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The molecular structure of thio-ether fatty acids influences PPAR-dependent regulation of lipid metabolism

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ABSTRACT

Thio-ether fatty acids (THEFAs), including the parent 2-(tetradecylthio)acetic acid (TTA), are modified fatty acids (FAs) that have profound effects on lipid metabolism given that they are blocked for β-oxidation, and able to act as peroxisome proliferator-activated receptor (PPAR) agonists. Therefore, TTA in particular has been tested clinically for its therapeutic potential against metabolic syndrome related disorders. Here, we describe the preparation of THEFAs based on the TTA scaffold with either a double or a triple bond. These are tested in cultured human skeletal muscle cells (myotubes), either as free acid or following esterification as phospholipids, lysophospholipids or monoacylglycerols. Metabolic effects are assessed in terms of cellular bioavailabilities in myotubes, by FA substrate uptake and oxidation studies, and gene regulation studies with selected PPAR-regulated genes. We note that the inclusion of a triple bond promotes THEFA-mediated FA oxidation. Furthermore, esterification of THEFAs as lysophospholipids also promotes FA oxidation effects. Given that the apparent clinical benefits of TTA administration were offset by dose limitation and poor bioavailability, we discuss the possibility that a selection of our latest THEFAs and THEFA-containing lipids might be able to fulfill the therapeutic potential of the parent TTA while minimizing required doses for efficacy, side-effects and adverse reactions.

1. Introduction

In the past decade, it has been found that structurally modified sulfur-containing thio-ether fatty acids (THEFAs) are more potent than natural fatty acids (FAs) in modulating critical steps in the regulation of lipid metabolism.[1] In particular, the THEFA known as 2-(tetradecylthio)acetic acid (TTA) **1** was found especially potent as a regulator of lipid metabolism (Fig. 1).[2,3] TTA **1** can be described as palmitic acid with a sulfur atom inserted between carbon atoms 2 and 3, or as septadecanoic acid with a sulfur atom in position 3 of the FA main chain, introduced as a means to limit β-oxidation. THEFAs studied with their sulfur atom positioned at higher odd number positions in the FA main chain were found more prone to β-oxidation than TTA **1**. Given the clear resistance

Abbreviations: THEFA, thio-ether fatty acid; FA, fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; TTA, 2-(tetradecylthio)acetic acid; TTA-PC, 1,2-di-[2-(tetradecylthio)acetyl]-*sn*-glycero-3-phosphocholine; TTA-MAG, 1-[2-(tetradecylthio)acetyl]-*sn*-glycerol; dTTA, 2-(tetradec-[7-enyl]thio)acetic acid; dTTA-PC, 1,2-di-[2-(tetradec-[7-enyl]thio)acetyl]-*sn*-glycero-3-phosphocholine; *lyso*-dTTA-PC, 1-[2-(tetradec-[7-enyl]thio)acetyl]-*sn*-glycero-3-phosphocholine; dTTA-MAG, 1-[2-(tetradec-[7-enyl]thio)acetyl]-*sn*-glycerol; tTTA, 2-(tetradec-[7-ynyl]thio)acetic acid; tTTA-PC, 1,2-di-[2-(tetradec-[7-ynyl]thio)acetyl]-*sn*-glycero-3-phosphocholine; *lyso*-tTTA-PC, 1-[2-(tetradec-[7-ynyl]thio)acetyl]-*sn*-glycero-3-phosphocholine; tTTA-MAG, 1-[2-(tetradec-[7-ynyl]thio)acetyl]-*sn*-glycerol; OA, oleic acid; *ANGPTL4*, angiopoietin-like 4; *FABP3*, fatty acid binding protein 3; *CD36*, fatty acid translocase; *CPT1A*, carnitine palmitoyltransferase 1A; NR, nuclear receptor; CA, cell-associated radioactivity.

