

Causes and prevention of tamoxifen-induced accumulation of triacylglycerol in rat liver

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Abstract Tamoxifen can induce hepatic steatosis in women. In this study, we wanted to elucidate the mechanism behind the tamoxifen-induced accumulation of triacylglycerol in liver in female rats, and we hoped to prevent this development by combination treatment with the modified fatty acid tetradecylthioacetic acid (TTA). The increased hepatic triacylglycerol level after tamoxifen treatment was accompanied by decreased acetyl-coenzyme A carboxylase (ACC) and FAS activities, increased glycerol-3-phosphate acyltransferase (GPAT) activity, and a tendency to increased diacylglycerol acyltransferase (DGAT) activity. The activities and mRNA levels of enzymes involved in β -oxidation, ketogenesis, and uptake of lipids from liver were unaffected by tamoxifen, whereas the uptake of lipoproteins was unchanged and the uptake of fatty acids was decreased. Combination treatment with tamoxifen and TTA (Tam+TTA) normalized the hepatic triacylglycerol level and increased the activities of ACC, FAS, GPAT, and DGAT compared with tamoxifen-treated rats. The activities and mRNA levels of enzymes involved in β -oxidation, ketogenesis, and uptake of lipids were increased after Tam+TTA treatment. **In conclusion,** tamoxifen increased the hepatic triacylglycerol level, probably as a result of increased triacylglycerol biosynthesis combined with unchanged β -oxidation. The tamoxifen-induced accumulation of triacylglycerol was prevented by cotreatment with TTA, through mechanisms of increased mitochondrial and peroxisomal β -oxidation.—Gudbrandsen, O. A., T. H. Rost, and R. K. Berge. Causes and prevention of tamoxifen-induced accumulation of triacylglycerol in rat liver. *J. Lipid Res.* 2006. 47: 2223–2232.

Supplementary key words steatosis • lipids • fatty acid catabolism • very low density lipoprotein • peroxisome proliferator-activated receptor

The anti-estrogen agent tamoxifen has been used as an effective treatment for estrogen receptor-positive breast cancers for several years (1). However, tamoxifen treatment is associated with an increased risk of developing fatty liver (steatosis) (2–4), and it is reported that as many as 43% of women with breast cancer treated with tamoxi-

fen may develop steatosis within the first 2 years of treatment (4). It has been hypothesized that impaired fatty acid β -oxidation could be the cause of fatty liver after tamoxifen treatment (5, 6).

The modified fatty acid tetradecylthioacetic acid (TTA) is a ligand for all peroxisome proliferator-activated receptors (PPARs) (7) and has shown promise in the prevention and treatment of common lipid disorders and insulin resistance (8). TTA increases both mitochondrial and peroxisomal β -oxidation in liver (9) and therefore may be a valuable tool for the treatment of fatty liver.

In this study, female rats with 7,12-dimethylbenz[a]anthracene-induced mammary tumors were treated with tamoxifen, TTA, or a combination of tamoxifen and TTA (Tam+TTA) for 14 days after palpable tumors developed. We had two aims in this study: first, we wanted to elucidate the mechanism behind the accumulation of triacylglycerol in liver after tamoxifen treatment in this rat model; second, we wanted to investigate whether cotreatment with TTA could abolish this undesirable side effect of tamoxifen. To clarify the mechanisms involved, we measured the activities and gene expression of enzymes controlling the hepatic biosynthesis, β -oxidation, secretion, and uptake of lipids and quantified several products of these pathways.

MATERIALS AND METHODS

Animals and diets

Thirty-two female Sprague-Dawley rats at the age of 3 weeks were treated with a single dose of 20 mg of 7,12-dimethylbenz[a]anthracene (D-3254; Sigma-Aldrich Norway AS, Oslo, Nor-

Abbreviations: AADA, arylacetamide deacetylase; ACC, acetyl-coenzyme A carboxylase; ACO, acyl-coenzyme A oxidase; CPT, carnitine palmitoyltransferase; DGAT, diacylglycerol acyltransferase; FAT/CD36, fatty acid translocase; GPAT, glycerol-3-phosphate acyltransferase; L-FABP, liver fatty acid binding protein; PPAR, peroxisome proliferator-activated receptor; SCD-1, stearoyl-coenzyme A desaturase-1; Tam+TTA, combination treatment with tamoxifen and tetradecylthioacetic acid; TTA, tetradecylthioacetic acid.

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way) from Taconic M&B A/S (Ry, Denmark). The rats were divided into four experimental groups of eight rats, each with comparable mean body weight (230–280 g). The rats were housed in a room maintained at a 12 h light/dark cycle and constant temperature of $20 \pm 3^\circ\text{C}$ with free access to tap water and pellet feed (Rat and Mouse Standard Diet No. 2; B&K Universal, Nittedal, Norway).

(Z)-1-(*p*-Dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene (tamoxifen, T-9262; Sigma-Aldrich Norway AS) and TTA [synthesized as described (10)] were added to commercial peanut oil and administered by orogastric intubation daily for 14 days at doses of 40 mg of tamoxifen, 300 mg of TTA, or a combination of 40 mg of tamoxifen and 300 mg of TTA per kilogram body weight. The control rats received corresponding amounts of peanut oil (2.8 ml/kg body weight). The treatment started when the rats were 10 weeks old and had palpable tumors. One rat in the control group was euthanized after 3 days of treatment because of severe illness, and one rat treated with Tam+TTA choked on the feeding tube. Both rats were excluded from the experiment. Otherwise, no mortality was observed. The percentage change in body weight during the experiment was comparable in all experimental groups ($+2.6 \pm 3.1$ for controls, -3.9 ± 4.1 for tamoxifen, $+1.7 \pm 2.7$ for Tam+TTA, and -0.2 ± 3.3 for TTA).

