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Tetradecylthioacetic acid attenuates dyslipidaemia in male patients with type 2 diabetes mellitus, possibly by dual PPAR-α/δ activation and increased mitochondrial fatty acid oxidation

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Aim: We previously demonstrated that a modified fatty acid, tetradecylthioacetic acid (TTA), improves transport and utilization of lipids and increases mitochondrial fatty acid oxidation in animal and cell studies. We conducted an exploratory study of safety and effects of this novel drug in patients with type 2 diabetes mellitus and investigated the mechanism of action in human cell lines.

Methods: Sixteen male patients with type 2 diabetes mellitus received 1 g TTA daily for 28 days in an open-labelled study, with measurement of parameters of lipid metabolism, glucose metabolism and safety (ClinicalTrials.gov NCT00605787). The mechanism of action was further investigated in a human liver cell line (HepG2) and in cultured human skeletal muscle cells (myotubes).

Results: Mean LDL cholesterol level declined from 4.2 to 3.7 mmol/l ($p < 0.001$), accompanied by increased levels of the HDL apolipoproteins A1 and A2, and a decline in LDL/HDL ratio from 4.00 to 3.66 ($p = 0.008$). Total fatty acid levels declined, especially the fraction of the polyunsaturated n-3 fatty acids docosahexaenoic acid (−13%, $p = 0.002$) and eicosapentaenoic acid (−10%, $p = 0.07$). Glucose metabolism was not altered and the drug was well tolerated. In cultured liver cells, TTA acted as a pan-PPAR agonist with predominant PPAR-α and PPAR-δ activation at low TTA concentrations. In myotubes, TTA and a PPAR-δ agonist, but not the PPAR-α or PPAR-γ agonists, increased the fatty acid oxidation.

Conclusions: We demonstrate for the first time that TTA attenuates dyslipidaemia in patients with type 2 diabetes mellitus. These effects may occur through mechanisms involving PPAR-α and PPAR-δ activation, resulting in increased mitochondrial fatty acid oxidation.

Keywords: dyslipidaemia, PPAR agonist(s), TTA, type 2 diabetes mellitus
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Introduction

Obesity and obesity-related diseases, often referred to as the metabolic syndrome, is a serious condition with clustering of risk factors and disorders like dyslipidaemia, hypertension, insulin resistance and type 2 diabetes mellitus, which is associated with increased mortality and premature heart disease. This condition is increasingly prevalent, mainly as a consequence of high caloric intake and physical inactivity. Although genetic disorders can cause obesity, most cases probably result from subtle interactions between genetic and environmental factors favouring deposition of excess energy as fat. Thus, novel therapies that increase energy expenditure should be considered.

Previously, we have shown that a modified fatty acid, tetradecylthioacetic acid (TTA), is of particular interest because of its beneficial effects on lipid transport and utilization. Results from animal and cell studies indicate that TTA causes significant reduction in plasma triacylglycerol, accompanied by enhanced mitochondrial and peroxisomal fatty acid oxidation in the liver [1]. TTA also increased the mitochondrial biogenesis and conferred mitochondrial uncoupling, that is dissipation of the membrane potential without ATP production [2].

Studies into the molecular basis of insulin resistance have focused on the PPAR family of nuclear receptors. PPAR-γ is a key regulator of adipocyte differentiation and lipid storage, and is involved in the formation of foam cells from macrophages [3]. The thiazolidinediones that are used to treat type 2 diabetes mellitus are predominantly PPAR-γ agonists [4]. PPAR-δ plays a pivotal role in the control of fatty acid oxidation and utilization in both adipose tissue and skeletal muscle [5–7]. PPAR-α is primarily expressed in the liver, brown adipose tissue and skeletal muscle, and controls the expression of numerous genes related to lipid metabolism, including genes involved in mitochondrial β-oxidation, fatty acid utilization and lipoprotein transport [8]. We have found evidence for both PPAR-α-independent and PPAR-α-mediated regulation by TTA [9]. In a study with obese Zucker rats, TTA reduced adiposity and improved insulin sensitivity through mechanisms independent of PPAR-γ target genes, such as lipoprotein lipase and glucose transporter 4, but dependent on PPAR-α target genes like carnitine palmitoyltransferase (CPT) and 3-hydroxy-3-methyl-glutaryl-CoA synthase [10]. We have hypothesized that the TTA-induced consumption of fatty acids in the liver reduces the flux of fatty acids to adipose tissue and skeletal muscles, thus improving insulin sensitivity in these organs; the fatty acid drainage hypothesis [1].

Based on previous observations of TTA-mediated effects in animal and cell studies, the aim of the present study was to determine whether TTA treatment of patients with type 2 diabetes and dyslipidaemia could improve lipoprotein profile and possibly blood glucose. To this end we carried out an open-labelled 4-week clinical exploratory study of TTA treatment in male patients with type 2 diabetes mellitus. To approach an understanding of TTA's mechanism of action, we performed *in vitro* studies in a human liver cell line (HepG2) and in cultured human skeletal muscle cells (myotubes).

