Modulation of rat liver apolipoprotein gene expression and serum lipid levels by tetradecylthioacetic acid (TTA) via PPARα activation

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Abstract 3-Thia fatty acids are modified fatty acids that promote hepatic peroxisome proliferation and decrease serum triacylglycerol, cholesterol and free fatty acid levels in rats. In vivo administration of tetradecylthioacetic acid (TTA) to rats led to a significant decrease in liver apolipoproteins apoA-I, A-II, A-IV, and C-III mRNA levels, and to an increase of liver acyl-CoA oxidase (ACO), carnitine palmitoyltransferase-II, and 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase) mRNA levels and activities. By contrast, no significant changes of lipoprotein lipase (LPL) mRNA levels were detected in rat epididymal adipose tissue. Liver carnitine palmitoyltransferase-I, apoB, apoE, and LDL receptor mRNA levels were not significantly affected. When tested in vitro, TTA increased rat ACO and carnitine palmitoyltransferase-I mRNA levels in primary rat hepatocytes and also LPL mRNA levels in 3T3-L1 preadipocytes. TTA also enhanced the transcriptional activity of chimeras containing the DNA binding domain of the yeast transcription factor Gal4 fused to the ligand binding domain of either human PPARα or human PPARγ. The effect depended on the concentration tested and the cell type. In conclusion, our data suggest that in vitro, TTA activates both PPARα and PPARγ, but the latter with much lower affinity. TTA affects serum lipid levels in vivo in rats by acting mainly on the liver via PPARα where it decreases the liver expression of genes involved in vascular lipid transport and increases the expression of genes involved in intracellular fatty acid metabolism.—Raspé, E., L. Madsen, A-M. Lefebvre, I. Leitersdorf, L. Gelman, J. Peinado-Onsurbe, J. Dallongeville, J-C. Fruchart, R. Berge, and B. Staels. **Modulation of rat liver apolipoprotein gene expression and serum lipid levels by tetradecylthioacetic acid (TTA) via PPARα activation.** ***J. Lipid Res.*** **1999.** 40: **2099–2110.**

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A group of modified fatty acids, in which a sulfur atom replaces a methylene group in a defined position of the carbon chain of an ordinary fatty acid (n-thia fatty acid), has been developed and extensively tested as hypolipidemic drugs (1). 3-Thia fatty acids such as tetradecylthioacetic acid or TTA, a saturated fatty acid where the 3 methylene group is substituted (CH_3-$(CH_2)_{13}$-S-CH_2-COOH), are non-β oxidizable but can undergo ω-oxidation (1). The other n-thia fatty acids can be metabolized both through β- or ω-oxidation and have different biological activities (1). When administered to rats, 4-thia fatty acids promote fatty liver development and increase plasma triacylglycerol concentrations most probably through inhibition of fatty acid oxidation, whereas 3-thia fatty acids promote hepatic peroxisome and mitochondria proliferation and decrease serum triacylglycerol, cholesterol and free fatty acid levels (2–4). Decreased triacylglycerol biosynthesis and secretion has also been described after feeding rats with TTA (5). In addition, 3-thia fatty acids are known to promote the expression and activity of acyl-CoA oxidase (ACO) (5, 6), carnitine palmitoyltransferase (CPT-II) (7), mitochondrial HMG-CoA synthase (7) and, only after acute challenge, CPT-I (8, 9). Other fatty acids of variable

Abbreviations: LPL, lipoprotein lipase; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; ACO, acyl-CoA oxidase; CPT-I, carnitine palmitoyltransferase-I; CPT-II, carnitine palmitoyltransferase-II; HMG-CoA synthase, 3-hydroxy-3-methylglutaryl coenzyme A synthase; TTA, tetradecylthioacetic acid; mRNA, messenger ribonucleic acid; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LTB4, leukotriene B4; 8(S)HETE, 8(S)-hydroxyeicosatetraenoic acid; PBS, phosphate buffered saline; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; cDNA, complementary deoxyribonucleic acid; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PPRE, peroxisome proliferator response element; CMC, carboxymethylcellulose; RAU, relative absorbance unit; GAPDH, glyceraldehydephosphate dehydrogenase; RXR, retinoid X receptor; SMRT, silencing mediator for RXR and TR; NCoR, nuclear receptor co-repressor; SRC-1, steroid receptor co-activator 1; kb, kilobase; CMV, cytomegalovirus.

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chain length but with a sulfur atom in the third position of the chain reproduce the TTA effect (7, 10).

Apolipoproteins are major protein constituents of lipoproteins which are responsible for plasma lipid transport. The functions of these apolipoproteins in plasma lipoprotein metabolism were unravelled by the phenotypic analysis of naturally occurring mutants or of strains of transgenic mice either overexpressing the human apolipoprotein genes or carrying mutations in endogenous apolipoprotein genes introduced by homologous recombination (11, 12). We mainly focused the present study on apoA-I, apoC-III, and lipoprotein lipase (LPL), important participants in high density lipoprotein (HDL) and triacylglycerol metabolism. ApoA-I is a major constituent of HDL, a lipoprotein involved in reverse cholesterol transport and in supply of cholesterol to steroidogenic tissues (13, 14). Plasma apoC-III concentrations have been shown to be positively correlated with plasma triacylglycerol levels, in normal individuals and in hypertriglyceridemic patients (15–17) as well as in strains of transgenic mice expressing the human apoC-III gene (18). Elimination of the endogenous apoC-III gene by homologous recombination in mice led to lowered plasma apoC-III and triacylglycerol concentrations and protected the animals against postprandial hypertriglyceridemia (19). Plasma triacylglycerol levels are also affected by the activity of LPL (20). LPL uses apoC-II as cofactor, is inhibited by apoC-III, and hydrolyzes triacylglycerols in triacylglycerol-rich lipoproteins and hence releases free fatty acids which are taken up by the underlying tissues. Apolipoprotein and LPL genes are therefore among potential target genes for hypolipidemic drugs.