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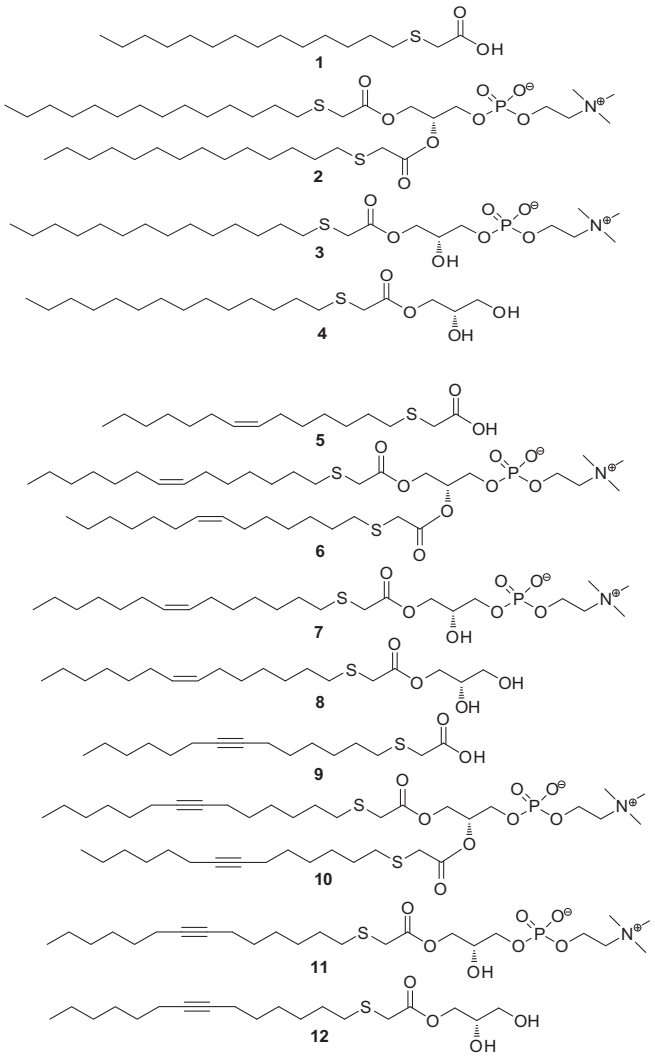


Figure 1. Chemical structures of THEFAs plus their corresponding glycerolipids synthesized and used here in the following biological studies. The first is TTA **1** with corresponding glycerolipids TTA-PC **2**, *lyso*-TTA-PC **3** and 1-[2-(tetradecylthio) acetyl]-*sn*-glycerol (TTA-MAG) **4**; plus 2-(tetradec-[7-enyl]thio)acetic acid (dTTA) **5** and corresponding glycerolipids 1,2-di-[2-(tetradec-[7-enyl]thio)acetyl]-*sn*-glycero-3-phosphocholine (dTTA-PC) **6**, 1-[2-(tetradec-[7-enyl]thio)acetyl]-*sn*-glycero-3-phosphocholine (*lyso*-dTTA-PC) **7**, 1-[2-(tetradec-[7-enyl]thio)acetyl]-*sn*-glycerol (dTTA-MAG) **8**; plus 2-(tetradec-[7-ynyl]thio)acetic acid (tTTA) **9** and corresponding glycerolipids 1,2-di-[2-(tetradec-[7-ynyl]thio)acetyl]-*sn*-glycero-3-phosphocholine (tTTA-PC) **10**, 1-[2-(tetradec-[7-ynyl]thio)acetyl]-*sn*-glycero-3-phosphocholine (*lyso*-tTTA-PC) **11**, 1-[2-(tetradec-[7-ynyl]thio)acetyl]-*sn*-glycerol (tTTA-MAG) **12**.

of TTA **1** to β-oxidation, this THEFA has been demonstrated to have many useful metabolic properties over the past 15 years.[1]

In mechanistic terms, TTA **1** has been found to act on mitochondria in cells. Broadband pleiotropic biological responses include increased hepatic and muscle mitochondrial FA oxidation in mammals, reduced body fat (as seen in rats), decreased plasma lipid levels, modification of plasma and tissue FA profiles, improved insulin sensitivity, potent antioxidant effects, and anti-inflammatory actions. Underlying many of these effects may be the fact that TTA **1** is a ligand for peroxisome proliferator-activated receptors (PPARs). Indeed, post reporter cell transfection, TTA **1** was found to induce reporter gene activities at concentrations comparable to those of the known PPARα ligand WY 14.643, but at concentrations markedly lower than required with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[1] PPARs have for the last decades been targeted in drug discovery for the treatment of metabolic diseases because of their critical role in the regulation of lipid metabolism and fat cell differentiation. Natural ligands of the PPARs include a wide variety of saturated and unsaturated FAs plus eicosanoid derivatives. As a PPAR ligand, TTA **1** is known to modulate the expression of lipid-metabolizing enzymes involved in catabolic pathways, all in a PPAR dependent manner.[4–7] This array of biological responses has suggested that TTA **1** could be a potent therapeutic agent matched to a variety of indications.[1] Accordingly, clinical trials have been carried out that indicate that TTA **1** could beneficially affect plasma lipid levels and inflammatory parameters in humans.[8–10] However, although TTA **1** operates by clear mechanisms involving PPARα agonism (and to some extent δ and γ agonism too) with modest acute toxicity,[11] the clinical development of this compound has been significantly impaired by low water solubility and poor bioavailability due to poor absorption, distribution, and excretion properties.[12] Hence, the ultimate clinical indication for TTA **1** use and the most appropriate form and means of administration remain to be established.

