reduction of these enzyme activities might, however, be less prominent and could be due to the reduction of cholesterol biosynthesis. Of interest is the observation that both HMG-CoA reductase and phosphatidate phosphohydrolase activities decreased during 3-thiadicarboxylic acid treatment. Previous studies have shown a link between these enzymes (16, 39, 40), demonstrating the importance of a concerted regulation of cholesterol and triglyceride biosynthesis. Indeed, a parallel regulation of these two metabolic pathways would be of importance for the observed reduction in VLDL secretion. As the composition of VLDL was not affected by drug treatment, it seems likely that 3-thiadicarboxylic acid treatment results in a general reduction in VLDL production involving both cholesterol and triglycerides rather than a selective decrease in triglyceride biosynthesis. Although the total liver content of cholesterol was not reduced, in contrast to triglycerides (Table 7), compartmentalization may nevertheless result in decreased availability of cholesterol for VLDL synthesis.

In summary, the present study demonstrates that 3-thiadicarboxylic acid treatment of normolipidemic rats decreases plasma triglycerides and cholesterol. Both effects seem to be primarily mediated on the synthetic level. These findings indicate that sulfur-substituted non-β-oxidizable fatty acid analogues might be a useful tool for studying, and possibly treating, metabolic disturbances characterized by increased lipoprotein synthesis.

The authors are grateful to Mr. Svein Krüger, Ms. Anne R. Alvestad, Ms. Anita Löfgren, Ms. Ulla Andersson, and Ms. Gunvor Nilsson for excellent technical assistance. The manuscript preparation by Ms. Vivianne Hanna is gratefully acknowledged. The work was supported by The Norwegian Council on Cardiovascular Disease (NCC), The Norwegian Research Council for Science and Humanities (NAVF), Norges Diabetesforbunds Forskningsfond, Familien Blix Forskningsfond, ODD Fellow Vitenskapelige Forskningsfond, The Norwegian Cancer Society (DNKF), The Swedish Medical Research Council (Projects 3141 and 10349), Nordisk Insulinfond, Swedish Hoechst, Hans and Loo Ostermans Fond, and by Svenska Margarinindustrins Förening för Näringsfysiologisk Forskning.

Manuscript received 29 September 1992 and in revised form 8 March 1993.