The effects of fibrates, lipid-lowering drugs used in clinical practice for over 25 years, have been well characterized on lipoprotein metabolism (21). They are most effective in lowering elevated plasma triacylglycerols both in human and animal models by reducing apoC-III gene expression (22). In addition, they reduce apoA-I, apoA-II, and apoA-IV gene expression in rodents and reduce plasma cholesterol levels (23–25). Fenofibrate, but not clofibrate or gemfibrozil, slightly reduces apoB gene expression and slightly induces LDL receptor gene expression (23). ApoE mRNA levels are also slightly reduced by fenofibrate but not by clofibrate or gemfibrozil after 1 day of treatment of rats but return back to normal levels after 3 days of treatment (23). Finally, fibrates enhance the expression of LPL in the liver of rats, with no effect on adipose tissue (26). Recently, fibrates have been shown to be ligands and activators of specific members of the peroxisome proliferator-activated receptor (PPAR) nuclear receptor subfamily (27, 28). To date, three different types of PPARs, PPARα, PPARδ (also termed NUC I, FAAR or PPARβ), and PPARγ have been identified in various species, each encoded by a different gene and exhibiting distinct tissue-specific expression patterns (29). After ligand activation and heterodimerization to the 9-*cis* retinoic acid receptor RXR (30, 31), PPARs bind to specific response elements, termed peroxisome proliferator response elements (PPREs), located in the regulatory regions of target genes (29). In addition to fibrates, the arachidonic acid metabolites LTB4 or 8(S)HETE have been identified as PPARα ligands (27, 32), whereas the insulin-sensitizing agents thiazolidinediones (such as the compound BRL 49653) and the arachidonic acid metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J 2 have been shown to be PPARγ ligands (33–35). Functional PPAR response elements have been identified in the promoters of genes involved in triglyceride metabolism such as the human apoC-III (36) and the mouse and human LPL genes (26), as well as in plasma HDL metabolism such as the human apoA-I (37) and apoA-II (38) genes. Finally, the action of fibrates on the expression of these genes was abolished in transgenic mice where the PPARα gene is inactivated by homologous recombination (39). Taken together, these data indicate that PPARα mediates the action of fibrates on apolipoprotein gene expression. In addition to lowering plasma lipid levels, fibrates also promote peroxisome proliferation in rodents (see ref. 29 and references therein). The recently reported absence of peroxisome proliferation in PPARα-deficient mice supports previous evidence suggesting the direct implication of this receptor in the action of peroxisome proliferators including fibrates (40). By contrast, thiazolidinedione PPARγ activators such as BRL49653 enhance epididymal adipose tissue LPL mRNA accumulation but do not affect liver apolipoprotein or ACO gene expression (41). Interestingly, simultaneous administration of BRL49653 and fenofibrate resulted in a more pronounced lowering of serum triacylglycerols than each drug alone, suggesting that drugs with combined PPARα and PPARγ agonist activity will display an increased hypotriglyceridemic activity (41). As fatty acids are PPARα and PPARγ activators (27, 28), 3-thia fatty acids are good pharmacological candidates for such combined action.

The first goal of this study was therefore to determine whether 3-thia fatty acids act by regulating the expression of genes encoding proteins involved in lipid transport and lipoprotein metabolism in hepatic and adipose tissue in vivo. Second, we aimed to determine whether the hypolipidemic effects of 3-thia fatty acids could be associated with activation of PPARα and/or PPARγ in vitro.

MATERIALS AND METHODS

Materials

Tetradecylthioacetic acid (TTA) was prepared as described earlier (42). BRL49653 and fenofibric acid were gifts respectively from Dr. J-J. Berthelon (Merck-Lipha, Lyon, France) and Dr. A. Edgar (Laboratoires Fournier, Daix, France). Pirixinic acid (Wy 14643) was from Chemsyn Science Laboratories (Lenexa, KS). The cationic lipid RPR 120535B (WO patent 97/18185) was a generous gift from Dr. G. Byk (Rhône-Poulenc-Rorer, Paris, France).

Animals

Male Wistar rats weighing approximately 150 g were housed in metal cages and maintained under 12-h light/dark cycles at a constant temperature of 20 ± 3°C. The animals were acclimatized for at least 1 week before the beginning of the experiments and randomized with respect to body weight. TTA was suspended in 0.5% sodium carboxymethylcellulose and administered by ga-

vage once a day for 7 days at a dose of 300 mg/day per kg body weight. The animals had free access to food and water. At the end of the experiment, the rats were weighed and killed by cardiac puncture under neuroleptic anaestesia. Blood was collected in EDTA-containing tubes. Plasma was prepared by centrifugation at 3000 rpm for 20 min. Liver and epididymal fat pads were removed immediately, weighed, and frozen in liquid nitrogen. All animals received appropriate care as outlined in the "Guide for the Care and Use of Laboratory Animals."

Serum lipid measurements

Serum lipid concentrations (total and free cholesterol, triacylglycerols, phospholipids) were measured colorimetrically using enzymatic test kits from Boehringer Mannheim (Mannheim, Germany).

For fast protein liquid chromatography (FPLC) size fractionation of lipoproteins, 300 μl of serum isolated from individual rats were injected on a Sepharose 6HR 10/30 prepacked column (Pharmacia, Uppsala, Sweden) and eluted at a constant flow rate of 0.2 ml/min with PBS pH 7.2. The effluent was monitored at 280 nm, collected in 0.3-ml fractions and cholesterol and triacylglycerol concentrations were determined in 0.1 ml of each fraction.

Cell culture

Rat hepatocytes were isolated by collagenase perfusion (25) of livers from male rats of mass ranging between 150 and 250 g. When cell viability judged by the Trypan Blue exclusion test was higher than 90%, the cells were seeded in 10-cm^2 Petri dishes (Falcon, Lincoln Park, NJ) and cultured as a monolayer (1.5×10^5 cells/cm^2) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The culture medium used was Leibowitz-15 medium (Gibco, Paisley, UK) supplemented with fetal calf serum (10% by vol), fatty acid-free BSA (0.2% mass/vol), $NaHCO_3$ (26 mm), l-glutamine (2 mm), glucose (3 g/l), dexamethasone (1 μm, from a 10^4-fold concentrated stock in ethanol), penicillin (100 U/ml), and streptomycin (100 μg/ml). Agents were dissolved in ethanol or DMSO (1000-fold concentrated stock) and added to the culture medium immediately after seeding. Control cells received vehicle only (0.1% by vol, final concentration). After 24 h incubation, the medium was removed, cells were quickly washed with ice-cold 0.15 m NaCl, 0.01 m sodium phosphate buffer, pH 7.2, lysed and scraped in 2 ml of ice-cold 4 m guanidinium isothiocyanate.

3T3-L1 cells obtained from E.C.A.C.C. (Porton Down, Salisbury, U.K.) were cultured as previously described (26) and incubated for the time indicated in the presence of tetradecylthioacetic acid. At the end of the experiment, cells were quickly washed with ice-cold 0.15 m NaCl, 0.01 m sodium phosphate buffer, pH 7.2, lysed and scraped in 2 ml of ice-cold 4 m guanidinium isothiocyanate.

Human hepatoma HepG2 cells were obtained from E.C.A.C.C. (Porton Down, Salisbury, U.K.). Cells were maintained in standard culture conditions (Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air). Medium was changed every 2 days.

Preparation of subcellular fractions

The livers from individual rats were homogenized in ice-cold sucrose medium (0.25 m sucrose, 10 mm HEPES (pH 7.4) and 2 mm EDTA). Postnuclear, mitochondrial, and peroxisomal fractions were prepared using preparative differential centrifugation as earlier described (43). The fractionation process was carried out at 0–4°C. Protein content of the homogenates was evaluated by the Bradford assay using the kit from Bio-Rad (Bio-Rad, München, Germany). BSA in distillated water was used as standard.