Following this, we reported fairly recently on a comparison of biological effects following peroral administration of free TTA **1**, TTA-containing phosphatidylcholine (1,2-di-[2-(tetradecylthio) acetyl]-*sn*-glycero-3-phosphocholine, TTA-PC) **2** and TTA-containing triacylglycerol (1,2,3-tri-[2-(tetradecylthio)acetyl]-*sn*-glycerol, TTA-TAG) to an obese, male Wistar rat model (Fig. 1).[13] Intriguingly, the TTA-PC **2** demonstrated metabolic properties (i.e., lowering triacylglycerol and cholesterol levels in plasma plus hepatic triacylglycerol levels; increasing rates of oxidation of palmitoyl-CoA; enhancing carnitine palmitoyltransferase II, 3-hydroxy-3-methylglutaryl-CoA synthase, and fatty acyl-CoA oxidase activities) that were much enhanced relative to the situation observed with 2 mol equiv of TTA **1**. On the other hand, effects of TTA-TAG were essentially indistinguishable from effects mediated by 3 mol equiv of TTA **1**. Therefore, administration of TTA-PC **2** appeared to be a very useful chemical formulation for TTA **1** in terms of improving TTA **1** bioavailability and TTA-mediated biological effects in particular FA oxidation and lipid lowering capacity. Subsequently, we were able to demonstrate that TTA-containing lysophosphatidylcholine (1-[2-(tetradecylthio)acetyl]-*sn*-glycero-3-phosphocholine, *lyso*-TTA-PC) **3** (Fig. 1) was even more potent as a PPARα agonist than TTA **1** itself when administered to MCF-7 cells in vitro, suggesting that *lyso*-TTA-PC **3** might be a key active form of TTA **1** in vivo either in terms of PPAR interactions or in terms of promoting TTA **1** bioavailability for intracellular PPAR interactions.[14]

One reason for these data could be that TTA **1** functions as any other FA and is readily incorporated in vivo into glycerolipids, with a preference for phospholipids over acylglycerols. In fact TTA **1** is frequently found esterified with cholesterol in lipoprotein particles.[15] Also, during intestinal digestion, lipids are typically metabolized in the gut into monoacylglycerols, lysophospholipids, and free FAs prior to absorption into enterocytes. Here we report on the next stage of investigation comparing biological effects of TTA **1** with effects mediated by other THEFAs and corresponding THEFA-containing lipids. These new THEFAs are prepared with double or triple bond unsaturations in their hydrocarbon chains. The reason for such inclusions is as follows. TTA **1** with a double bond is a metabolite formed in vivo when feeding animals with this analog.[15] Therefore, we speculated that inclusion. of such a double bond unsaturation might be beneficial to biological effects by providing an additional structural chemical block to β-oxidation, and potentially enhance PPAR binding in order to promote PPAR agonism. Furthermore, we speculated that inclusion of a triple bond might further potentiate PPAR agonism by providing the chemical structural means to attenuate β-oxidation even more effectively and enhance PPAR interactions. Here we describe synthetic routes to these THEFAs and for the preparation of

corresponding THEFA-containing phospholipids and monoacylglycerols (see Fig. 1). Thereafter, we describe the testing of these compounds in vitro in cultured human myotubes, a well-established cell system for characterization of FA metabolism such as FA uptake and oxidation.[16] Data suggest that *lyso*-TTA-PC **3** and triple bond THEFA structures could be useful as next generation TTA **1** replacements for use in clinical scenarios in due course.

2. Results

2.1. Synthetic chemistry

New THEFAs and THEFA-containing glycerolipids (**2–12)** were prepared for comparison with TTA **1** as shown (Schemes 1–3).

Following previous precedent, synthesis of TTA **1** followed a simple S_N2 mechanism with tetradecyl bromide **13** as the electrophile and thioglycolic acid **14** as the nucleophile, and was carried out as previously described.[17] The synthesis of TTA-PC **2** and *lyso*-TTA-PC **3** has also been reported earlier, by activation of TTA **1** using 1,1′-carbonyldiimidazole (CDI) prior to combining with L-α-glycerolphosphocholine (L-α-GPC).[13] This procedure was however replaced by a one-pot reaction using ester-coupling conditions with dicyclohexyldiimide (DCC) and dimethylamin opyridine (DMAP) as coupling reagents in dry dimethylformamide (DMF) to obtain TTA-PC **2** in 50% yield and *lyso*-TTA-PC **3** in 20% yield as white solid compounds (Scheme 1). TTA-MAG **4** was synthesized by means of ester-coupling between TTA **1** and D-1,2-isopropylideneglycerol (Solketal), making use of 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) with DMAP as coupling reagents to yield a diol-protected ester intermediate **15**. This was smoothly converted using trifluoroacetic acid (TFA) to TTA-MAG **4** recovered as a white solid in 55% yield (Scheme 1).