The rats were anesthetized with 2–5% isoflurane (Forene; Abbott Scandinavia AB, Solne, Sweden) mixed with nitric oxide and oxygen under nonfasting conditions. Blood was drawn from the heart and collected in BD Vacutainer tubes containing heparin or no additive (Becton Dickinson and Co., Plymouth, UK), and the liver was removed immediately. The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Quantification of lipids, transaminases, and fatty acids

Lipids and serum transaminases were measured on the Hitachi 917 system (Roche Diagnostics GmbH, Mannheim, Germany) using the following kits: triglyceride (Bayer, Tarrytown, NY), total cholesterol (Bayer), free cholesterol (Wako Chemicals, Dalton, GA), choline-containing phospholipids (PAP150; BioMerieux, Lyon, France), nonesterified fatty acids (Wako Chemicals), alanine transaminase (Roche), and aspartate transaminase (Roche). The amount of cholesteryl esters in plasma was calculated as the difference between total cholesterol and free cholesterol. Liver lipids were extracted (11), evaporated under nitrogen, and redissolved in isopropanol before analysis. The fatty acid composition in liver was analyzed as described previously (12, 13).

Enzyme activities

The livers were homogenized and fractionated (14), and the palmitoyl-CoA oxidation (measured as acid-soluble products) (15) and the activities of ACAT (16), acetyl-coenzyme A carboxylase (ACC) (17), fatty acyl-coenzyme A oxidase (ACO) (18), carnitine palmitoyltransferase (CPT)-I and CPT-II (19), diacylglycerol acyltransferase (DGAT) (20), FAS (21, 22), and glycerol-3-phosphate acyltransferase (GPAT) (23) were measured in the postnuclear fraction. HMG-CoA synthase activity (24) was measured in the mitochondrial fraction.

Ketone bodies, Krebs cycle intermediates, and short CoAs

Ketone bodies and Krebs cycle intermediates were measured in plasma (25). Short CoAs were measured in liver (12).

Real-time quantitative RT-PCR

Total RNA was purified from frozen liver using the RNeasy Midi Kit (Qiagen GmbH, Germany). Primers and Taqman probes

for rat $\Delta 5$ desaturase, $\Delta 6$ desaturase, and glyceraldehyde-3-phosphate dehydrogenase were designed using Primer Express (Applied Biosystems, Foster City, CA), and gene expression was determined using Taqman probes. The following sequences were used: $\Delta 5$ desaturase forward (5'-TGG ATC TTT GGA ACT TCC TTG GT-3'), reverse (5'-CAA AGT CAT GCT GTA GCC AAC CT-3'), and probe (5'-CAG TTC AGG CCC AGG C-3'); $\Delta 6$ desaturase forward (5'-CAG CGG GCA CCT CAA TTT-3'), reverse (5'-TGC TTG GCG CAG AGA GAC T-3'), and probe (5'-CAG ATT GAG CAC CAC CTC TTC CCC AC-3'); And glyceraldehyde-3-phosphate dehydrogenase forward (5'-TGC ACC ACC AAC TGC TTA GC-3') reverse (5'-CAG TCT TCT GAG TGG CAG TGA TG-3') and probe (5'-TGG AAG GGC TCA TGA CCA CAG TCC A-3').

Arylacetamide deacetylase (AADA; Rn00571934_m1), ACO (subtype Acox-1; Rn00569216_m1), apolipoprotein B (Rn01499049_g1), CPT-I (subtype CPT-Ia; Rn00580702_m1), CPT-II (Rn00563995_m1), DGAT (subtype DGAT-1; Rn00584870_m1), fatty acid translocase (FAT/CD36; Rn00580728_m1), LDL receptor (Rn00598438_m1), liver fatty acid binding protein (L-FABP; subtype FABP-1; Rn00664587_m1), mitochondrial HMG-CoA synthase (subtype Hmgcs2; Rn00597339_m1), PPAR α (Rn00566193_m1), PPAR δ (Rn00565707_m1), PPAR γ (Rn00440945_m1), stearoyl-coenzyme A desaturase-1 (SCD-1; Rn00594894_g1), and VLDL receptor (Rn00565784_m1) are "Assay-on-Demand" designed by Applied Biosystems. FAS (4327124F) was purchased from Applied Biosystems. Real-time RT-PCR was carried out in triplicate on an ABI 7900 sequence detection system (Applied Biosystems). Results were normalized to 18S rRNA (RT-CKFT-18S from MedProbe, Oslo, Norway) and glyceraldehyde-3-phosphate dehydrogenase, but only results normalized to 18S are shown because both reference genes gave similar results.

Statistical analysis

The results are presented as mean values \pm SD for seven (control group and the combination treatment group) or eight (TTA and tamoxifen groups) rats per group. The data were evaluated by one-way ANOVA and Tukey's test with the level of statistical significance set at $P < 0.05$ (GraphPad Prism version 3.0; GraphPad, San Diego, CA).

RESULTS

Cotreatment with TTA reduced hepatic triacylglycerol and serum alanine transaminase levels in tamoxifen-treated rats

It is well known that tamoxifen treatment is associated with an increased risk of developing nonalcoholic fatty liver (steatosis) (2–4). In this work, tamoxifen treatment increased the hepatic triacylglycerol level by $>50\%$ (Fig. 1A) but did not change the hepatic amounts of cholesterol (Fig. 1B) or choline-containing phospholipids (Fig. 1C). Tam+TTA decreased the hepatic levels of triacylglycerol (Fig. 1A) and cholesterol (Fig. 1B) and increased the phospholipid level (Fig. 1C) compared with tamoxifen treatment. Serum transaminases are regarded as reliable markers of hepatic steatosis and liver damage, and indeed, tamoxifen treatment increased the serum levels of alanine transaminase (Fig. 2A) and aspartate transaminase (Fig. 2B). Tam+TTA reduced serum alanine transaminase level (Fig. 2A), whereas the aspartate trans-