Methods

Design and Participants

The study was designed as a 28-day open-labelled phase II exploratory clinical study. Inclusion criteria were age 30–60 years, and type 2 diabetes mellitus with haemoglobin A1c (HbA1c) 8.0–12.0%, fasting S-triacylglycerol 2.0–10.0 mmol/l, body mass index 25–40 kg/m^2 and/or waist/hip ratio >0.90. Patients with fasting total cholesterol >10 mmol/l, blood pressure ≥170/110 mmHg or other significant disease were excluded. Any corticosteroid, anticoagulant or lipid-lowering drug had to be discontinued 2 weeks before inclusion. Blood glucose >16 mmol/l during the study was withdrawal criterion. Thirty-three male patients with type 2 diabetes mellitus were screened and gave written consent. Sixteen patients were found eligible and included in the study. They entered a 2-week washout period of oral hypoglycaemic agents and lipid-lowering agents, before they entered a 28-day treatment period with a daily morning dose of 1 g TTA. The patient population was heterogeneous with respect to lipid and glucose levels and treatment, ranging from mild type 2 diabetes (patient 13) to severe metabolic syndrome (patient 15) (Table 1, patient characteristics before washout). Three patients withdrew from the study after 2 weeks of treatment (patients 7 and 16 because of hyperglycaemia; patient 12 for unknown reason). Unused tablets were counted for evaluation of adherence to therapy.

The study was conducted in accordance with the Declaration of Helsinki and consistent with Good Medical Practice and applicable regulatory requirements. Preclinical toxicology studies with TTA in dogs and rats (unpublished data) and a phase I clinical study in humans [11] did not reveal toxic effects, and the Norwegian Medicines Agency approved the compound for testing in humans. The Regional Ethics Committee approved the study.

Table 1 Baseline patient characteristics

Patient no.	Age (years)	DM2 duration (years)	HbA1c (%)	Antidiabetic drugs	Total cholesterol (mmol/l)	Lipid-lowering drugs	BMI (kg/m^2)	Blood pressure (mmHg)
1	55	4	7.1	Metformin 500 mg × 2	4.4	None	30.3	160/90
2	68	4	6.9	Glipizid 5 mg × 1	4.9	None	25.4	160/80
3	66	4	7.6	Metformin 500 mg × 2	5.1	Atorvastatin 10 mg × 1	30.7	150/80
4	62	5	7.4	Diet	5.6	Simvastatin 10 mg × 1	26.4	160/100
5	49	2	8.7	Diet	6.7	None	28.4	125/85
6	55	5	9.3	Diet	5.3	Omega 3	26.8	170/85
7	58	5	7.9	Daonil 3.5 + 1.75	6.9	None	28.2	140/90
8	55	4	7.4	Metformin 500 mg × 3	4.6	None	35.3	140/85
9	54	4	7.2	Glimepirid	4.3	None	34.0	140/85
10	62	8	7.1	Metformin 500 mg × 2	6.1	None	32.6	140/80
11	52	3	8.4	Diet	7.9	None	24.7	130/85
12	44	3	8.4	Glimepirid 2 mg × 1	6.2	None	31.9	145/100
13	42	2	6.6	Diet	5.8	None	26.5	120/80
14	46	3	6.9	Metformin 500 mg × 2	6.2	None	24.4	125/90
15	50	4	9.8	Metformin 500 mg × 2	6.4	Simvastatin 20 mg × 1	35.9	140/90
16	42	3	7.6	Metformin 500 mg × 4	5.7	None	25.1	110/80

DM2, type 2 diabetes mellitus; HbA1c, haemoglobin A1c; BMI, body mass index.

Laboratory Analyses and Assays

At all visits, the patients met in the morning to give fasting blood samples. Clinical biochemistry routine samples (Table 2) were analysed in serum by standard methods at the Department of Clinical Biochemistry and the Hormone Laboratory, Haukeland University Hospital. For analysis of fatty acid composition, lipids were extracted from EDTA plasma samples using a mixture of chloroform and methanol [12]. Heneicosanoic acid was added to the extracts as internal standard. The extracts were transesterified using boron fluoride–methanol [13]. To remove neutral sterols and non-saponifiable material, the extracts were heated in 0.5 M KOH in ethanol–water solution (9 : 1). Recovered fatty acids were re-esterified using boron fluoride–methanol. The methyl esters were quantified by gas chromatography equipped with a mass spectrometer as previously described [14]. Triacylglycerol, total cholesterol, HDL cholesterol and LDL cholesterol in serum were measured enzymatically on a modular instrument from Roche (Basel, Switzerland). Apolipoprotein B was measured in serum by an immunological nephelometric assay on a Nephelometer from Dade Behring (Marburg, Germany). The serum levels of apolipoproteins A1 and A2 were determined with a LINCOplex assay (LINCO, St Charles, MO, USA) on a Bioplex 200 instrument (Bio-Rad, Hercules, CA, USA). The level of vitamin E (α-tocopherol) in plasma was measured by high-performance liquid chromatography as previously described [15].

Table 2 Clinical biochemistry routine analyses in serum at start (day 0) and end (day 28) of TTA treatment, mean (s.d.)