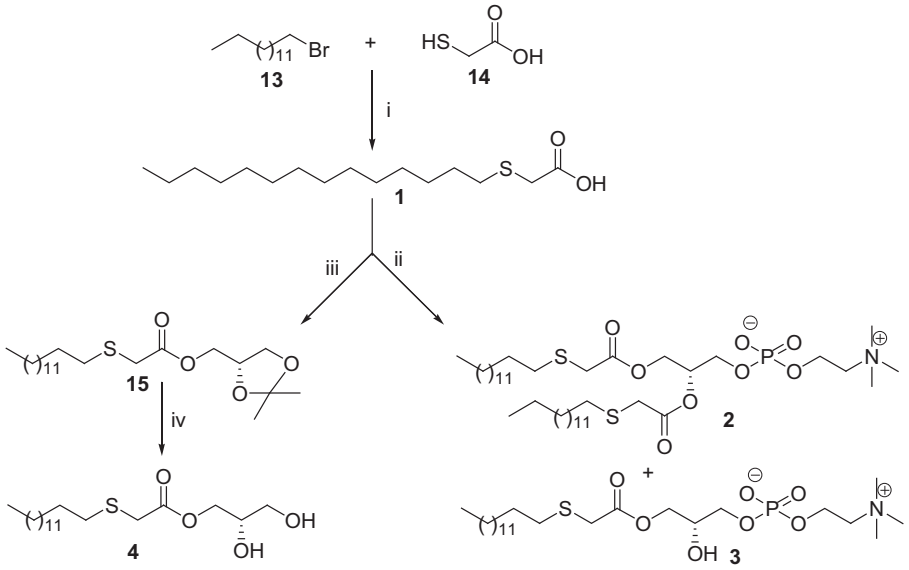
Synthesis of dTTA **5** was carried out by employing commercially available 1,7-heptanediol **16** as a starting material, which was combined with 2,3-dihydro-2*H*-pyran (DHP) in the presence of *p*-toluene sulfonic acid (PTSA) to give a mono pyran-protected 7-(2-tetrahydropyranyloxy)heptane-1-ol **17**. Iodination of **17** by reaction with iodine led to compound **18**, which were treated with triphenyl phosphine (TPP, PPh_3) in the presence of calcium carbonate to give phosphonium salt **19**. A highly stereo-selective Wittig condensation between **19** and heptaldehyde was then performed in dry tetrahydrofuran (THF), in the presence of potassium *t*-butoxide ($KOBu^t$), in order to obtain *cis* and *trans* compounds **20** in 50% and 10% yield, respectively. Product **20** was then combined with triphenyl phosphine dibromide (PPh_3Br_2) in dry CH_2Cl_2 to give bromo compound **21** that was then coupled with thioglycolic acid **14** to give the desired compound dTTA **5** as white solid in 65% yield (Scheme 2).

THEFA-containing lipids dTTA-PC **6** and *lyso*-dTTA-PC **7** were synthesized by similar methods to **2** and **3**. Firstly, dTTA **5** was activated with DCC and DMAP as coupling reagents in dry CH_2Cl_2, then coupled to L-α-GPC to give crude product that was purified by flash column chromatography using two different solvent mixtures: firstly, CH_2Cl_2/MeOH/H_2O (345:90:10 v/v/v); and secondly, CH_2Cl_2/MeOH/H_2O in the ratio 65:25:4 (v/v/v). The product dTTA-PC **6** was obtained in 50% yield and *lyso*-dTTA-PC **7** in 35% yield, both as white solids (Scheme 2). Finally, dTTA-MAG **8** was prepared as for **4** by coupling of Solketal to dTTA **5**, using HBTU and DMAP as coupling reagents. The resulting diol-protected ester intermediate **22** that was then subject to TFA deprotection leading to the preparation of **8** as white solid in 50% yield (Scheme 2).