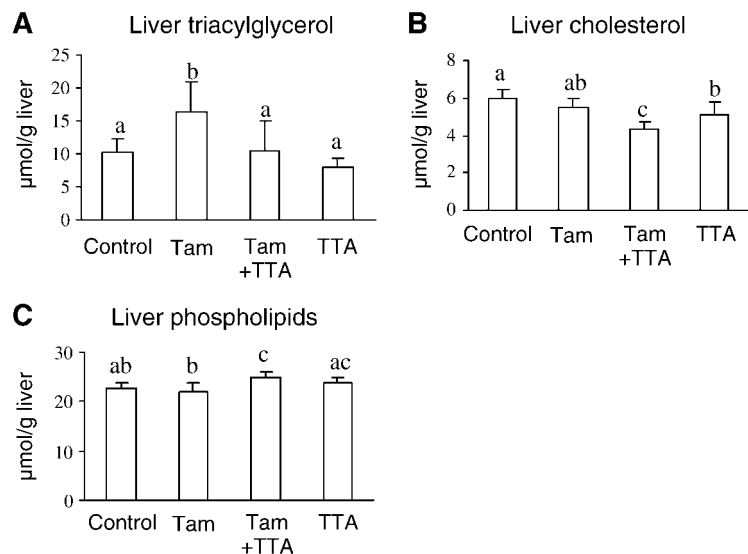


Fig. 1. Hepatic levels of triacylglycerol (A), cholesterol (B), and phospholipids (C) in control rats and rats treated with tamoxifen (Tam), tamoxifen and tetradecylthioacetic acid (Tam+TTA), or tetradecylthioacetic acid (TTA). Values are means \pm SD. Bars without a common letter are significantly different ($P < 0.05$).

aminase level was similar to that in the tamoxifen group (Fig. 2B).

Cotreatment with TTA increased hepatic β -oxidation and ketogenesis in tamoxifen-treated rats

Tamoxifen treatment did not affect the palmitoyl-CoA β -oxidation measured as acid-soluble products in the liver (Fig. 3A). Also, no change was seen in the activities of CPT-I (Fig. 3B) and CPT-II (Fig. 3C) or in the mRNA levels of CPT-I and CPT-II (Table 1). In addition, tamoxifen did not change the plasma levels of the Krebs cycle intermediates pyruvate, citrate, malate, and α -ketoglutarate (Table 2) or the hepatic levels of the β -oxidation degradation products acetyl-CoA (Fig. 4A) and propionyl-CoA (Fig. 4B). Parallel with this, the activity and mRNA level of mitochondrial HMG-CoA synthase in liver (Fig. 3D, Table 1) and the plasma levels of the ketone bodies acetoacetate and β -hydroxybutyrate (Table 2) were not changed; thus, the ketogenesis appeared not to be influenced by tamoxifen treatment. The peroxisomal β -oxidation, measured as the activity of ACO (Fig. 3E), and the mRNA levels of ACO, PPAR α , and PPAR δ (Table 1) were not affected by tamoxifen treatment.

The β -oxidation of palmitoyl-CoA was markedly increased in liver from rats treated with Tam+TTA compared with rats treated with tamoxifen alone (Fig. 3A). This was accompanied by increased activities of CPT-II (Fig. 3C), HMG-CoA synthase (Fig. 3D), and ACO (Fig. 3E) and in-

creased mRNA levels of CPT-I, CPT-II, HMG-CoA synthase, and ACO (Table 1), whereas the CPT-I activity (Fig. 3B) was decreased compared with tamoxifen-treated rats. The plasma levels of pyruvate, citrate, malate, and acetoacetate (Table 2) and the hepatic levels of acetyl-CoA (Fig. 4A) and propionyl-CoA (Fig. 4B) were increased in Tam+TTA rats compared with tamoxifen-treated rats, but no changes were seen in the mRNA levels of the PPARs (Table 1).

Cotreatment with TTA increased hepatic lipogenesis and triacylglycerol biosynthesis in tamoxifen-treated rats

Tamoxifen treatment decreased the ACC activity (Fig. 5A), the FAS activity (Fig. 5B), and the FAS mRNA level (Table 1), indicating a reduced biosynthesis of fatty acids in these rats, but it did not affect the hepatic level of malonyl-CoA (Fig. 4C). The increased PPAR γ mRNA level (Table 1) and GPAT activity (Fig. 5C) indicated increased esterification of fatty acids to glycerolipids. The biosynthesis of triacylglycerol from diacylglycerol may have been increased, as the activity of DGAT tended to increase (Fig. 5D) ($P = 0.06$, tamoxifen vs. control); however, the mRNA level of DGAT was unchanged by tamoxifen treatment (Table 1). The decreased ACAT activity (Fig. 5E) after tamoxifen treatment suggested a reduced capacity for esterification of cholesterol in these rats.

When rats were treated with Tam+TTA, the activities of ACC (Fig. 5A), FAS (Fig. 5B), GPAT (Fig. 5C), and DGAT

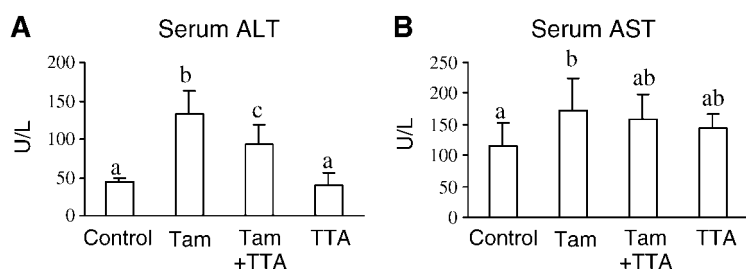


Fig. 2. Serum levels of alanine transaminase (ALT; A) and aspartate transaminase (AST; B) in control rats and rats treated with tamoxifen (Tam), Tam+TTA, or TTA. Values are means \pm SD. Bars without a common letter are significantly different ($P < 0.05$).