	Start of treatment (n = 16)	End of treatment (n = 16)	p value
Fasting blood glucose, nmol/l	10.5 (1.65)	10.6 (1.75)	>0.20
Haemoglobin A1c, %	8.1 (0.94)	8.3 (1.00)	0.17
Insulin C-peptide, mmol/l	0.71 (0.35)	0.98 (0.33)	0.003
Haemoglobin, g/dl	16.1 (0.83)	15.3 (0.94)	0.001
Leucocyte count, 10^{-12}/l	6.18 (1.66)	6.07 (1.60)	>0.20
Thrombocyte count, 10^{-9}/l	232 (50.5)	238 (48.2)	0.08
Micro-CRP, mg/l	2.7 (2.5)	1.9 (1.4)	>0.20
Fibrinogen, g/l	3.24 (0.41)	2.95 (0.49)	0.04
INR	0.93 (0.07)	0.98 (0.07)	>0.20
Creatinine, μmol/l	87 (7.6)	89 (7.7)	>0.20
Urea, mmol/l	5.2 (1.38)	6.2 (1.34)	0.02
Sodium, mmol/l	140 (1.8)	141 (1.5)	>0.20
Potassium, mmol/l	4.5 (0.25)	4.4 (0.31)	>0.20
Calcium, mmol/l	2.50 (0.42)	2.31 (0.06)	0.005
Phosphate, mmol/l	1.07 (0.15)	0.96 (0.13)	0.004
Uric acid, μmol/l	345 (18.6)	333 (18.3)	>0.20
Aspartate transaminase, U/l	30 (12.2)	28 (9.5)	>0.20
Alanine transaminase, U/l	47 (22.6)	41 (21.6)	>0.20
γ-glutamyl transpeptidase, U/l	47 (46.5)	40 (28.0)	>0.20
Alkaline phosphatase, U/l	172 (49.0)	158 (49.1)	>0.20
Bilirubin, μmol/l	13.6 (3.8)	11.1 (2.3)	>0.20
Albumin, g/l	47 (1.7)	45 (1.8)	0.01
Creatinine kinase, mmol/l	147 (105.4)	170 (111.7)	0.08

P values from Wilcoxon matched pairs signed rank sum test.
INR, international normalized ratio (prothrombin time).

Culturing and Transient Transfection of Human Hepatoma Cells

The human hepatoma cell line, HepG2, was cultured in Eagle's minimum essential medium (Bio Whittaker, Walkersville, MD, USA) supplemented with 10% foetal bovine serum, L-glutamine (2 mmol/l), penicillin (50 IU/ml), streptomycin (50 μg/ml) and non-essential amino acids. They were kept at 37 °C in a humidified incubator with 5% CO_2. HepG2 cells were seeded in a 12-well plate (Corning Costar Corp., Cambridge, MA, USA; 10^5 cells/well) and transiently transfected the following day by the SuperFect transfection procedure according to the manufacturer's protocol (Qiagen, Hilden, Germany). Transfection was carried out with 0.9 μg PPAR response element (PPRE)x3-LUC reporter plasmid, 0.15 μg pcDNA3.1 hPPAR-α/δ/γ and 0.95 μg empty vector pCMV-5. Twenty-four hours after transfection, the cells were treated with specific PPAR agonists (PPAR-α; 30 μmol/l WY14.643, PPAR-δ; 0.5 μmol/l L165.041, PPAR-γ; 0.5 μmol/l rosiglitazone) or 10, 30 or 75 μmol/l TTA for 24 h. The cells were then washed with phosphate-buffered saline (PBS) and lysed by 80 μl of a lysis buffer containing 25 mmol/l Tris–acetate–EDTA buffer, 2 mmol/l dithiothreitol, 10% (v/v) glycerol and 1% (v/v) Triton X-100. The cell extracts were assessed for luciferase activity on a LUCY-1 luminometer (Anthos, Salzburg, Austria). The luciferase assay was performed in accordance with the protocol of the Luciferase Assay Kit (BIO Thema AB, Dalarö, Sweden). Each transfection was performed in duplicate.

The PPREx3-LUC reporter construct, containing three copies of the PPRE in the promoter of a luciferase gene, as well as the expression vectors encoding PPAR-α, PPAR-δ and PPAR-γ_1 were a kind gift from Dr K. Kristiansen, University of Odense, Denmark. The empty vector pCMV-5 was kindly provided by Dr S. Anderson, Stockholm University, Sweden.

Measurement of Fatty Acid Oxidation in Human Skeletal Muscle Cells

A cell bank of satellite cells has previously been established from muscle biopsies of the *musculus obliquus internus abdominis* of healthy donors obtained after informed consent and approved by ethics committee [16]. Human myoblasts were grown and differentiated in 96-well plates as previously described [17,18]. The cells were allowed to differentiate to myotubes at a physiological concentration of insulin (25 pmol/l) and glucose (5.5 mmol/l) for 5 days before they were preincubated with either vehicle [bovine serum albumin (BSA) 0.133–133 μmol/l] or TTA (0.32–320 μmol/l) for 24 h. Myotubes were then exposed to PBS with Ca^{2+} and Mg^{2+} supplemented with 20 mmol/l HEPES (pH 7.4 at 37 °C), 5 mmol/l glucose, 40 μmol/l BSA, 1 mmol/l L-carnitine and [1-^{14}C]oleic acid (1 μCi/ml, 100 μmol/l) for 4 h with trapping of [1-^{14}C]CO_2 produced from oxidation [18]. The level of [1-^{14}C]CO_2 relative to total uptake of [1-^{14}C]oleic acid (cell associated and oxidized) was used as a measure of fatty acid oxidation. Similar protocols were used to study the effects of the selective agonists of PPAR-α (WY14.643 and clofibrate), PPAR-δ (GW501516) and PPAR-γ (rosiglitazone).