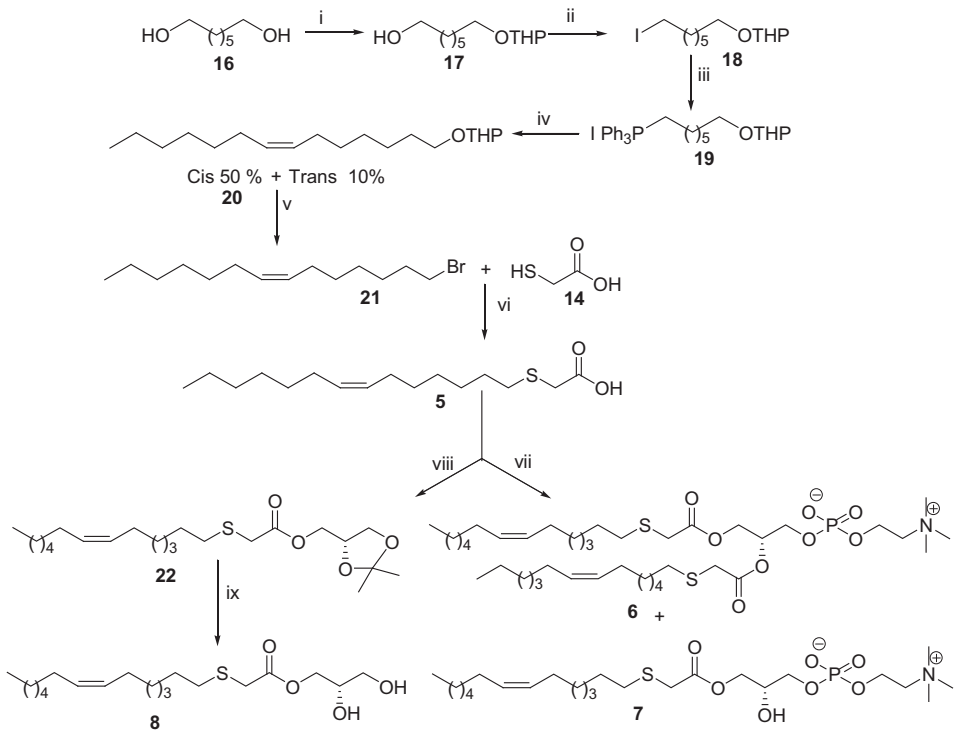
In the final set of synthetic activities, tTTA **9** was synthesized using 6-bromohexyl-1-ol **23** as starting material. Initial THP protection was followed by halogen exchange using NaI in acetone followed by alkynylation with 1-octyne, in a THF-hexamethylphosphoramide (HMPA) solvent mixture, resulting in THP-protected alkyne intermediate **26**. Afterwards **26** was combined with PPh_3Br_2 to give bromo compound **27** in excellent yield, followed by coupling with thioglycolic acid **14** to yield tTTA **9** (Scheme 3). Thereafter, tTTA-PC **10** and *lyso*-tTTA-PC **11** were synthesized by similar methods to **2** and **3**. In brief, tTTA **9** was activated with DCC and DMAP and coupled to L-α-GPC in dry DMF resulting in tTTA-PC **10** and *lyso*-tTTA-PC **11** in 60% and 20% yield, respectively (Scheme 3). tTTA-MAG **12** was prepared as **4**, through coupling of Solketal to afford **28** using HBTU and DMAP as coupling reagents, followed by TFA deprotection in methanol, resulting in tTTA-MAG **12** as a white solid in a yield of 60% (Scheme 3).

2.2. Cellular uptake of THEFAs, lipids and fatty acid composition in myotubes

The FA composition in cultured myotubes was determined after incubating the cells for 96 h with a selection of seven THEFAs and



Scheme 1. Reagents and conditions: (i) NaOH/MeOH, rt, 72 h, 75%; (ii) L-α-GPC, DCC, DMAP, dry DMF, N_2, rt, 16 h, **2** 50%, **3** 20%; (iii) HBTU, DMAP, Solketal, dry CH_2Cl_2, rt, 19 h, 80%; (iv) TFA, MeOH, at 0 °C–rt, 30 min, 55%.



Scheme 2. Reagents and conditions: (i) DHP, PTSA, dry CH_2Cl_2, rt, 24 h, 80%; (ii) PPh_3, imidazole, dry THF, iodine, −10 °C–rt, 30 min, 75%; (iii) PPh_3, $CaCO_3$, CH_3CN, reflux 85 °C, 22 h, 93%; (iv) $KOBu^t$, 1-heptaldehyde, dry THF, −5 °C, 3 h, rt, 5 h, *cis* 50%, *trans* 10%; (v) PPh_3Br_2, PPh_3, dry CH_2Cl_2, 0 °C, 15 min, 90%; (vi) 25% NaOH/MeOH, rt, 72 h, 65%; (vii) L-α-GPC, DCC, DMAP, dry CH_2Cl_2, N_2, rt, 16 h, **6**: 50%, **7**: 35%; (viii) HBTU, DMAP, Solketal, dry CH_2Cl_2, rt, 19 h, 83%; (ix) TFA, MeOH, at 0 °C–rt, 30 min, 50%.