Measurement of enzyme activities

Fatty acyl-CoA oxidase activity in the peroxisomal fraction was measured as previously described (5). Acid-soluble products were measured using palmitoyl-CoA as substrate (44). Briefly, the assay mix (0.3 ml) contained 12 mm HEPES buffer (pH 7.3), 11 mm $MgCl_2$, 12 mm dithiothreitol, 5.6 mm ADP, 0.2 mm NAD^+, 0.6 mm EDTA, 125 mm KCl, and 1.0 mg mitochondrial protein. Palmitoyl-CoA oxidation was measured with 80 μm [1-^{14}C]palmitoyl-CoA supplemented with 1 mm l-carnitine. After 2 min incubation at 30°C, the reaction was terminated with 150 μl 1.5 m KOH. Then, 2.5 mg BSA and 500 μl 4 m $HClO_4$ were added. The test tubes were centrifuged at 1880 *g* for 10 min and 500 μl of the supernatant was assayed for radioactivity. Carnitine palmitoyltransferase (CPT)-I and -II activity was measured essentially as described by Bremer (45). The assay for CPT-I contained 20 mm HEPES, pH 7.5, 70 mm KCl, 5 mm KCN, 100 μm palmitoyl-CoA, 10 mg BSA/ml, and 0.6 mg mitochondrial protein/ml. The reaction was started with 200 μm [methyl-^{14}C]-l-carnitine (200 cpm/nmol). Assay conditions for CPT-II were identical except that BSA was omitted and 0.01% Triton X-100 was included. Mitochondrial protein concentration was 0.1 mg/ml. Mitochondrial HMG-CoA synthase was measured according to Clinkenbeard et al. (46).

RNA analysis

Total RNA was isolated from liver and epididymal adipose tissue or from cell culture extracts by the acid guanidinium thiocyanate/phenol/chloroform method (47). Northern and dot blot analyses of total cellular RNA were performed as described (23). Rat apoA-I, apoA-II, apoA-IV, apoC-III, apoE, apoB, LDL receptor, acyl-CoA oxidase (ACO), and human LPL cDNA clones were used as probes (22, 23, 48). cDNA clones for β-actin (49) and 36B4 (50) (encoding the human acidic ribosomal phosphoprotein PO (51)) were used as control probes. A partial fragment of 0.7 kb BamHI-SalI of rat CPT I cDNA, covering the region corresponding to positions 1272–2020 of the rat mRNA CPT I (52) was amplified by PCR as described (53). A 1.4 kb XhoI-BamHI fragment from cDNA clone (pBKS-CPTII.4) for rat CPT II (54), a specific 1.5 kb probe corresponding to the KpnI-KpnI fragment of the cDNA for rat mitochondrial HMG-CoA synthase, which had been subcloned in pBluescript (55) were used as probes. All probes were labeled by random primed labeling (Boehringer Mannheim). Filters were hybridized to 1.5×10^6 cpm/ml of each probe as described (23). They were washed once in 500 ml 75 mm NaCl, 7.5 mm sodium citrate, and 0.1% SDS for 10 min at room temperature and twice for 30 min at 65°C and subsequently exposed to X-ray film (X-OMAT-AR, Kodak). Autoradiograms were analyzed by quantitative scanning densitometry (Bio-Rad GS670 Densitometer) as described (23).

Cloning and construction of recombinant plasmids

The plasmid pTkpGL3 was constructed by PCR-amplification of a fragment of the thymidine kinase promoter of the Herpes simplex virus using the plasmid pBLCAT4 (56) as template and the following oligos (5′-CGA CTC TAG AAG ATC TTG CCC CGC CCA GCG-3′ and 5′-TCG CCA AGC TTC TCG TGA TCT GCG GCA-3′), followed by restriction of the PCR product with HindIII and BglII and insertion into the corresponding sites of the pGL3 vector (Promega, Madison, WI). The plasmid pGal5-TkpGL3 was obtained by inserting five copies of the 17m Gal4 DNA binding site (57) in front of the thymidine kinase promoter of the pTkpGL3 plasmid.

The plasmid pGal4-ϕ used as negative control was constructed by subcloning the yeast transcription factor Gal4 DNA binding domain present in the plasmid pBD-Gal4 (Stratagene, La Jolla, CA) into the HindIII-EcoRI sites of the vector pCDNA3 (In Vitrogen, Carlsbad, CA). The plasmid pGal4-hPPARγDEF was con-

structed by first subcloning a blunted BamHI fragment of the plasmid pSG5-hPPARγ_2 (58) into the blunted EcoRI site of the plasmid pBD-Gal4 and subcloning the chimera into the HindIII-XbaI sites of the pCDNA3 vector. The expressed chimera contains the amino acids 1 to 147 of the Gal4 transcription factor and the amino acids 182 to 505 of hPPARγ_2. The plasmid pGal4hPPARαDEF was constructed by PCR-amplifying the hPPARα DEF domains using the plasmid pSG5-hPPARα (59) as template with the following oligos (5′-ACG GTT CTC GAG GTC ACA CAA CGC GAT TCG TTT TGG ACG AAT G-3′, 5′-ACG AAC TCT AGA TCA GTA CAT GTC CCT GTA GAT CTC-3′), cloning the fragment in the blunted EcoRI site of pBD-Gal4 and subcloning the chimera into the HindIII-XbaI sites of the pCDNA3 vector. The expressed chimera contains the amino acids 1 to 147 of the Gal4 transcription factor and the amino acids 166 to 467 of hPPARα. All constructs were validated by sequencing before use.

Transient transfections assays

Cells were seeded in 24-well plates at a density of 10^5 cells/well or 10^4 cells/well for HepG2 or 3T3-L1 cells, respectively, and incubated at 37°C for 16 h prior to transfection. Cells were transfected in culture medium without serum for 2 h at 37°C using the cationic lipid RPR 120535B. The transfection protocol (cell density and time course of the experiment) was optimized for each cell line. Similar amounts of DNA were transfected in both cell lines. The plasmid pRL-CMV (Promega, Madison, WI) which drives the expression of the Renilla luciferase reporter gene under the control of the cytomegalovirus promoter/enhancer was used as control of transfection efficiency, whereas the plasmid pBluescript (Stratagene, La Jolla, CA) was used as carrier DNA to set the final amount of DNA to 500 ng/well. The plasmids (pG5TkpGL3 at 100 ng/well (reporter); pGal4-hPPARαDEF, pGal4-hPPARγDEF and pGal4-ϕ at 10 ng/well (expression vectors) and pRL-CMV at 1 ng/well) were dissolved in serum-free DMEM supplemented with NaCl (150 mm), sodium bicarbonate (50 mm) and the cationic lipid (6 nmol/μg DNA), vortexed, incubated for 30 min at room temperature, and added to the cells. After 2 h, cells were washed with 0.15 m NaCl, 0.01 m sodium phosphate buffer, pH 7.2, and incubated for 36 h in fresh medium containing 10% fetal calf serum and the compounds tested or vehicle (DMSO 0.1% by vol). At the end of the experiment, the cells were washed once with ice-cold 0.15 m NaCl, 0.01 m sodium phosphate buffer, pH 7.2, and the luciferase activity was measured with the Dual-Luciferase™ Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. All transfection experiments were performed at least 3 times. Protein content of the extract was evaluated by the Bradford assay using the kit from Bio-Rad (Bio-Rad, München, Germany). Concentration–action curves were analyzed by fitting to the Michaelis-Menten or the Hill equation ($A=(Amax*S^n)/(K+S^n)$ where S is the concentration of agonist, A the measured activity, and Amax, K, and n are constants) by a Marquardt-Levenberg algorithm (based on a least square method). As a better fit was generally obtained with the Hill equation, this equation and the fitted parameters were used to estimate the EC_{50}.