Myotubes were treated with TTA or vehicle (BSA) for 96 h, then RNA was isolated and real-time quantitative PCR performed with conditions and reagents as specified by Gaster *et al.* [17]. Gene expression was determined by SYBR Green (Applied Biosystems, Foster City, CA, USA) with the following primers designed by Primer Express (Applied Biosystems): CD36/fatty acid translocase (CD36/FAT; accession no. L06850; F: AGTCACTGCGACATGATTAATGGT and R: CTGCAATACCTGGCTTTTCTCAA) and CPT-I (accession no. L39211; F: CGGTGGAACAGAGGCTGAA and R: CGAGGCGATACATATGCTGATG). The expression was quantified in quadruplicate and carried out in a 25 μl reaction volume according to the supplier protocol. All assays were run for 40 cycles (95 °C for 12 s followed by 60 °C for 60 s). The transcription levels were normalized to the housekeeping control gene β-actin (accession no. M28424; F: ACCGAGCGCGGCTACA and R: TCCTTAATGTCACGCACGATTT).

Statistical Analysis

All efficacy variables and safety variables in the clinical study were analysed by Wilcoxon matched pairs signed rank sum test comparing the levels before and after the treatment period, considered statistically significant when $p < 0.05$, and 95% confidence intervals for the mean effects were estimated. In case of missing values (withdrawn patients), the last measurement available was carried forward. HepG2 and myotube data are presented as mean ± s.d. The *in vitro* data were evaluated by two-tailed Student's *t*-test.

Results

Effects of TTA on Circulating Levels of Lipoproteins, Lipids and Fatty Acids

Four-week TTA treatment of male patients with type 2 diabetes mellitus significantly reduced the serum levels of total cholesterol (figure 1A; mean change: −0.41 mmol/l, 95% CI: −0.56 to −0.24, $p = 0.0002$ by

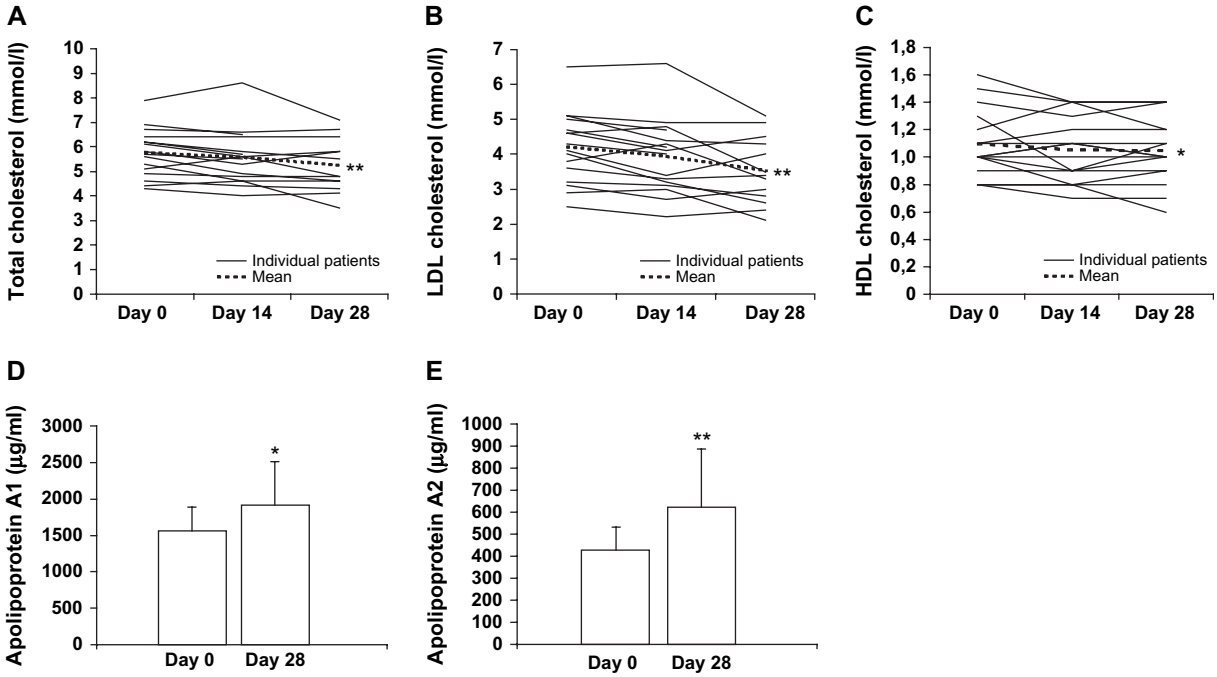

Fig. 1 Changes during 14 and 28 days after tetradecylthioacetic acid treatment in serum/plasma levels of (A) total cholesterol; (B) LDL cholesterol; (C) HDL cholesterol; (D) apolipoprotein A1 and (E) apolipoprotein A2, mean concentrations and s.d. *$p < 0.05$, **$p < 0.01$ compared with baseline values (Wilcoxon matched pairs signed rank sum test).