REFERENCES

1. Aarsland, A. N. Aarsaether, J. Bremer, and R. K. Berge. 1989. Alkylthioacetic acids (3-thia fatty acids) as non-β-oxidizable fatty acid analogues: a new group of hypolipidemic drugs. III. Dissociation of cholesterol- and triglyceride-lowering effects and the induction of peroxisomal β-oxidation. *J. Lipid Res.* **30:** 1711–1718.
2. Berge, R. K., A. Aarsland, H. Kryvi, J. Bremer, and N. Aarsaether. 1989. Alkylthioacetic 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.
3. Berge, R. K., A. Aarsland, H. Kryvi, J. Bremer, and N. Aarsaether. 1989. 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.
4. Skorve, J., D. Asiedu, A. C. Rustan, C. A. Drevon, A. Al-Shurbaji, and R. K. Berge. 1990. Regulation of fatty acid oxidation and triglyceride and phospholipid metabolism by hypolipidemic sulfur-substituted fatty acid analogues. *J. Lipid Res.* **31:** 1627–1635.
5. Gibbons, G. F. 1990. Assembly and secretion of hepatic very-low-density lipoprotein. *Biochem. J.* **268:** 1–13.
6. Khan, B., H. G. Wilcox, and M. Heimberg. 1989. Cholesterol is required for secretion of very low density lipoprotein by rat liver. *Biochem. J.* **258:** 807–816.
7. Cianflone, K. M., Z. Yasruel, M. A. Rodriguez, D. Vas, and A. D. Sniderman. 1990. Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. *J. Lipid Res.* **31:** 2045–2055.
8. Björkhem, I., and A. Kallner. 1976. Hepatic 7α-hydroxylation of cholesterol in ascorbate-deficient and ascorbate-supplemented guinea pigs. *J. Lipid Res.* **17:** 360–365.
9. Aarsland, A., R. K. Berge, J. Bremer, and N. Aarsaether. 1990. The hypolipidemic peroxisome proliferating drug bis (carboxy methylthio)1,10 decane, a dicarboxylic metabolite of tiadenol, is activated to an acylcoenzyme A thioester. *Biochim. Biophys. Acta.* **1033:** 176–183.
10. Spydevold, Ø., and J. Bremer. 1989. Induction of peroxisomal β-oxidation in 7800 Morris hepatome, cells in steady state by fatty acids and fatty acid analogues. *Biochim. Biophys. Acta.* **1003:** 72–79.
11. Berglund, L., I. Björkhem, and K. Einarsson. 1982. Apparent phosphorylation-dephosphorylation of soluble phosphatidic acid phosphatase in rat liver. *Biochem. Biophys. Res. Commun.* **105:** 288–295.
12. Humble, E., and L. Berglund. 1991. Stimulation and inhibition of the activity of rat liver cytosolic phosphatidate phosphohydrolase by various phospholipids. *J. Lipid Res.* **32:** 1869–1872.
13. Mavis, R. D., J. N. Finkelstein, and B. P. Hall. 1978. Pulmonary surfactant synthesis. A highly active microsomal phosphatidate phosphohydrolase in the lung. *J. Lipid Res.* **19:** 467–477.
14. Bates, E. J., and D. A. Saggerson. 1977. A selective decrease in mitochondrial glycerol phosphate acyltransferase activity in livers from streptozotocin-diabetic rats. *FEBS Lett.* **84:** 229–232.
15. Daae, L. N. W., and J. Bremer. 1970. The acylation of glycerol phosphate in rat liver: a new assay procedure for glycerophosphate acylation, studies on its subcellular and submitochondrial localization and determination of the reaction products. *Biochim. Biophys. Acta.* **210:** 92–104.
16. Bell, R. M., and R. M. Coleman. 1980. Enzymes of glycerolipid synthesis in eucaryotes. *Annu. Rev. Biochem.* **49:** 459–487.
17. Al-Shurbaji, A., C. Larsson-Backström, L. Berglund, G. Eggertsen, and I. Björkhem. 1991. Effect of n-3 fatty acids on the key enzymes involved in cholesterol and triglyceride turnover in rat liver. *Lipids.* **26:** 385–389.
18. Rose-Kahn, G., and J. Bar-Tana. 1989. Inhibition of lipid synthesis by $\beta^1\beta^1$-tetramethyl-substituted C_{14}-C_{22}, α,ω-dicarboxylic acids in cultured rat hepatocytes. *J. Biol. Chem.* **260:** 8411–8415.
19. Berge, R. K., A. Nilsson, and A. M. Huseby. 1988. Rapid stimulation of liver palmitoyl-CoA synthetase, carnitine palmitoyltransferase and glycerophosphate acyltransferase compared to peroxisomal β-oxidation and palmitoyl-CoA hydrolase in rats fed high-fat diets. *Biochim. Biophys. Acta.* **960:** 417–426.
20. Berge, R. K., T. Flatmark, and E. N. Christiansen. 1987. Effect of a high-fat diet with partially hydrogenated fish oil on long-chain fatty acid metabolizing enzymes in subcellular fractions of rat liver. *Arch. Biochem. Biophys.* **252:** 269–276.
21. Asiedu, D., A. Aarsland, J. Skorve, A. M. Svardal, and R. K. Berge. 1990. Fatty acid metabolism in liver of rats treated with hypolipidemic sulphur-substituted fatty acid analogues. *Biochim. Biophys. Acta.* **1044:** 211–221.
22. Tanabe, T., S. Nakanishi, T. Hashimoto, J-I. Nikowa, and S. Numa. 1981. Acetyl CoA carboxylase from rat liver. *Methods Enzymol.* **71:** 5–16.
23. Roncari, D. A. 1981. Fatty acid synthase from human liver. *Methods Enzymol.* **71:** 73–79.
24. Brown, M. S., J. L. Goldstein, and J. M. Dietschy. 1979. Active and inactive forms of 3-hydroxy-3-methylgluteryl coenzyme A reductase in the liver of the rat: comparison with the rate of cholesterol synthesis in different physiological states. *J. Biol. Chem.* **254:** 5144–5149.
25. Field, F. J., E. Albright, and S. Mathur. 1991. Inhibition of acyl coenzyme A: cholesterol acyltransferase activity by PD 128042. Effect on cholesterol metabolism and secretion in CaCo-2 cells. *Lipids.* **26:** 1–8.
26. Nilsson-Ehle, P., and R. Ekman. 1977. Rapid, simple and specific assays for lipoprotein lipase and hepatic lipase. *Artery.* **3:** 194–209.
27. Carlson, K. 1973. Lipoprotein fractionation. *J. Clin. Pathol.* **26:** 32–37.
28. Lopez-Virella, M. F., P. Stone, S. Ellis, and J. A. Colwell. 1977. Cholesterol determination in high-density lipoproteins separated by three different methods. *Clin. Chem.* **23:** 882–884.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
30. Bartlett, G. 1958. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
31. Otway, S., and D. S. Robinson. 1967. A non-ionic detergent (Triton WR 1339) to determine rates of triglyceride entry into the circulation of the rat under different physiological conditions. *J. Physiol.* **190:** 321–323.
32. Al-Shurbaji, A., L. Berglund, and I. Björkhem. 1990. The effect of Acipimox on triacylglycerol metabolism in rat. *Scand. J. Clin. Lab. Invest.* **50:** 203–208.
33. Shimizu, S., K. Yasui, Y. Tani, and H. Yamada. 1979. Acyl-