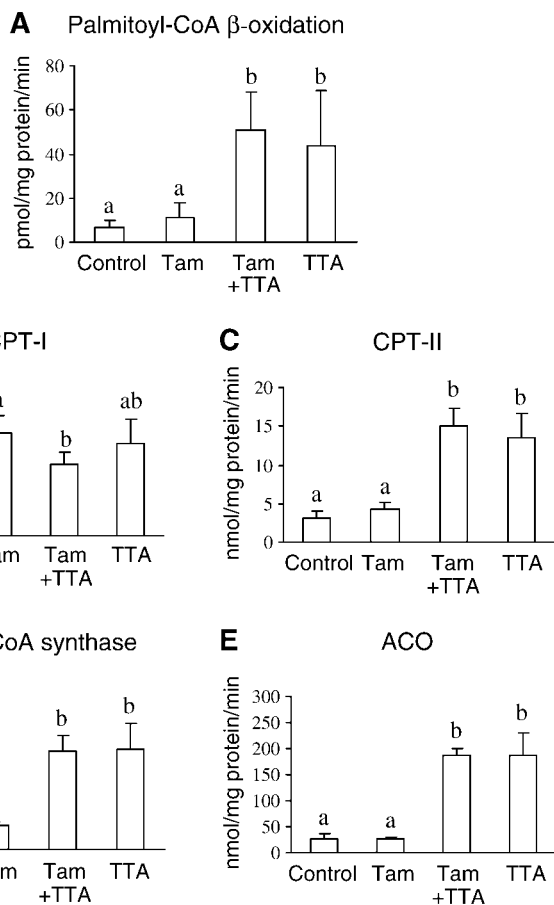


Fig. 3. Palmitoyl-CoA oxidation (measured as acid-soluble products) (A) and the activities of carnitine palmitoyltransferase (CPT)-I (B), CPT-II (C), HMG-CoA synthase (D), and acyl-coenzyme A oxidase (ACO) (E) in liver from control rats and rats treated with tamoxifen (Tam), Tam+TTA, or TTA. Values are means \pm SD. Bars without a common letter are significantly different ($P < 0.05$).

(Fig. 5D) and the mRNA levels of FAS and DGAT were increased compared with the tamoxifen group, whereas the malonyl-CoA level (Fig. 4C) was unchanged. The esterification of cholesterol seemed unaffected, as the ACAT activity was similar in the tamoxifen and Tam+TTA groups (Fig. 5C).

Cotreatment with TTA increased the hepatic uptake of fatty acids and VLDL and increased the serum triacylglycerol level in tamoxifen-treated rats

Tamoxifen did not change the mRNA levels of FAT/CD36, LDL receptor, or VLDL receptor but markedly decreased the mRNA level of L-FABP (Table 1), indicating

TABLE 1. Hepatic mRNA levels in control rats and rats treated with tamoxifen, Tam+TTA, or TTA

Variable	Control	Tamoxifen	Tam+TTA	TTA
Arylacetamide deacetylase	1.00 \pm 0.30 a,b	1.16 \pm 0.20 a	1.08 \pm 0.08 a,b	0.82 \pm 0.23 b
Acyl-CoA oxidase	1.00 \pm 0.16 a	1.35 \pm 0.25 a	7.22 \pm 1.37 b	6.58 \pm 3.04 b
Apolipoprotein B	1.00 \pm 0.22 a	0.95 \pm 0.10 a	1.22 \pm 0.10 a	1.00 \pm 0.26 a
Carnitine palmitoyltransferase-I	1.00 \pm 0.41 a	0.82 \pm 0.38 a	1.81 \pm 0.37 b	2.05 \pm 0.56 b
Carnitine palmitoyltransferase-II	1.00 \pm 0.42 a	1.39 \pm 0.27 a	5.93 \pm 0.94 b	3.40 \pm 0.95 c
Δ 5 desaturase	1.00 \pm 0.17 a	0.57 \pm 0.14 a	2.38 \pm 0.84 b	1.98 \pm 0.44 b
Δ 6 desaturase	1.00 \pm 0.16 a	0.69 \pm 0.25 a	4.41 \pm 1.06 b	3.30 \pm 0.56 c
Diacylglycerol acyltransferase-1	1.00 \pm 0.13 a	1.07 \pm 0.09 a,b	1.69 \pm 0.43 c	1.41 \pm 0.45 b,c
FAS	1.00 \pm 0.20 a	0.35 \pm 0.12 b	1.06 \pm 0.49 a	0.85 \pm 0.35 a
Fatty acid translocase	1.00 \pm 0.40 a	0.75 \pm 0.13 a	1.66 \pm 0.33 b	1.68 \pm 0.19 b
HMG-CoA synthase (mitochondrial)	1.00 \pm 0.16 a	1.25 \pm 0.18 a	2.27 \pm 0.28 b	2.35 \pm 0.69 b
Liver fatty acid binding protein	1.00 \pm 0.22 a	0.47 \pm 0.07 b	2.02 \pm 0.34 c	2.71 \pm 0.33 d
LDL receptor	1.00 \pm 0.27 a,b	1.57 \pm 0.60 a	1.52 \pm 0.43 a,b	0.92 \pm 0.32 b
PPAR α	1.00 \pm 0.27 a	2.11 \pm 0.97 a,b	3.12 \pm 1.46 b	1.93 \pm 1.18 a,b
PPAR δ	1.00 \pm 0.48 a	0.60 \pm 0.33 a,b	0.46 \pm 0.24 b	0.69 \pm 0.33 a,b
PPAR γ	1.00 \pm 0.25 a	2.71 \pm 0.26 b	2.49 \pm 0.37 b	1.01 \pm 0.17 a
Stearoyl-coenzyme A desaturase-1	1.00 \pm 0.55 a	0.21 \pm 0.11 a	3.89 \pm 1.39 b	1.82 \pm 0.85 a
VLDL receptor	1.00 \pm 0.21 a	1.14 \pm 0.58 a	3.11 \pm 1.01 b,c	2.23 \pm 1.26 a,c

PPAR, peroxisome proliferator-activated receptor; Tam+TTA, combination treatment with tamoxifen and tetradecylthioacetic acid; TTA, tetradecylthioacetic acid. The mRNA levels are shown relative to 18S rRNA and normalized to controls. Means in a row without a common letter are significantly different ($P < 0.05$).