Wilcoxon matched pairs signed rank sum test) and LDL cholesterol [figure 1B; −0.52 mmol/l (−0.74 to −0.30), $p < 0.001$]. The serum level of HDL apolipoproteins A1 and A2 increased significantly (figures 1D, E), whereas the HDL cholesterol level showed a minor decline of borderline statistical significance [figure 1C; −0.05 mmol/l (−0.09 to −0.01), $p = 0.05$]. The LDL/HDL ratio declined significantly from 4.00 to 3.66 [absolute ratio reduction −0.34 (−0.55 to −0.14), $p = 0.008$].

The serum triacylglycerol level tended to decrease after TTA treatment (figure 2A; −0.27 mmol/l [−0.53 to 0.00], $p = 0.09$) and circulating apolipoprotein B (figure 2B; −0.08 µg/dl, $p = 0.01$) and vitamin E (figure 2C; −2.8 µmol/l, $p = 0.02$) declined significantly following TTA treatment.

The total fatty acid level in plasma declined after TTA treatment (figure 3A), statistically significant when one obvious outlier (patient 15) was excluded from the statistical analysis ($p = 0.023$). The relative reduction was largest for the polyunsaturated n-3 fatty acids docosahexaenoic acid (DHA) (−13%, $p = 0.023$; figure 3B) and eicosapentaenoic acid (EPA) (−10%, $p = 0.25$; figure 3C). TTA was recovered in plasma of all the patients during treatment [39 µmol/l (s.d. 32)]. Apart from patient 15 (140 µmol/l) the interindividual variation was minor [mean 31 µmol/l (s.d. 13)]. The delta-9 desaturated metabolite of TTA, TTA:1 n-8, was also found in plasma of these patients.

Effects of TTA on Glucose Metabolism, Inflammation, Bodyweight and Blood Pressure

Blood glucose levels did not increase significantly during the washout period [10.1 (2.1) to 10.5 (2.7), $p > 0.20$]. As shown in Table 2, fasting blood glucose levels and HbA1c did not change during 4 weeks of TTA treatment, whereas mean insulin C-peptide value increased significantly, within the normal range; reaching a plateau after 2 weeks of treatment. Overall, inflammation as evaluated by micro-C-reactive protein (micro-CRP) was unchanged (Table 2). Bodyweight did not change during treatment [96.3 (15.9) to 96.3 (15.9) kg, $p > 0.20$]. Diastolic blood pressure fell significantly during TTA therapy [88 (10.0) to 83 (8.3) mmHg, $p = 0.008$], whereas systolic blood pressure remained unchanged [136 (16.8) to 133 (18.9) mmHg, $p > 0.20$].

Safety

All the safety parameters were within normal range before and during treatment with TTA, as shown in Table 2. Haemoglobin levels declined significantly and CK

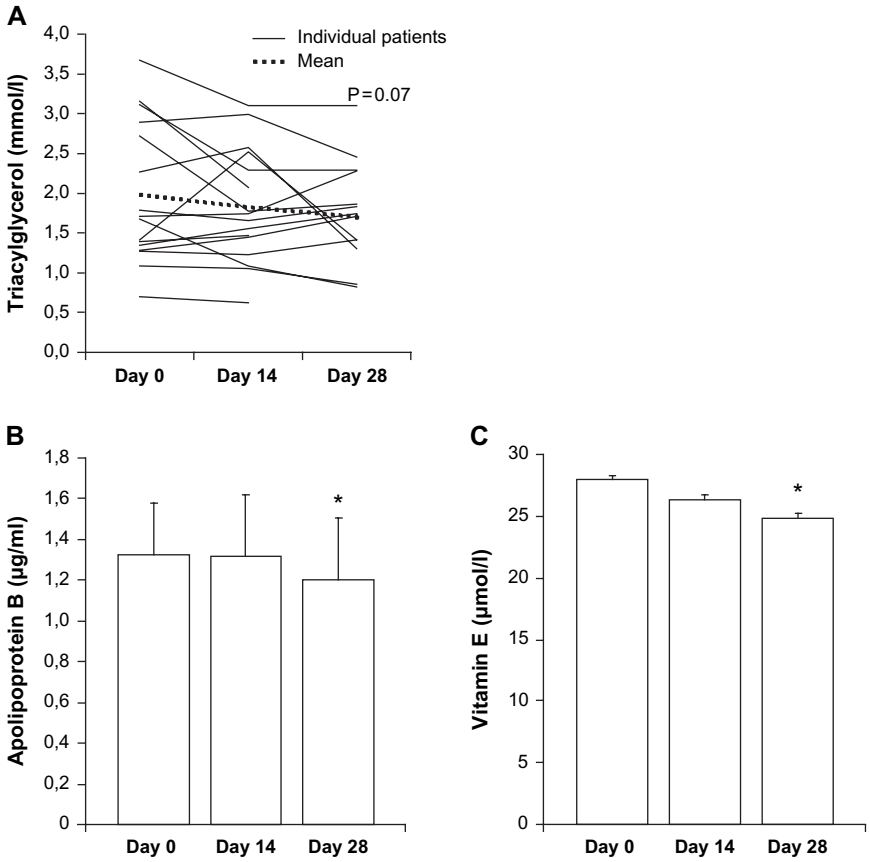

Fig. 2 Changes during 14 and 28 days after tetradecylthioacetic acid treatment in serum/plasma levels of (A) triacylglycerol; (B) apolipoprotein B and (C) vitamin E, mean concentrations and s.d. *$p < 0.05$, **$p < 0.01$ compared with baseline values (Wilcoxon matched pairs signed rank sum test).

increased with borderline significance, although both well within the reference range. The haemoglobin fall was because of a significant reduction in erythrocyte count, whereas mean cell haemoglobin levels, markers of haemolysis (haptoglobin, lactate dehydrogenase, bilirubin, reticulocytes, data not shown) and leucocyte or thrombocyte counts remained unaltered. There was a moderate but significant increase in urea levels, but creatinine levels were unaltered. Both calcium (also albumin corrected calcium, data not shown) and phosphate levels declined significantly. No adverse events with likely association with TTA intake were reported. Overall, TTA was well tolerated.