RESULTS

Effects of TTA treatment on liver and adipose tissue gene expression and plasma lipid levels

In a first set of experiments, male Wistar rats were treated for 7 days with TTA at a dose known to induce peroxisome proliferation and to decrease plasma lipid levels (300 mg/kg/day). Subsequently, plasma lipid levels and apolipoprotein and LPL gene expression were measured.

TTA treatment significantly increased liver weight (**Table 1**) without significantly affecting the body weight or the food intake (result not shown). A significant decrease in total serum triacylglycerols (52%), phospholipids (60%), and cholesterol (73%) was observed (Table 1). Palmitic acid was without effect (Table 1). As these results are similar to those previously described (5), we further characterized the effect of TTA on the lipoprotein profile.

Serum samples from control or treated animals were separated by fast protein liquid chromatography (FPLC) size fractionation and each collected fraction was analyzed for triacylglycerol and cholesterol. **Figure 1** shows that the decrease of total triacylglycerols after TTA challenge is mainly observed in large particles (VLDL). Treatment of rats with TTA decreases the cholesterol content in both large and smaller particles (VLDL and HDL).

As shown in **Fig. 2**, TTA treatment of rats was followed by a pronounced increase of the ACO gene expression in liver, concomitant to an increase in liver ACO activity (Table 1). A substantial reduction of apoA-I, apoA-II, apoA-IV, and apoC-III liver mRNA levels was observed after TTA feeding whereas apoB and control β-actin mRNA levels remained unchanged. Similar results were obtained in two independent experiments. Palmitic acid was inactive. Rat

TABLE 1. Effects of TTA on rat blood parameters, liver weight, mitochondrial palmitoyl-CoA oxidation, ACO, HMG-CoA synthase, CPT-I, and CPT-II activities

Treatment	Liver Weight	Triacylglycerol Level	Total Cholesterol Level	Phospholipid Level	Mitochondrial Palmitoyl-CoA Oxidation	Mitochondrial HMG-CoA Synthase	Mitochondrial CPT-I	Mitochondrial CPT-II	Microsomal ACO Activity
	g	*g/l*	*g/l*	*g/l*	*nmol/mg protein/min*				
CMC	3.36 ± 0.18	0.45 ± 0.14	0.67 ± 0.1	1.06 ± 0.1	0.94 ± 0.09	10.84 ± 2.05	1.35 ± 0.12	22 ± 3	17.1 ± 2.8
Palm	3.28 ± 0.20	0.39 ± 0.04	0.66 ± 0.09	1.06 ± 0.13	1.26 ± 0.31	8.91 ± 3.24	1.26 ± 0.17	26 ± 2	16.6 ± 1.4
TTA	5.05 ± 0.48[a]	0.22 ± 0.35[b]	0.18 ± 0.09[a]	0.42 ± 0.07[a]	2.41 ± 0.56[b]	24.33 ± 3.64[b]	1.19 ± 0.18	68 ± 12[b]	200.8 ± 52.8[a]

Liver and serum were collected from adult male rats treated for 7 days with TTA (300 mg/kg/day) or palmitic acid at the same dose suspended in 0.5% carboxymethylcellulose (CMC). Triacylglycerol, total cholesterol, and phospholipid levels were determined by colorimetric enzymatic test kit (Boehringer Mannheim). Liver peroxisomal ACO activity was measured as previously described (5). Mitochondrial palmitoyl-CoA oxidation, mitochondrial HMG-CoA synthase, mitochondrial CPT-I, and mitochondrial CPT-II activities were measured as previously described (5, 45, 46). Values represent mean ± SD from four animals.

[a] $P \leq 0.005$.

[b] $P \leq 0.05$.

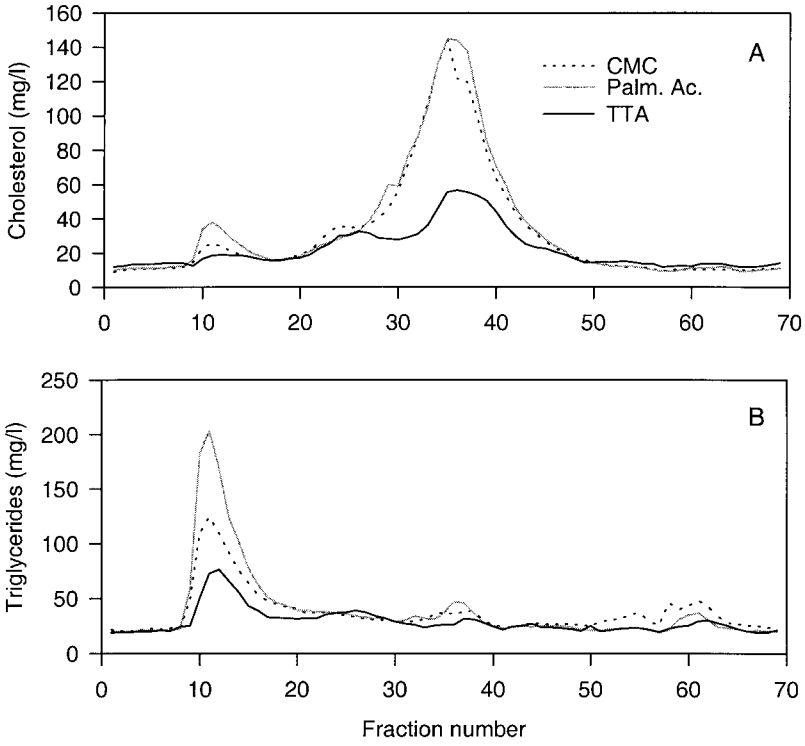

Fig. 1. Effects of TTA on serum lipoprotein profiles in rats. Serum was collected from adult male rats treated for 7 days with TTA (300 mg/kg/day) or palmitic acid (isocaloric control; Palm. Ac.) at the same dose suspended in 0.5% carboxymethylcellulose (CMC). Serum lipoproteins were separated by gel filtration chromatography and triacylglycerol and cholesterol concentrations (median value of 4 animals) were measured in the isolated fractions using colorimetric enzymatic test kits (Boehringer Mannheim).

liver LDL-receptor mRNA levels were generally weak but remained unaffected by TTA in most experiments (five out of seven). Feeding rats with TTA produced variable effects on apoE expression: in some experiments, TTA was inactive whereas in others, as illustrated in Fig. 2, we observed a slight decrease albeit smaller than the effect on other apolipoprotein genes. In addition to liver apolipoprotein gene expression, we also investigated whether liver mitochondrial HMG-CoA synthase, CPT-I and CPT-II mRNA levels and activities were also modified by TTA feeding. We observed, in accordance to previously published data (7), that HMG-CoA synthase and CPT-II mRNA levels and activities where elevated by TTA whereas CPT-I mRNA level was almost unaffected. Finally, in most experiments (seven out of nine), LPL gene expression in epididymal adipose tissue was not affected by TTA (**Table 2**), whereas it was slightly increased in the two remaining experiments.