TABLE 2. Krebs cycle intermediates and ketone bodies in plasma of control rats and rats treated with tamoxifen, Tam+TTA, or TTA

Variable	Control	Tamoxifen	Tam+TTA	TTA
Pyruvate	347 ± 73 a	264 ± 26 a	549 ± 102 b	578 ± 142 b
Citrate	220 ± 38 a,b	199 ± 27 a	272 ± 56 b,c	329 ± 67 c
Malate	22 ± 9 a	24 ± 5 a	49 ± 8 b	54 ± 11 b
α-Ketoglutarate	30 ± 9 a,b	37 ± 4 a	32 ± 4 a,b	29 ± 2 b
Acetoacetate	90 ± 19 a	122 ± 28 a	189 ± 28 b	177 ± 41 b
β-Hydroxybutyrate	240 ± 51 a	335 ± 59 a	339 ± 94 a	356 ± 108 a

Means in a row without a common letter are significantly different ($P < 0.05$).

that the accumulation of triacylglycerol in the liver could not be explained by an increased hepatic uptake of lipids from the circulation. The gene expression of AADA and apolipoprotein B, which play important roles for the assembly of VLDL (26), was not changed in liver after tamoxifen treatment (Table 1). The serum levels of triacylglycerol (Fig. 6A), nonesterified fatty acids (Fig. 6B), total cholesterol (Fig. 6C), cholesteryl esters (Fig. 6D), and phospholipids (Fig. 6E) were decreased after tamoxifen treatment.

Tam+TTA treatment increased the mRNA levels of FAT/CD36, L-FABP, and VLDL receptor compared with tamoxifen treatment but had no significant effect on the gene expression of the LDL receptor, AADA, or apolipoprotein B (Table 1). The combination treatment increased the serum triacylglycerol level (Fig. 6A) but did not change the amounts of the other serum lipids compared with the tamoxifen group (Fig. 6B–E).

Cotreatment with TTA increased the gene expression of desaturases and changed the fatty acid composition in liver from tamoxifen-treated rats

Because administration of tamoxifen and Tam+TTA had an impact on the biosynthesis of saturated fatty acids by reducing the activities of ACC (Fig. 5A) and FAS (Fig. 5B), it was of interest to determine whether these

drug treatments also could affect the capacity for insertion of double bonds and consequently the fatty acid composition of the liver. SCD-1 is the rate-limiting enzyme in the synthesis of $\Delta 9$ desaturated fatty acids from saturated fatty acids, whereas the $\Delta 6$ and $\Delta 5$ desaturases are involved in the biosynthesis of long-chain n-3 and n-6 polyunsaturated fatty acids. Tamoxifen did not change the mRNA levels of SCD-1, $\Delta 6$ desaturase, or $\Delta 5$ desaturase (Table 1) or the 18:1n-9/18:0 ratio (Table 3), but it decreased the 20:4n-6/18:2n-6 and 20:5n-3/18:3n-3 ratios (Table 3). In contrast, Tam+TTA treatment increased the mRNA levels of SCD-1, $\Delta 6$ desaturase, and $\Delta 5$ desaturase compared with tamoxifen treatment (Table 1). No change was seen in the 18:1n-9/18:0 ratio, but the 20:4n-6/18:2n-6 and 20:5n-3/18:3n-3 ratios were increased in Tam+TTA rats compared with the tamoxifen group (Table 3).

TTA treatment did not affect hepatic triacylglycerol level but increased hepatic β -oxidation, lipogenesis, and triacylglycerol biosynthesis

The effects of treating rats with TTA in the absence of tamoxifen were in most cases similar to those seen after Tam+TTA treatment, but certain differences were observed. Compared with the Tam+TTA group, TTA treatment had less pronounced effects on the mRNA levels of CPT-II, $\Delta 6$ desaturase, PPAR γ , and SCD-1 (Table 1) but

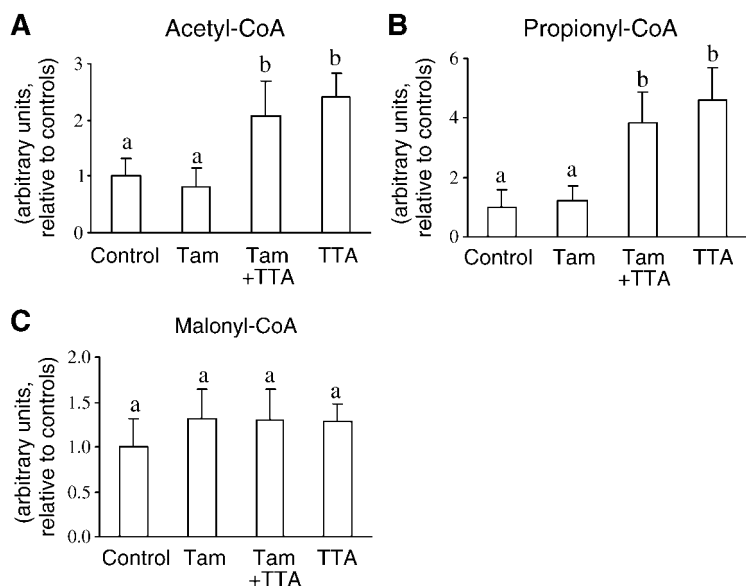


Fig. 4. Hepatic levels of acetyl-CoA (A), propionyl-CoA (B), and malonyl-CoA (C) in control rats and rats treated with tamoxifen (Tam), Tam+TTA, or TTA. Values are means \pm SD. Bars without a common letter are significantly different ($P < 0.05$).