In Vitro Mechanistic Studies of TTA

HepG2 cells were transiently transfected with expression vectors encoding PPAR-α, PPAR-δ or PPAR-γ, as well as a luciferase driven reporter, enabling determination of how various doses of TTA affected the PPAR-dependent transcriptional activity (figure 4). At the lowest TTA concentration (10 µmol/l), there was a significant increase in PPAR-α and PPAR-δ activities, the former being more prominent. The activity of all PPARs was similarly affected by 30 µmol/l of TTA, although only the PPAR-δ effect was statistically significant. A significant activation of PPAR-γ required the highest TTA concentration (75 µmol/l).

In cultured human skeletal muscle cells (myotubes), 24-h preincubation with TTA significantly enhanced production of [1-^{14}C]CO_2 from oxidation of [1-^{14}C]oleic acid in a dose-responsive manner (figure 5A). The change in mitochondrial oxidation induced by TTA showed a similar maximal level of efficacy as with the selective PPAR-δ agonist GW501516 (figure 5B). However, the specific agonists of PPAR-α, clofibric acid and WY14.643, did not increase mitochondrial fatty acid oxidation in the myotubes, except for a modest increase with the highest concentration of WY14.643 (figure 5B). Likewise, the PPAR-γ agonist rosiglitazone did not

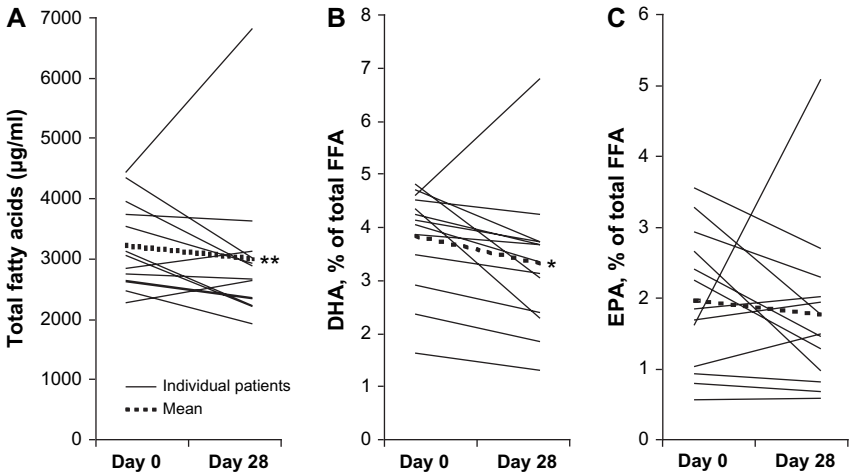

Fig. 3 Plasma fatty acid changes during 14 and 28 days after tetradecylthioacetic acid treatment. (A) Total fatty acids (one outlier excluded from statistical analysis); (B) docosahexaenoic acid (DHA) and (C) eicosapentaenoic acid (EPA) levels. *$p < 0.05$, **$p < 0.01$ compared with baseline values (Wilcoxon matched pairs signed rank sum test).

affect [1-^{14}C]CO_2 production (data not shown). Furthermore, incubation with TTA for 96 h increased the expression of the established PPAR target genes, CD36/FAT and CPT-I (figure 5C).

Discussion

A few PPAR agonists are currently approved and used for treatment of hyperlipidaemia and type 2 diabetes mellitus. The fibrates and thiazolidinediones are potent agonists, which are selective activators of PPAR-α and PPAR-γ, respectively; but no specific PPAR-δ agonist is yet approved for treatment of humans [3,4,8]. Several compounds with dual PPAR activation have been tested in clinical trials but have not come to clinical use [19]. TTA is a pan-PPAR agonist of moderate potency with predominant PPAR-α and PPAR-δ activation, hence representing a novel and promising treatment strategy for dyslipidaemia. In this short-term exploratory clinical study in male patients with type 2 diabetes mellitus, we