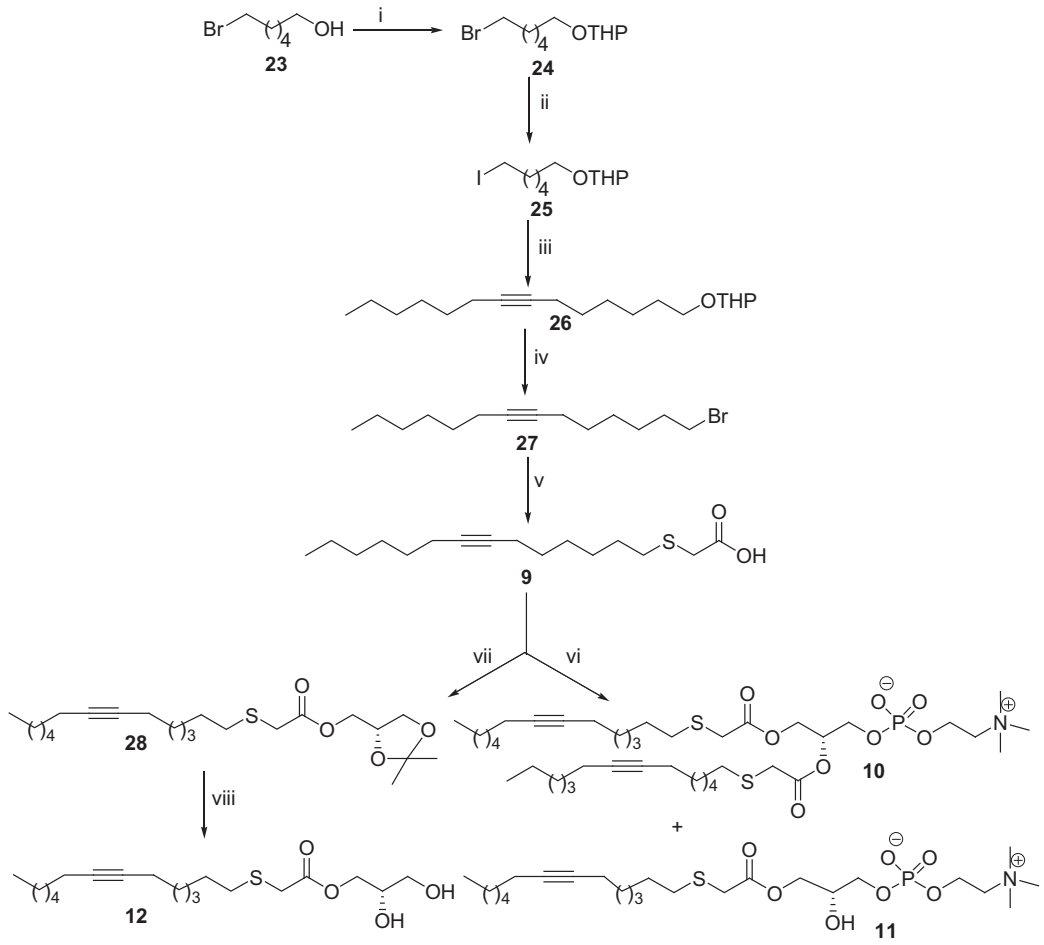
THEFA-containing lipids (**1**, **2**, **3**, **4**, **6**, **9**, **11**, syntheses of which are described above). After harvesting and lipid extraction of the cells, levels of recovered FAs were determined as described in the experimental section. Two samples with dimethyl sulphoxide (DMSO) (0.1%) but no THEFA added were also analyzed as controls. The levels of free TTA **1**, dTTA **5** or tTTA **9** were used as a measure for the cellular uptake of THEFAs or corresponding THEFA-containing lipids from the medium (Table 1). The uptake of THEFAs or THEFA-containing lipids by the myotubes was found in each case to be mainly concentration-dependent, thus 3-fold increases in extracellular THEFA or THEFA-containing lipid concentrations, from 10 to 30 μM, often resulted in approximately 3-fold increases in the measured intracellular levels of corresponding THEFAs in myotubes. In this, TTA **1** was the most bioavailable of all the THEFAs or THEFA-containing lipids administered, while uptake of the TTA-containing lipids *lyso*-TTA-PC **3** and TTA-MAG **4** resulted in intracellular levels of TTA **1** 30–40% lower than those same levels observed following treatment with equimolar amounts of TTA **1** (Table 1). Similarly, uptake of tTTA **9** also resulted in lower intracellular levels of tTTA **9** than those levels observed following treatment with equimolar amounts of TTA **1** (Table 1). In contrast, a 3-fold increase in extracellular *lyso*-tTTA-PC **11** concentration only resulted in a doubling of the intracellular levels of the corresponding tTTA **9**, while a tripling of the extracellular concentration of TTA-PC **2** resulted in no significant increase in the corresponding intracellular levels of TTA **1**.