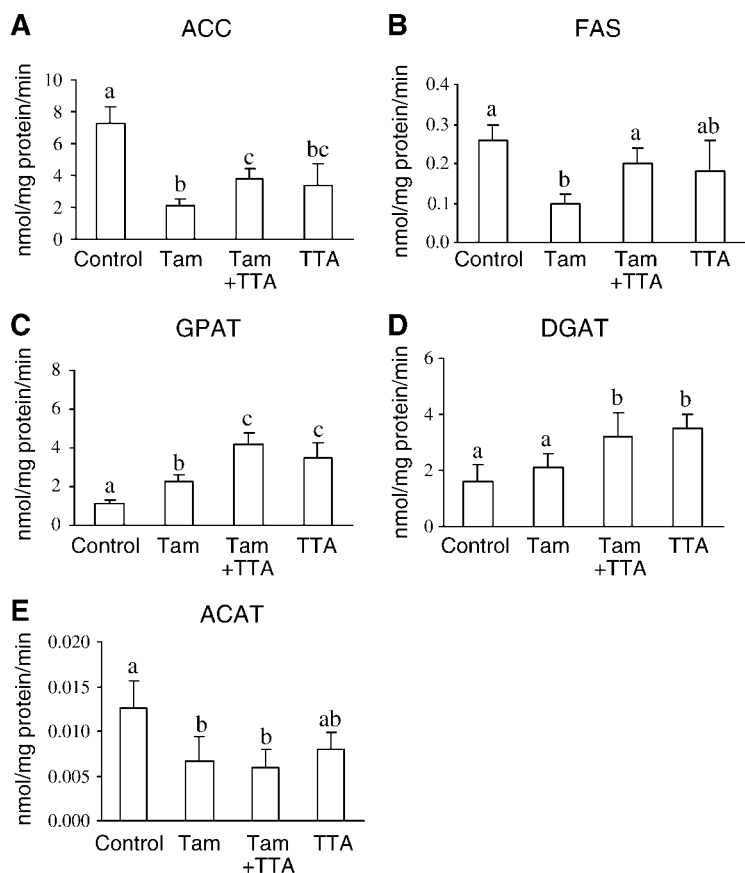


Fig. 5. Hepatic activities of acetyl-coenzyme A carboxylase (ACC; A), FAS (B), glycerol-3-phosphate acyltransferase (GPAT; C), diacylglycerol acyltransferase (DGAT; D), and ACAT (E) in control rats and rats treated with tamoxifen (Tam), Tam+TTA, or TTA. Values are means \pm SD. Bars without a common letter are significantly different ($P < 0.05$).

had a stronger impact on the mRNA level of L-FABP and the ratio of 20:4n-6 to 18:2n-6 (Table 3) compared with controls. The cholesterol levels in liver (Fig. 1B) and serum (Fig. 6C) and the serum levels of cholesteryl ester (Fig. 6D), nonesterified fatty acids (Fig. 6B), and phospholipids (Fig. 6D) were higher in TTA-treated rats compared with rats treated with Tam+TTA. However, the activities of enzymes involved in β -oxidation, lipogenesis, and lipid biosynthesis (Figs. 3, 5) and the liver and serum levels of triacylglycerol (Figs. 1A, 6A) were similar when rats were treated with TTA alone or in combination with tamoxifen. In addition, TTA treatment alone did not affect the serum alanine transaminase level (Fig. 2A).

DISCUSSION

For >25 years, tamoxifen has been the gold standard for endocrine treatment of all stages of estrogen receptor-positive breast cancer. However, tamoxifen treatment is associated with an increased risk of the development of nonalcoholic fatty liver (steatosis) (2–4), and it is reported that as many as 43% of women with breast cancer treated with tamoxifen may develop steatosis within the first 2 years of treatment (4). In this study, we had two aims: first, we wanted to elucidate the mechanisms behind the accumulation of triacylglycerol in liver of female rats

treated with tamoxifen; second, we wanted to investigate whether cotreatment with the modified fatty acid TTA could abolish this undesirable side effect of tamoxifen.

The increased hepatic triacylglycerol level after tamoxifen treatment was accompanied by increased serum levels of transaminases, indicating hepatocellular injury, and is in agreement with findings in breast cancer patients who developed fatty liver after tamoxifen therapy (2, 4). Interestingly, combination treatment with tamoxifen and TTA normalized the hepatic triacylglycerol level and partially restored the level of serum alanine transaminase, which is a specific marker of liver damage, indicating that cotreatment with TTA may have potential in the prevention of liver damage induced by tamoxifen.

The increased triacylglycerol content in liver in tamoxifen-treated rats may be caused by increased biosynthesis, reduced β -oxidation, increased uptake, or reduced secretion of lipids, or by a combination of these factors. Because the activities of ACC and FAS were reduced by tamoxifen, possibly as a compensatory response to the increased level of triacylglycerol in the liver, the increased hepatic triacylglycerol level is probably not attributable to increased lipogenesis, as also has been demonstrated by others (6). However, the amount of malonyl-CoA, the important inhibitor of CPT-I, was not changed, and no change was seen in the capacity for fatty acid β -oxidation or in the activities and mRNA levels of enzymes involved in mitochondrial