Evaluation of PPAR isoform activation by TTA

As simultaneous activation of both PPARα and PPARγ could lead to synergistic action on plasma triacylglycerol levels (41), and as the actions of TTA are similar to those of PPAR activating substances, we evaluated the respective effects of TTA on PPARα and γ transactivation by transient transfection assays. To avoid interference with endogenous nuclear receptors, we used two chimeras comprising the DNA-binding domain of the yeast transcription factor Gal4 fused to the ligand binding domain of either human PPARα or PPARγ and a reporter vector containing five copies of the Gal4 response element cloned in front of the Herpes simplex thymidine kinase promoter and the luciferase reporter gene as described previously (34). The advantage of this assay is that the background is low as the chimeras and the Gal4 transcription factor are not expressed in non-transfected target cells.

When cotransfected with the appropriate reporter vector in HepG2 cells, the Gal4-hPPARα chimera was activated by the reference fibrate Wy14643 but not by the thiazolidinedione BRL 49653 while the negative control Gal4-ϕ was not affected by either Wy14643 or BRL49653 (**Fig. 3A**). By contrast, the Gal4-hPPARγ chimera was weakly affected by Wy14643 but strongly stimulated by BRL49653 (Fig. 3A). The effects of Wy14643 and BRL49653 were concentration-dependent: the respective estimated EC_{50}s are reported in **Table 3**. TTA also activated the Gal4-hPPARα chimera in a concentration-dependent manner (Fig. 3B, Table 3). A significant activation was already noticed at a concentration as low as 1 μm. The maximal effect (40-fold induction) was reached at 3 μm but was less pronounced than the effect of Wy14643 (about 150-fold activation when tested at 30 μm). On the other hand, TTA was a very poor activator of the Gal4-hPPARγ chimera in HepG2 cells: a small 10-fold activation was observed only above 60 μm (Fig. 3B). This effect has to be compared to the 50-fold activation of this chimera by BRL49653 tested at a concentration of 1 μm.

When 3T3-L1 cells were cotransfected with the appropriate reporter and the Gal4-hPPARα expression vector, an enhancement of reporter activity was also observed in response to Wy14643 and TTA, whereas BRL49653 was inactive (**Fig. 4A**). On the other hand, the Gal4-hPPARγ chimera was mainly activated by BRL49653 and TTA tested at high concentrations (above 10 μm) and modestly by Wy14653 (only 6-fold stimulation at 100 μm) (Fig. 4A), while the negative control Gal4-ϕ chimera activity remained unchanged with all compounds. The effects of these agents

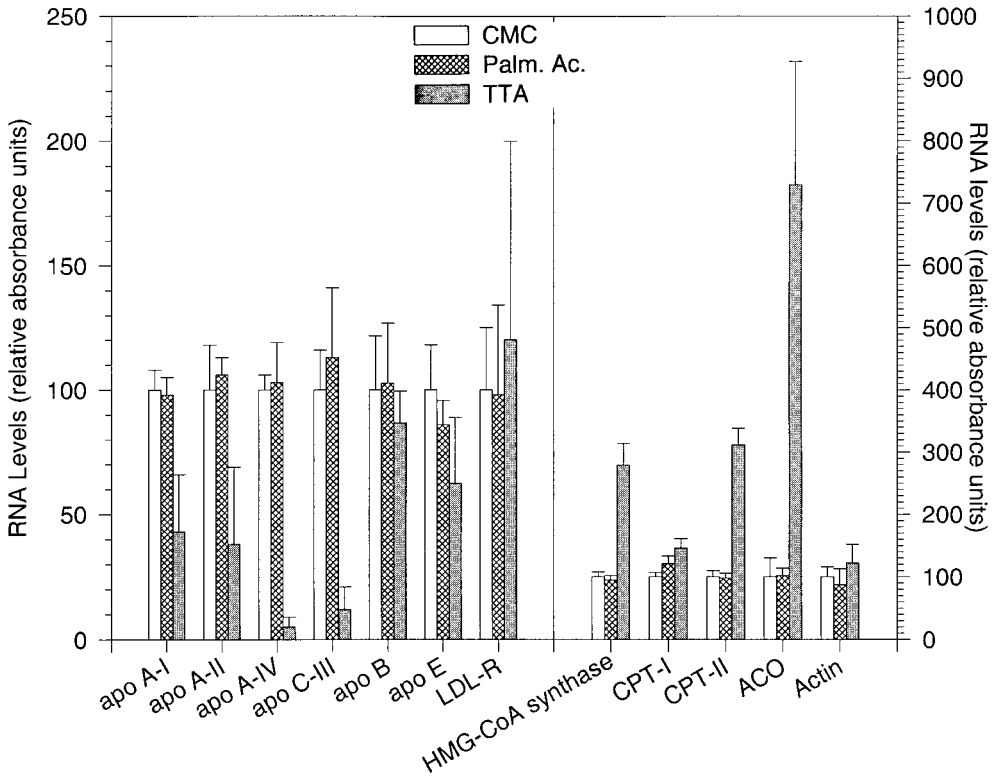

Fig. 2. Effects of TTA on rat liver mRNA levels. Livers were collected from adult male rats treated for 7 days with TTA (300 mg/kg/day) or palmitic acid (isocaloric control) at the same dose suspended in 0.5% carboxymethylcellulose (CMC). Total RNA was extracted from frozen tissue and apoA-I, apoA-II, apoA-IV, apoC-III, apoB, apoE, LDL-receptor, HMG-CoA synthase, CPT-I, CPT-II, LPL, or β-actin mRNA levels were quantitated by dot or Northern blotting using specific probes. RNA values are expressed in relative absorbance units, taking the control CMC value as 100%. Values represent mean ± SD from 4 animals.

were concentration-dependent: the estimated respective EC_{50}s are reported in Table 3. When the activation of the Gal4-hPPARα and Gal4-hPPARγ chimeras by Wy14643, BRL49653, and TTA are compared in the two cell lines, four differences can be pointed out. First, the EC_{50}s for Gal4-hPPARα and Gal4-hPPARγ activation by TTA are slightly but significantly lower in 3T3-L1 versus HepG2 cells ($P < 0.05$). Second, the efficiency of Gal4-hPPARα activation is lower in 3T3-L1 cells, especially when cells are stimulated by Wy14643. Third, the maximal stimulation of the Gal4-hPPARα and Gal4-hPPARγ chimeras by TTA is comparable in 3T3-L1 cells whereas the effect on the Gal4-hPPARα chimera is more intense than the effect on the Gal4-hPPARγ chimera in HepG2 cells. Finally, when compared to the effect of BRL49653 tested at 1 μm, the activation of the Gal4-hPPARγ chimera by TTA tested at 30 μm is more important in 3T3-L1 cells than in HepG2 cells (about 30% of the BRL49653 effect in 3T3-L1 cells versus 10% in HepG2).

Our data therefore suggest that, in vitro and in the appropriate cell context, TTA can activate both PPARα and PPARγ. However, the activation of PPARγ occurs only at higher concentrations than those required for PPARα activation.

TABLE 2. Effects of TTA on rat epididymal adipose tissue mRNA levels

Treatment	LPL	GAPDH
CMC	100 ± 26	100 ± 24
Palmitic Acid	160 ± 37	143 ± 22
TTA	110 ± 56	105 ± 28

Epididymal fat depots were collected from adult male rats treated for 7 days with TTA (300mg/kg/day) or palmitic acid (isocaloric control) at the same dose suspended in 0.5% carboxymethylcellulose (CMC). Total RNA was extracted from frozen tissue as described previously (41). LPL or GAPDH mRNA levels were quantitated by dot blotting. RNA values are expressed in relative absorbance units (RAU), taking the control CMC value as 100%. Values represent mean ± SD from four animals.