The FA composition in the myotubes was further analyzed and compared with control myotube cultures in which no THEFAs or THEFA-containing lipids were added (Table 1). The main impact of the treatment with THEFAs or THEFA-containing lipids was a decrease in the relative content of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), mainly oleic acid. The largest decrease in SFAs (up to 50%) was observed in the myotubes incubated with 30 μM TTA **1**. Incubation with *lyso*-TTA-PC **3** and TTA-MAG **4** resulted in similar SFA decreases although levels of incorporation were generally lower and peaked at a 38% decrease. In contrast, treatment with THEFAs and THEFA-containing lipids with double and triple bonds resulted in minimal effects on relative SFA levels, but dTTA-PC **6**, tTTA **9** and *lyso*-tTTA-PC **11** treatment did impact on the relative MUFA levels in a concentration-dependent manner (up to 21%). Finally, treatment with TTA **1**, *lyso*-TTA-PC **3** and TTA-MAG **4** also resulted in comparable reductions in levels of MUFAs. Cell protein levels and total fatty acid levels per mg of protein were constant between controls, 10 μM and 30 μM of analogs, indicating no toxic effect on the skeletal muscle cells.

2.3. Fatty acid oxidation

Myotube mediated uptake of [^{14}C]oleic acid (OA) and oxidative capacity of cells for FAs in the myotubes were assessed during 4 h incubation after pre-incubation of cells for 96 h with 10 and 30 μM THEFAs and THEFA-containing lipids or 10 nM of the PPARδ agonist GW501516 as positive control. For negative control purposes, myotubes were treated with DMSO (0.1%). Initially, we noted that the extent of intracellular [^{14}C]OA uptake was unaffected by extracellular concentration rises of 10–30 μM in extracellular THEFAs and THEFA-containing lipid concentrations (Fig. 2A). On the other hand, [^{14}C]OA oxidation was increased in myotubes in a dose-dependent manner following treatment with TTA **1**, *lyso*-TTA-PC **3**, TTA-MAG **4** and *lyso*-tTTA-PC **11** (Fig. 2B).

In particular, we observed that TTA **1** treatment (30 μM) resulted in a nearly 3-fold increase in [^{14}C]OA oxidation above control, in keeping with previous observations.[18] Furthermore, treatment with *lyso*-TTA-PC **3** and TTA-MAG **4** resulted in [^{14}C]OA oxidation levels approximately 2-fold higher than those levels observed following treatment with an equimolar (30 μM)



Scheme 3. Reagents and conditions: (i) DHP, PTSA, dry CH_2Cl_2, rt, 16 h, 95%; (ii) NaI, acetone, reflux, 15 min, 89%; (iii) 1-octyne, *n*BuLi, dry THF, HMPA, N_2, 0 °C–rt, 50 h, 72%; (iv) PPh_3Br_2, PPh_3, dry CH_2Cl_2, N_2, 0 °C, 15 min, 90%; (v) thioglycolic acid, NaOH/MeOH, rt, 72 h, 85%; (vi) L-α-GPC, DCC, DMAP, dry DMF, N_2, rt, 16 h, **10**: 60%, **11**: 20%; (vii) HBTU, DMAP, Solketal, dry CH_2Cl_2, rt, 19 h, 81%; (viii) TFA, MeOH, 0 °C–rt, 15 min, 60%.

amount of TTA **1**. This outcome is also in keeping with previous data showing *lyso*-TTA-PC **3** to be more efficient as a PPAR agonist relative to TTA **1**.[14] This efficacy difference is made more striking considering that the measured intracellular levels of TTA **1** in myotubes following administration of TTA **1** alone were 30–40% higher than the intracellular levels of TTA **1** observed following administration of either *lyso*-TTA-PC **3** or TTA-MAG **4** (Table 1). Furthermore, administration of *lyso*-tTTA-PC **11** was also able to increase [^{14}C]OA oxidation to levels approximately 2-fold higher than those levels observed following treatment with an equimolar (30 μM) amount of TTA **1**. This 2-fold enhancement was also all the more striking given that the intracellular levels of tTTA **9** post administration of *lyso*-tTTA-PC **11** were approximately 20% or less than the corresponding intracellular levels of TTA **1** in myotubes following administration of TTA **1** alone (Table 1). One surprising result here was that TTA-PC **2** was found to be less effective at stimulating lipid oxidation than the other glycerolipids in spite of the significantly greater efficacy of TTA-PC **2** in comparison to TTA **1** when used in vivo.[13] This lack of efficacy may be related to the fact that extent of cellular uptake of TTA-PC **2** was unexpectedly low. Indeed the intracellular levels of TTA **1** following administration of TTA-PC **2** were only a fraction of the corresponding intracellular levels of TTA **1** observed in myotubes following administration of TTA **1** alone at the same extracellular concentration (Table 1).