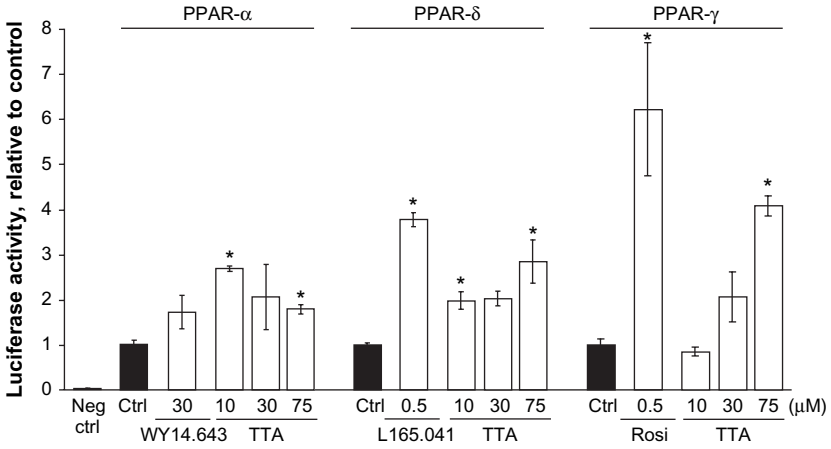

Fig. 4 Stimulation of PPAR-dependent transcriptional activity was measured by a luciferase-based reporter assay in transiently transfected HepG2 cells. The negative control (neg ctrl) includes the transfection of reporter plasmid but no PPAR expression vector. The cells were treated with vehicle (ctrl), specific PPAR agonists (PPAR-α; 30 µmol/l WY14.643, PPAR-δ; 0.5 µmol/l L165.041, PPAR-γ; 0.5 µmol/l rosiglitazone) or tetradecylthioacetic acid (10, 30 and 75 µmol/l) for 24 h. Data are given as mean ± s.d. of duplicate transfections from one of three representative experiments. *Significantly different from control, $p < 0.05$. Rosi; rosiglitazone.

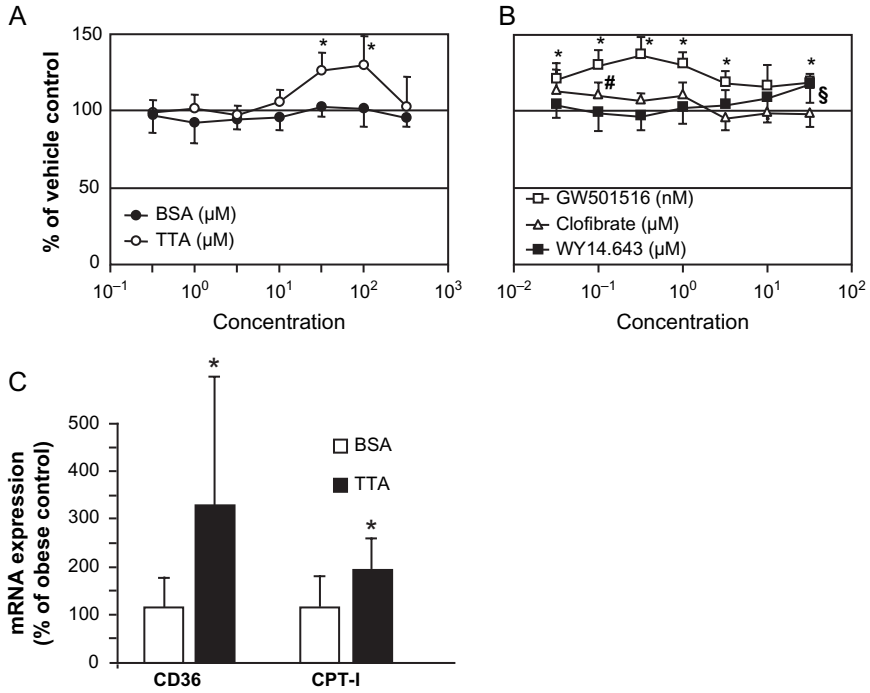

Fig. 5 Fatty acid oxidation in cultured human skeletal muscle cells. Four hours [1-^{14}C]-CO_2 production from oxidation of 100 μmol/l [1-^{14}C]-OA after 24-h incubation with different concentrations of (A) bovine serum albumin and tetradecylthioacetic acid (TTA), (B) GW501516, clofibrate and WY14.643. Data are expressed as mean ± s.d. (n = 6) relative to (A) differentiation medium and (B) vehicle control (set to 100%) of [1-^{14}C]-CO_2 production normalized against total uptake of [1-^{14}C]-oleic acid (accumulated and oxidized). (C) Myotube expression of the PPAR target genes CD36/fatty acid translocase and carnitine palmitoyltransferase-I (CPT-I) after 96-h treatment with bovine serum albumin (BSA) or TTA. *, Significantly different from control; #, clofibrate significantly different from control; §, WY14.643 significantly different from control, $p < 0.05$.

show for the first time that TTA attenuates diabetes dyslipidaemia. These results are in agreement with findings of improved lipid and lipoprotein profiles in experimental animal models [1] and in short-term clinical studies in human immunodeficiency virus-infected patients on highly active antiretroviral therapy [20]. Furthermore, our results add to the phase I study results indicating that short-term exposure of the drug is well tolerated [11].

TTA reduced the serum levels of cholesterol in our patients, in particular LDL cholesterol, resulting in a clinically relevant reduction of the LDL/HDL cholesterol ratio. The LDL-lowering effect was further supported by a reduction of apolipoprotein B, which is the principal apolipoprotein of the LDL particle. Conversely, the levels of apolipoproteins A1 and A2 increased, indicating increased reverse cholesterol transport. The initial steps of this transport include transfer of cholesterol via apolipoprotein A1 from peripheral cells to nascent premature HDL, with a subsequent maturation of the HDL particle, where both apolipoproteins A1 and A2 are needed. It is possible that the short time-span of the study is the reason why HDL cholesterol did not increase. Apolipoprotein B is also involved in assembly of the VLDL particle, which is rich in triacylglycerol. Vitamin E is transported in plasma in association with lipoproteins, especially in VLDL [21], and its concentration is reported to correlate with the concentration of plasma lipids [22]. Therefore, decreased level of apolipoprotein B and vitamin E supports the observed tendency of reduced serum triacylglycerol level in these patients after TTA treatment.