In vitro effects of TTA on gene expression in rat hepatocytes in primary culture and in 3T3-L1 cells

To evaluate whether TTA could directly affect gene expression in cells of hepatic or adipose origin and activate the endogenous PPARs, rat hepatocytes in primary culture or 3T3-L1 cells were incubated with TTA and harvested. Total RNA was extracted and analyzed with specific probes for genes known to be targets for PPARs and often used as marker for PPAR activation. The expression of the rat ACO gene, which is strongly induced by fibrates or other PPARα activators (25), increased in a concentration-dependent manner after TTA treatment of primary rat

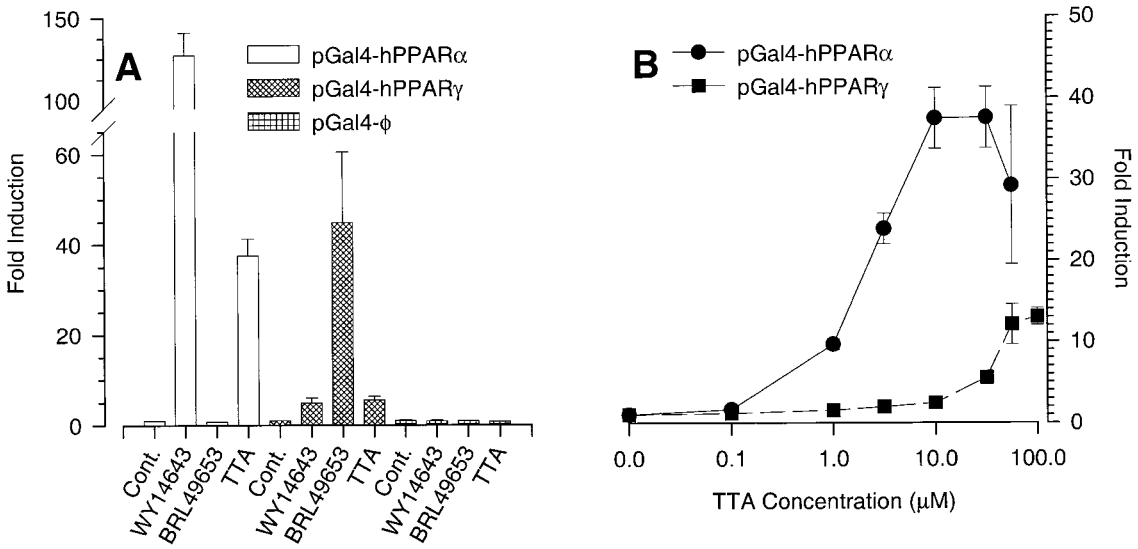

Fig. 3. Effects of TTA on the activity of PPARα or PPARγ chimeras in HepG2 cells. HepG2 cells were cultured and transfected with expression plasmids for the Gal4-hPPARα and Gal4-hPPARγ chimera and the reporter plasmid pGal5TkpGL3 comprising five copies of the DNA binding element of the yeast transcription factor Gal4 cloned in front of the thymidine kinase promoter and the luciferase reporter gene as described under Materials and Methods. Cells were treated for 36 h with either vehicle (0.1% vol/vol DMSO), TTA (30 μm), Wy14643 (30 μm) or BRL49653 (1 μm) (panel A) or TTA at the indicated concentrations (panel B). Cell extracts were subsequently analyzed for luciferase gene activity and protein content as described under Materials and Methods.

hepatocytes (**Fig. 5**). These data suggest that TTA acts directly on the rat hepatocyte and activates endogenous PPARα. In order to see whether, in addition to peroxisomal β-oxidation, mitochondrial fatty acid catabolism may also be affected in vitro by TTA, we measured the accumulation of CPT-I and CPT-II mRNAs after challenge of primary hepatocytes with TTA. A strong concentration-dependent increase in CPT-I mRNA accumulation was observed (Fig. 5). A smaller 2-fold increase in CPT-II mRNA level was also seen (data not shown).

On the other hand, a time-dependent induction of LPL mRNA accumulation was observed after incubation of 3T3-L1 cells with 100 μm TTA (**Fig. 6**), a concentration which maximally enhanced Gal4-hPPARγ chimera activity (Fig. 4B). This effect was similar to the reported effects of thiazolidinediones (41). This suggests that, at least in vitro, TTA acts directly on the adipocyte and could activate PPARγ in these cells.

TABLE 3. EC_{50} of activation by various agonists of the Ga14-hPPARα and Ga14-hPPARγ chimeras in HepG2 and 3T3-L1 cells

Agent	PPAR Type	HepG2 Values	3T3-L1 Values
TTA	PPARα	1.8 ± 0.3 μm	1.3 ± 0.4 μm
TTA	PPARγ	40 ± 8 μm	18.4 ± 11.7 μm
BRL 49653	PPARγ	147 ± 82 nm	68.8 ± 23.0 nm
Wy 14643	PPARα	21.9 ± 10.5 μm	28.6 ± 3.1 μm
Wy 14643	PPARγ	61.9 ± 23.4 μm	>100 μm

Concentration-action curves (6 concentrations/curve) of various agonists on Gal4-hPPARα and Gal4-hPPARγ chimeras in HepG2 and 3T3-L1 cells were fitted as described in Materials and Methods by a least squares based algorithm. The reported values are means ± SD of values estimated using three independent experiments.

DISCUSSION

3-Thia fatty acids are modified fatty acids that are reported to promote hepatic peroxisome proliferation and to decrease serum triacylglycerol, cholesterol, and free fatty acid levels when administered to rats (1). In order to determine their action mechanism on plasma lipoprotein metabolism, we characterized the effects of TTA, one of the most potent 3-thia fatty acids, on liver apolipoprotein gene expression in rats treated for 7 days with the drug. We observed a pronounced increase of ACO gene expression in liver together with a substantial reduction of apoA-I, apoA-II, apoA-IV, and apoC-III liver gene expression. Because these effects, as well as the peroxisome proliferation capacity of TTA, are similar to those of fibrates (29), our results therefore suggest that TTA shares at least some common mechanisms of action with fibrates.