CoA oxidase from *Candida tropicalis*. *Biochem. Biophys. Res. Commun.* **91:** 108–113.
34. Mulder, C., J. A. Schouten, and C. Popp-Snijders. 1983. Determination of free fatty acids: a comparative study of the enzymatic versus the gas chromatographic and colorimetric method. *J. Clin. Chem. Biochem.* **21:** 823–827.
35. Grundy, S. M., H. Y. I. Mok, L. Zech, and M. Berman. 1981. Influence of nicotinic acid on metabolism of cholesterol and triglycerides in man. *J. Lipid Res.* **22:** 24–36.
36. Grundy, S. M. 1988. HMG CoA reductase inhibitors for treatment of hypercholesterolemia. *N. Engl. J. Med.* **319:** 24–33.
37. Tobert, J. A., G. D. Bell, J. Birtwell, I. James, W. R. Kukovetz, J. S. Pryor, A. Buntinx, I. B. Holmes, Y-S. Chao, and J. A. Bolognese. 1982. Cholesterol-lowering effect of mevinolin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, in healthy volunteers. *J. Clin. Invest.* **69:** 913–919.
38. Reihnér, E., M. Rudling, D. Ståhlberg, L. Berglund, S. Ewerth, I. Björkhem, K. Einarsson, and B. Angelin. 1990. Influence of pravastatin, a specific inhibitor of HMG CoA reductase, on hepatic metabolism of cholesterol. *N. Engl. J. Med.* **323:** 224–228.
39. Björkhem, I., B. Angelin, L. Berglund, K. Einarsson, S. Ewerth, and A. Lewenhaupt. 1985. Effect of flux of lymph and bile acids on the hepatic phosphatidic acid phosphatase in rat liver. *In* Proceedings of the Workshop on Coordinate Regulation of Cholesterol Metabolism. A. Sanghvi, editor. 262–279.
40. Björkhem, I., and L. Berglund. 1987. Stimulatory effect of mevinolin on rat liver phosphatidic acid phosphatase. *Biochim. Biophys. Acta.* **920:** 20–25.