We have previously shown that TTA improves insulin sensitivity and reduces adiposity in rats [10]. In the present short-term study in humans, we did not observe any effects on glucose metabolism but the plasma concentrations of TTA in the rats were much higher than those yielded in humans. However, drainage of fatty acids by the liver is shown to be an important effect of TTA in animals [1], lowering the level of fatty acids in the blood stream and relieving the flux of fatty acids to skeletal muscle, where fatty acids inhibit glucose uptake and utilization [1,23]. In this article, we

demonstrate reduction in plasma total fatty acids, in particular EPA and DHA, after TTA treatment. We have previously shown that after TTA treatment of rats, a higher oxidation rate of n-3 fatty acids may cause decreased levels of hepatic EPA and DHA [24], which is in accordance with the reduction in plasma levels of these fatty acids observed here. We also demonstrate increased capacity of fatty acid oxidation after incubation of cultured human skeletal muscle cells with low concentrations of TTA. Being the major tissues of glucose uptake, increased fatty acid oxidation in liver and muscle suggests that long-term TTA treatment might improve insulin sensitivity, but this remains to be investigated in further studies.

Preclinical studies in rodents have indicated that TTA acts through mechanisms that at least partly involve PPAR-α activation [1]. In this clinical study, many of the effects also point in direction of PPAR-α activation, with a similar pattern of lipid-lowering effects as observed in type 2 diabetic patients after treatment with fenofibrate, which is a selective PPAR-α agonist [25]. Furthermore, the reduction of fibrinogen levels and no short-term glucose-lowering effect are consistent with a PPAR-α-activating mode of action *in vivo* [8]. In addition, PPAR-α is known to increase transcription of apolipoproteins A1 and A2, as well as to increase hydrolysis of VLDL particles [8], as observed in our patients.

Both PPAR-α and PPAR-δ play important roles in lipid metabolism in liver and skeletal muscle [5–8], and share some target genes, such as CPT-I and FAT/CD36, controlled by PPAR-α in liver and by PPAR-δ in muscle [26–28]. Interestingly, a recent report by Risérus *et al.* [29] demonstrates that a reduction of LDL cholesterol and apolipoprotein B in moderately obese men could be assigned to the effect of a PPAR-δ agonist but not a PPAR-α agonist. Furthermore, our *in vitro* studies show that TTA acts as a pan-PPAR ligand in human liver cells, which is in accordance with previous studies in cell lines other than HepG2 [30,31]. However, the potency of TTA-mediated PPAR transactivation and which of the PPAR subtypes being most potently activated by TTA vary with cell type. In HepG2 cells, we observed a predominant PPAR-α and PPAR-δ activation at the lowest TTA concentrations. In cultured human skeletal muscle cells, both TTA and the PPAR-δ agonist GW501516 increased fatty acid oxidation, whereas both PPAR-α agonists did not, except for a modest increase with the highest concentration of WY14.643. Indeed, incubation with TTA significantly upregulated the PPAR target genes CPT-I and FAT/CD36, which are central regulators of the mitochondrial fatty acid oxidation, and regarded as PPAR-δ target genes in skeletal muscle. Thus, it is tempting to hypothesize that the lipid-lowering effect of TTA, as observed previously in animals [1] and now in patients with type 2 diabetes mellitus, involves a dual action of both PPAR-α and PPAR-δ, with possible increase in fatty acid oxidation both in liver and skeletal muscle.

The evaluation of the clinical effects in the current study is limited by the study design. The open-labelled design was chosen because safety was as an important issue and aim only to explore the clinical effects in humans. As this was the first exposure of TTA to patients, the allowed duration of TTA treatment was restricted to 28 days. The washout of other hypoglycaemic and lipid-lowering drugs before the study did not have major impact on glucose and lipid levels, but further studies should be conducted in a more stable metabolic condition. As this and other studies [20] have now indicated clinically relevant effects on lipid metabolism and that the drug is well tolerated [11,20], a double-blind placebo-controlled trial of longer duration including also females is clearly warranted to further establish the true effects of TTA treatment in diabetes dyslipidaemia.

There has been much concern about the safety of PPAR agonists as illustrated by a recent report of possible association of treatment with the PPAR-γ agonists rosiglitazone, with increased risk of myocardial infarction and death from cardiovascular causes [32]. Trials with fibrates have indicated adverse cardiovascular effects when combined with statins [33]. Furthermore, trials in humans with combined PPAR-α/γ agonists were discontinued because of reports of urinary tract cancers in rodents [19]. Although short-term studies in humans have shown no adverse effects TTA at this stage, long-term safety of TTA treatment must be closely monitored in further trials.

In conclusion, we have demonstrated for the first time that TTA attenuates dyslipidaemia in patients with type 2 diabetes mellitus during a 4-week period, and that the treatment is well tolerated. The results suggest that these effects may occur through mechanisms involving PPAR-α and/or PPAR-δ activation, resulting in increased mitochondrial fatty acid oxidation. Further studies will have to address long-term effects on glucose metabolism, body composition and blood pressure, as well as to unravel the mechanism of action.

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