The observation that TTA increases ACO gene expression not only in vivo but also in vitro in rat hepatocytes maintained in primary culture indicates that the effects of TTA on liver gene expression are direct. As illustrated in Fig. 1, in rats, the majority of plasma cholesterol is carried in HDL lipoproteins. Therefore, the decreased expression of liver apolipoproteins A-I, A-II, and A-IV, which are components of HDL (13, 60), most likely contribute to the cholesterol-lowering effect of TTA. In addition to its effect on apolipoprotein gene expression, TTA also affects the liver activity of HMG-CoA reductase, the rate-limiting

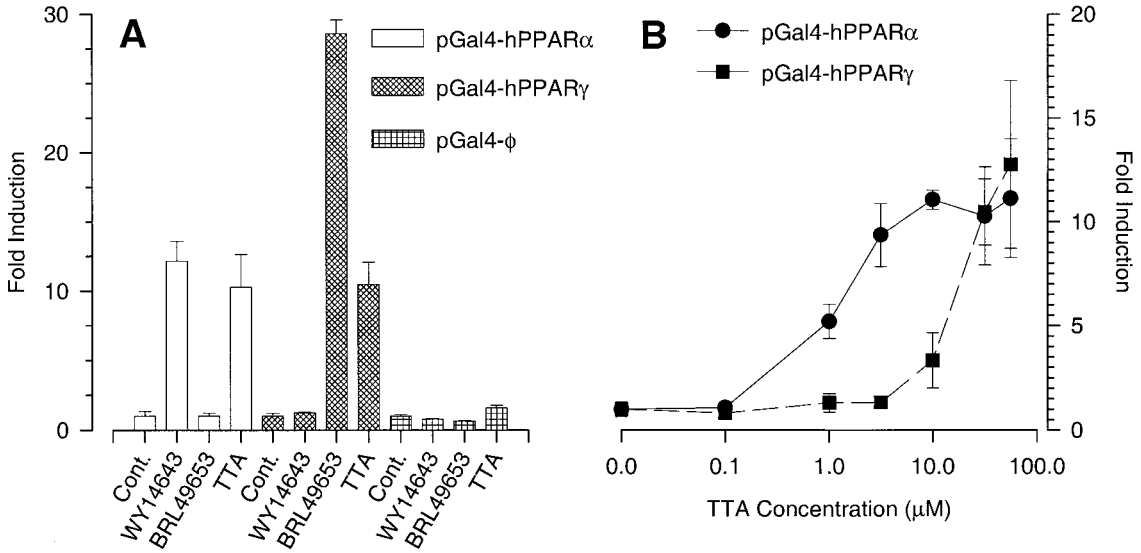

Fig. 4. Effects of TTA on the activity of PPARα or PPARγ chimeras in 3T3-L1 cells. 3T3-L1 cells were cultured and transfected as described in Material and Methods. Cells were treated for 36 h with either vehicle (0.1% vol/vol DMSO), TTA (30 μm), Wy14643 (30 μm) or BRL 49653 (1 μm) (panel A) or TTA at the indicated concentrations (panel B). Cell extracts were subsequently analyzed for luciferase gene activity and protein content as described under Materials and Methods.

enzyme in cholesterol synthesis (5). The decrease in plasma cholesterol probably results, therefore, from combined effects on transport and synthesis of cholesterol. Plasma triacylglycerol levels depend on the balance between triacylglycerol synthesis, secretion, and catabolism by the liver as well as on its intravascular catabolism. Intravascular catabolism of triacylglycerol-rich VLDL particles is a consequence of triacylglycerol hydrolysis by LPL followed by receptor-mediated VLDL or VLDL remnant clearance (20, 61). Both processes are inhibited by apoC-III which therefore plays a central role in the control of plasma triacylglycerol levels (62–66). As liver apoB or LDL receptor and adipose tissue LPL gene expressions are not significantly affected by TTA and as only minor effects compared to the effects on other apolipoproteins are observed on apoE gene expression in some but not all experiments, the strong reduction in liver apoC-III expression evoked by TTA feeding likely accounts for a large part of its effect on intravascular triacylglycerol catabolism. In addition to its effect on intravascular triacylglycerol catabolism, TTA

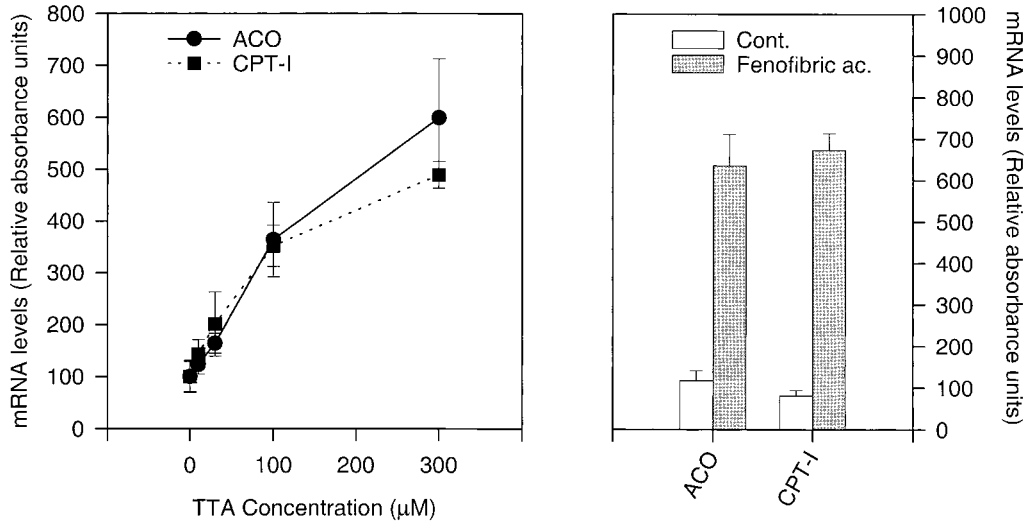

Fig. 5. Effects of TTA on ACO and CPT-I mRNA levels in primary rat hepatocytes. Rat hepatocytes were isolated, cultured as previously described (25), and treated for 24 h with TTA at the indicated concentrations, fenofibric acid (250 μm), or vehicle (0.1% vol/vol ethanol for TTA, 0.1% vol/vol DMSO for fenofibric acid). Total cellular RNA was extracted and ACO and CPT-I mRNA levels were quantitated by dot blotting as previously described (25). RNA values (mean ± SD of three replicate values) are expressed in relative absorbance units, taking the respective ethanol value as 100%.

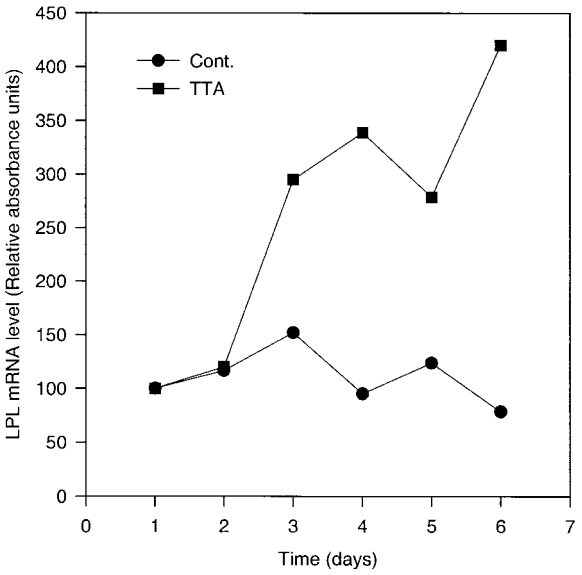

Fig. 6. Effects of TTA on LPL mRNA levels in 3T3-L1 preadipocytes cells in culture. 3T3-L1 cells were cultured as previously described and incubated for the indicated time in the presence of TTA (100 μm) and LPL mRNA level was determined as previously described (41). RNA values are expressed in relative absorbance units (RAU), taking the control ethanol value as 100%.