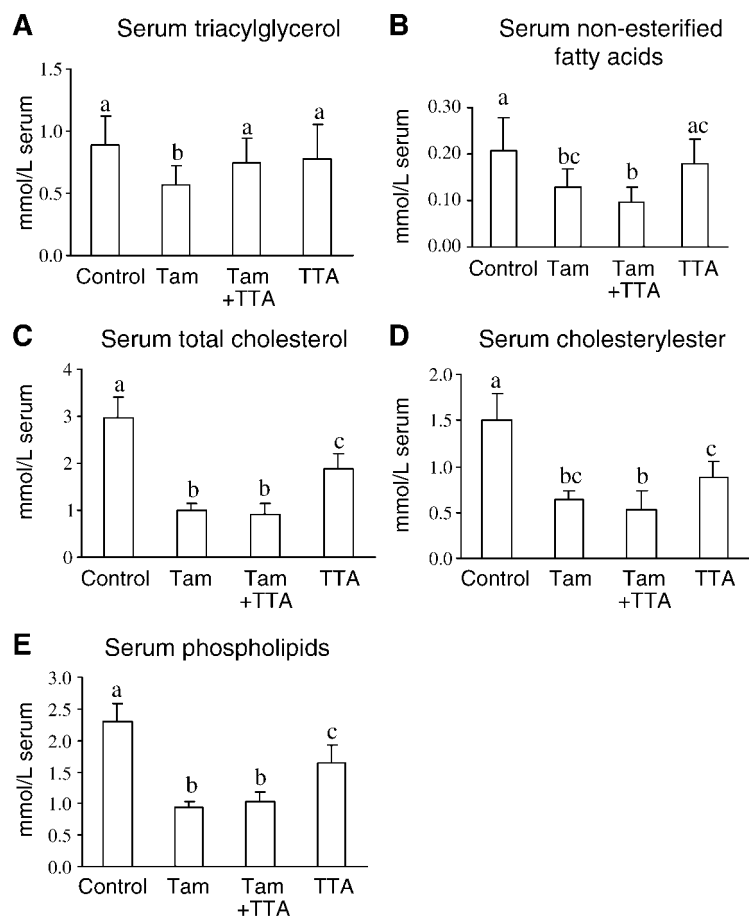


Fig. 6. Serum levels of triacylglycerol (A), nonesterified fatty acids (B), total cholesterol (C), cholesteryl esters (D), and phospholipids (E) in control rats and rats treated with tamoxifen (Tam), Tam+TTA, or TTA. Values are means \pm SD. Bars without a common letter are significantly different ($P < 0.05$).

and peroxisomal β -oxidation or ketogenesis, or in the levels of degradation products and metabolites thereof, in tamoxifen-treated rats. This strongly indicates that the accumulation of triacylglycerol in liver observed after tamoxifen treatment could not be ascribed to a reduced oxidation of fatty acids.

Tamoxifen treatment seemed to increase the synthesis of glycerolipids, as PPAR γ gene expression and GPAT activity were increased. In accordance with others (27), we found that tamoxifen reduced the activity of ACAT, suggesting that the fatty acids were directed toward the synthesis of triacylglycerol and phospholipids instead of cholesteryl esters. In view of the fact that no change was seen in the hepatic phospholipid level but the triacylglycerol level in liver was increased, together with the strong tendency to increased DGAT activity ($P = 0.06$),

it appeared that triacylglycerol synthesis may be favored over the synthesis of phospholipids after tamoxifen treatment.

The increased hepatic level of triacylglycerol after tamoxifen treatment was accompanied by decreased levels of triacylglycerol, cholesteryl esters, and phospholipids in serum; therefore, we suspected that the hepatic secretion and/or uptake of fatty acids and lipids could be affected. The secretion of VLDL from the liver was not measured directly, but the unchanged mRNA levels of AADA and apolipoprotein B indicated that tamoxifen did not affect VLDL secretion, resulting in an accumulation of newly synthesized triacylglycerol in the liver. However, increased secretion of VLDL has been observed by others without any change in AADA mRNA content (26), and only direct measurements of VLDL secretion can answer

TABLE 3. Fatty acid ratios in liver of control rats and rats treated with tamoxifen, Tam+TTA, or TTA

Variable	Control	Tamoxifen	Tam+TTA	TTA
18:1n-9/18:0	0.65 \pm 0.13 a	1.01 \pm 0.31 a	1.12 \pm 0.29 a	0.99 \pm 0.33 a
20:4n-6/18:2n-6	1.77 \pm 0.37 a	0.86 \pm 0.17 b	1.92 \pm 0.21 a	2.58 \pm 0.52 c
20:5n-3/18:3n-3	0.78 \pm 0.24 a	0.28 \pm 0.09 b	2.32 \pm 0.95 c	2.14 \pm 1.05 c

Means in a row without a common letter are significantly different ($P < 0.05$).

whether tamoxifen affects the secretion of triacylglycerol-rich lipoproteins.

The mRNA levels of genes involved in the uptake of lipoproteins from the circulation, such as LDL receptor and VLDL receptor, were not affected by tamoxifen treatment, suggesting that the uptake of triacylglycerol-containing lipoproteins was not changed. On the other hand, the unchanged mRNA level of FAT/CD36 and the decreased gene expression of L-FABP implied that the uptake of nonesterified fatty acids from the circulation was decreased. Thus, the increased triacylglycerol content in liver of tamoxifen-treated rats was probably not caused by increased uptake of lipids and fatty acids.

The described changes in fatty acid metabolism after tamoxifen treatment were expected to influence the fatty acid composition in liver. The mRNA level of SCD-1 is reported to be a determinant of the biosynthesis of triacylglycerol and cholesteryl esters, because 18:1n-9 is the preferred substrate for esterification (28). However, although ACAT activity was decreased, GPAT activity was increased, and DGAT activity tended to increase ($P = 0.06$) after tamoxifen treatment, no change was seen in the mRNA level of SCD-1. Tamoxifen did not fully induce fatty liver in this experiment, but we found that tamoxifen treatment decreased the 20:5n-3/18:3n-3 and 20:4n-6/18:2n-6 ratios, as has been reported in severe fatty liver induced by ethanol, obesity, or the hyperlipidemic drug tridecylthiopropanoic acid (13, 29, 30). These changes in fatty acids ratios suggested that the activities of the desaturases involved in the biosynthesis of long-chain polyunsaturated fatty acids were decreased, although no changes were seen in the gene expressions of $\Delta 6$ and $\Delta 5$ desaturases.