has been reported to reduce triacylglycerol synthesis and secretion (5) and to enhance its intracellular catabolism. Indeed, TTA potently enhances the expression and activity of ACO, along with increased peroxisome proliferation (5, 6). Moreover, increased mitochondrial proliferation (3) and mitochondrial fatty acid oxidation (5) occurs after TTA feeding of rats. Mitochondrial long chain fatty acid catabolism requires the transport of long chain fatty acids across the outer and inner mitochondrial membrane via the CPT-I and CPT-II shuttle systems. CPT-I, the outer mitochondrial membrane CPT, is considered as the rate-limiting system. Acute challenge with TTA, but not long exposure to the drug, increases the expression of CPT-I (7, 9). By contrast, the expression of CPT-II, the inner form of mitochondrial membrane CPT, is induced by long-term treatment with TTA (7). The enhancement of CPT-I expression in hepatocytes in primary culture but not in vivo in rats after 1 week of treatment with TTA is consistent with these previously reported data (7, 9). Moreover, our data indicate that the short-term TTA effect on CPT-I expression is due to a direct effect on the hepatocyte. Additionally, our observations on CPT-II expression are also consistent with previously described data (7, 9). Acetyl-CoA produced by mitochondrial β-oxidation can be used as a carbon source for anabolic processes as, for example, the synthesis of ketone bodies. Mitochondrial HMG-CoA synthase plays a key regulatory role in this process (67). Previously, we described that the expression and the activity of this enzyme are increased by TTA feeding in rats (7). The data presented here confirm these observations. Thus, in conclusion, TTA provokes a pleiotropic spectrum of actions on intravascular transport and catabolism as well as on intracellular catabolism and synthesis of triacylglycerols. These actions then combine to result in a strong decrease of plasma triacylglycerol level. The relative contributions of each possible mechanism to the overall hypotriglyceridemic effect of TTA remain, however, to be determined.

As the role of PPARα in the actions of fibrates on liver apolipoprotein gene expression is clearly established (29) and as TTA has effects similar to those of fibrates, we used transient transfection assays to evaluate whether TTA could activate PPARα. A potent concentration-dependent activation of the Gal4-hPPARα chimera by TTA was observed in HepG2 cells. Although the maximal effect of TTA is lower than the effects of reference fibrates such as Wy14643, the TTA EC_{50} for Gal4-hPPARα activation is ten times lower than that of Wy14643. This increased affinity suggests that TTA could be of pharmacological interest. The reported activation of ACO gene expression in primary rat hepatocytes (25), an effect of fibrates which is strictly dependent on PPARα activation (40), strongly suggests that TTA is also active on the endogenous wild-type PPARα. Activation of wild-type mouse PPARα by TTA has indeed been reported in CV1 cells transiently transfected with an ACO PPRE driven reporter plasmid and a mouse PPARα expression vector (28). Taken together, these data indicate that TTA acts on liver gene expression by activating PPARα.

In addition to its PPARα activation profile, we investigated whether TTA, as other fatty acids, might also activate PPARγ, which should result in a more potent triacylglycerol-lowering activity (41). However, the lack of effect of TTA on epididymal adipose tissue LPL expression observed in most of our experiments contrasts with the effect of the thiazolidinedione BRL49653 on rat adipose tissue (41) and argues against any in vivo activation of PPARγ in normal rats. This result suggests that the liver is the main TTA site of action in vivo. This is supported by the observation of a limited activation of the Gal4-hPPARγ chimera (only about 10% of the maximal effect of the reference compound BRL49653) when HepG2 cells are challenged with TTA at 30 μm, a 3- to 10-fold higher concentration than the one required for maximal effect on the Gal4-hPPARα chimera and by the lack of activity of TTA on wild-type mouse PPARγ activity measured in transient transfection experiment using CV1 cells (28). In addition, the maximal stimulation of the Gal4-hPPARα chimera by TTA in HepG2 cells was 3-fold higher than the maximal effect of the drug on the Gal4-hPPARγ chimera.

Interestingly, a significant activation of the Gal4-hPPARγ chimera (30% of the maximal effect of the reference compound BRL49653) was observed when tested in another cell context (the 3T3-L1 preadipocyte cell line), albeit at a concentration 30-fold higher than the one required for maximal activation of the Gal4-hPPARα chimera. Moreover, in these cells and in contrast to HepG2 cells, maximal activation of both chimeras by TTA was similar. The induction by TTA of the expression of the PPARγ target gene LPL in 3T3-L1 cells indicates that TTA

is able to activate the endogenous PPARγ in these cells. The discrepancies between, on the one hand, the in vitro activation by TTA of the Gal4-hPPARγ chimera and the induction of LPL expression in 3T3-L1 cells and, on the other hand, the lack of in vitro activation by TTA of the wild-type mouse PPARγ in CV1 cells (28) and the lack of in vivo effect of TTA on adipose tissue LPL gene expression can therefore not be attributed to in vitro artefacts associated with the use of chimeras lacking the A, B, and C domains of PPARs. Therefore, one likely explanation is that at the doses chosen, the bioavailability of TTA is too low in adipose tissue to activate PPARγ. Indeed, TTA has been detected in epididymal adipose tissue of rats but its concentration in this tissue was 6 times lower compared to its hepatic concentration (68). Moreover, at a concentration of 10 μm where Gal4-hPPARα activation is maximal in HepG2 cells, Gal4-hPPARγ activation is only 10% of the maximal effect of BRL 49653 in 3T3-L1 cells (compare Fig. 4A and Fig. 4B). In addition, it is likely that the efficiency of PPAR activation by a given ligand differs among tissues or cells. This is illustrated by the reduced responsiveness to Wy14643 of the Gal4-hPPARα chimera in 3T3-L1 cells relative to HepG2 cells reported in this study and by differences in transactivation by PPAR ligands in different cells previously reported (69). Such cell type difference in response to ligands might be due to cell type differences in transport or metabolism of the compounds and / or in expression of cofactors which transmit the ligand-activated PPAR signal to the transcription machinery (for review see [70, 71]). Such variations in cofactor expression levels have indeed been reported for SRC-1, p300, SMRT, and N-CoR in different tissues or cell lines (72). Finally, we cannot exclude that TTA modulates other nuclear factors which could be differently expressed in rat adipose tissue or 3T3-L1 and CV-1 cells and which could interfere with PPARγ signalling. For example, TTA also activates PPARδ when tested in CV1 cells (28).

In conclusion, the results presented in this study suggest that the main site of the lipid lowering action of TTA, a 3-thia fatty acid, is the liver where it modulates the expression of apolipoproteins and enzymes involved in lipid transport and metabolism. This reduction of apolipoprotein gene expression could, in combination with the effects of the drug on intracellular peroxisomal and mitochondrial fatty acid metabolism, account for the hypolipidemic effect of TTA. Our in vitro data indicate that TTA mainly activates PPARα, when tested at low concentrations, although a significant activation of PPARγ could be observed at high concentrations and in the appropriate cell context. Therefore, our data suggest that the lipid lowering efficiency of TTA in vivo in rats cannot be attributed to combined activation of both PPARα and PPARγ. Moreover, our data provide evidence that the activation of PPARs is dependent on the cellular context. The screening for PPAR activators should therefore be performed using cell models relevant for the in vivo target tissue. Furthermore, it should be possible to develop compounds with specific target tissue activity.

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