Combination treatment with TTA normalized the hepatic triacylglycerol level compared with tamoxifen treatment alone, and the β -oxidation of palmitoyl-CoA was increased parallel with this. It is generally accepted that β -oxidation in liver is regulated through CPT-I, but in this experiment we found that the increased palmitoyl-CoA β -oxidation after treatment with Tam+TTA was accompanied by a decreased activity of CPT-I and an unchanged hepatic level of malonyl-CoA, combined with increased activities of CPT-II and HMG-CoA synthase. This suggested that the β -oxidation could be regulated beyond CPT-I, as we showed previously (31), and that CPT-II and HMG-CoA synthase are potential control sites for mitochondrial fatty acid β -oxidation and ketogenesis in liver of rats treated with Tam+TTA. Although the mRNA levels of PPAR α and PPAR δ were similar in rats treated with tamoxifen alone and in combination with TTA, the expression of the PPAR α target genes CPT-I, CPT-II, and ACO was markedly increased in the latter group, further supporting an increased β -oxidation of fatty acid. The clearance of the mitochondrial metabolites pyruvate, citrate, and malate was not measured, but the increased plasma levels of these metabolites and the increased hepatic levels of the β -oxidation degradation products acetyl-CoA and propionyl-CoA, together with the increased β -oxidation, suggested an increased combustion


of fatty acids. Thus, cotreatment with TTA may prevent the tamoxifen-induced accumulation of triacylglycerol in liver through increased β -oxidation.

The biosynthesis of fatty acids, triacylglycerol, and phospholipids seemed to be increased after cotreatment with TTA, as the activities of ACC, FAS, GPAT, and DGAT were increased compared with those in tamoxifen-treated rats, although the mRNA level of PPAR γ was similar in the two groups. As the hepatic level of triacylglycerol was decreased and that of phospholipids was increased, it seems that the fatty acids were directed toward esterification as phospholipids rather than as triacylglycerol after cotreatment with TTA. The ACAT activity after Tam+TTA treatment was not significantly different from that in tamoxifen-treated rats, indicating that the biosynthesis of cholesteryl esters was similar in these groups.

The increased β -oxidation reduced, whereas the increased triacylglycerol synthesis increased, the availability of triacylglycerol for VLDL synthesis and secretion after cotreatment with TTA, and the comparable mRNA levels of AADA and apolipoprotein B in rats treated with tamoxifen or Tam+TTA suggested that the secretion of VLDL was similar in these groups. The gene expression of the LDL receptor was also similar in the two treatment groups, in parallel with equal serum cholesterol and cholesteryl ester levels. Tam+TTA increased the gene expression of FAT/CD36, L-FABP, and VLDL receptor compared with tamoxifen treatment, suggesting that the uptake of nonesterified fatty acids and VLDL was increased. However, the serum levels of nonesterified fatty acids and phospholipids were similar in these groups, whereas serum triacylglycerol was significantly higher after Tam+TTA compared with tamoxifen treatment. These findings indicate that the liver triacylglycerol-lowering effect of Tam+TTA treatment did not abolish the tamoxifen-induced accumulation of triacylglycerol through the mechanisms of reduced biosynthesis of fatty acids and triacylglycerol, increased secretion of VLDL, or reduced uptake of fatty acids and lipoproteins.

Peroxisome proliferators are reported to increase the activities of SCD-1 and $\Delta 6$ and $\Delta 5$ desaturases (32, 33) and thereby increase the biosynthesis of long-chain unsaturated fatty acids, including 18:1n-9, 20:4n-6, and 20:5n-3. TTA is a peroxisome proliferator (8), and in this experiment we show that Tam+TTA increased the mRNA levels of SCD-1 and $\Delta 6$ and $\Delta 5$ desaturases compared with tamoxifen treatment. This was accompanied by increased 20:4n-6/18:2n-6 and 20:5n-3/18:3n-3 ratios in the Tam+TTA-treated rats, whereas no change was seen in the 18:1n-9/18:0 ratio. It has been speculated that the reason for the increased formation of unsaturated fatty acid by peroxisome proliferators is to supply phospholipids with fatty acids with proper chain length and desaturation for the building of membranes of the rapidly proliferating peroxisomes (33). This is in agreement with the increased GPAT activity and the increased hepatic phospholipid level observed after Tam+TTA treatment, which suggest that the biosynthesis of not only triacylglycerol but also phospholipids could be increased.

Treatment of rats with TTA alone showed many of the same effects as Tam+TTA compared with controls. Surprisingly, whereas Tam+TTA treatment reduced the hepatic triacylglycerol level and increased β -oxidation and lipid biosynthesis compared with tamoxifen-only treatment, no decrease of hepatic triacylglycerol level was observed after TTA treatment compared with controls, although the β -oxidation and the biosynthesis of lipids were increased. We recently presented similar results in a study of male Wistar rats, in which TTA treatment alone did not affect the hepatic triacylglycerol level but significantly improved the fatty liver induced by tridecylthiopropionic acid (34). From this, it seemed that TTA may have the capacity to normalize rather than to reduce the hepatic triacylglycerol content under certain circumstances, but the mechanism behind this remains to be solved.

In conclusion, the tamoxifen-induced accumulation of triacylglycerol in liver of rats in this study is possibly a consequence of increased triacylglycerol biosynthesis combined with unchanged β -oxidation, VLDL secretion, and uptake of lipids to the liver. We also present evidence that tamoxifen-induced triacylglycerol accumulation could be prevented by combination treatment with TTA, through mechanisms of increased mitochondrial and peroxisomal β -oxidation. 

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