2.4. Increased expression of PPAR regulated genes

The impact of THEFAs and THEFA-containing lipids on regulated gene expression was investigated when myotubes were incubated for 96 h with THEFAs and THEFA-containing lipids (30 μM) followed by qPCR of selected genes. Since TTA **1** is a known PPAR agonist, genes that are PPAR regulated were primarily selected for study.[16] The genes that were examined were angiopoietin-like 4 (*ANGPTL4*), fatty acid binding protein 3 (*FABP3*), fatty acid translocase (*CD36*) and carnitine palmitoyltransferase 1A (*CPT1A*). The encoded protein corresponding with *ANGPTL4* functions as a hormone that regulates glucose homeostasis and lipid metabolism.[19] The *FABP3* gene encodes the fatty acid-binding protein found in liver. The encoded protein corresponding with *CD36* is a FA transporter involved with long-chain FA uptake as well as signaling.[20] The *CPT1A* gene encodes a mitochondrial enzyme responsible for the formation of acyl carnitines.[21] Within experimental error, administration of THEFAs and THEFA-containing lipids all induced broadly similar levels of gene activation with the exception of TTA-PC **2** (Fig. 3A, B and D). On closer examination, *ANGPTL4* was induced the strongest, up to 35-fold over the control by tTTA **9** (Fig. 3A), followed by up to 25-fold over control by *lyso*-tTTA-LPC **11** and up to 20-fold over control by positive control TTA **1**. Similarly the *CPT1A* gene was induced the strongest up to 10-fold over the control by tTTA **9** (Fig. 3D), followed by up to 7-fold over control by *lyso*-tTTA-PC **11** and up to approximately 5-fold over

Table 1
Fatty acid composition in human myotubes after 96 h incubation with different THEFAs and THEFA-containing lipids as indicated

	THEFAs added to the culture medium															
	Control I	Control II	TTA-1		TTA-PC 2		lyso-TTA-PC 3		TTA-MAG 4		dTTA-PC 6		tTTA 9		lyso-tTTA-PC 11	
			THEFA concentrations (μM)													
			10	**30**	**10**	**30**	**10**	**30**	**10**	**30**	**10**	**30**	**10**	**30**	**10**	**30**
Fatty acids			Intracellular fatty acid concentration (mol %)													
TTA			13.02	31.81	5.50	5.90	7.99	19.51	7.17	20.04	0.01	0.40	0.06	0.32	0.43	1.02
dTTA			1.11	1.28	0.17	0.34	0.76	0.91	0.49	0.91	1.87	6.08				
tTTA													3.01	11.34	2.16	4.15
SFA	36.16	31.41	26.24	18.25	35.14	31.79	30.15	23.57	29.36	22.30	32.26	32.82	38.85	36.30	32.02	33.52
MUFA	41.04	49.95	35.09	28.44	37.48	39.28	37.12	33.15	38.46	33.11	48.17	43.41	36.30	32.32	46.19	40.64
PUFA n-3	7.47	5.16	7.86	6.62	6.98	7.30	7.68	7.56	8.42	8.21	4.74	4.63	8.41	7.09	5.18	5.68
PUFA n-6	13.41	11.46	14.34	11.78	12.71	13.31	13.96	13.20	14.15	13.63	11.29	11.45	13.93	11.63	12.40	13.42
PUFA n-9	1.78	1.89	2.20	1.73	1.88	1.95	2.21	1.97	1.81	1.68	1.53	1.42	1.32	0.87	1.48	1.41
Total FA	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
% change SFA			−27.2	−49.4	−2.5	−11.8	−16.3	−34.6	−18.5	−38.1	2.7	4.5	2.2	0.7	0.7	4.5
% change MUFA			−14.6	−30.8	−8.8	−4.4	−9.6	−19.3	−6.4	−19.4	−3.6	−13.1	−11.6	−21.3	−21.3	−13.1

Values are given as mol % of free THEFAs and selected groups of fatty acids. % change in SFA and MUFA are given relative to DMSO control (0.1%); for THEFAs **1**–**4** and **9** % change was relative to control I, and for THEFAs **6** and **11** it was relative to control II. Concentrations of THEFAs added to the myotube medium are given in bold.

control by the positive control TTA **1**. On the other hand, administration of THEFAs and THEFA-containing lipids with double and triple bonds did appear to modestly but significantly stimulate *CD36* gene expression compared with TTA **1** and other TTA-containing lipids (Fig. 3C). Curiously, *FABP3* was induced best by TTA-MAG **4** followed by *lyso*-tTTA-PC **11** (Fig. 3B).

These stimulatory effects of tTTA **9** and *lyso*-tTTA-LPC **11** are particularly interesting given previous observations that measured intracellular levels of tTTA **9** post administration of either tTTA **9** or *lyso*-tTTA-PC **11** were less than 20% of the corresponding level of TTA **1** in myotubes following administration of TTA **1** alone at the same extracellular concentration (Table 1). Overall, the data shown (Figs. 2 and 3) do provide some support for the notion that tTTA **9** and other tTTA containing lipids could surpass TTA **1** in their capacities to stimulate FA oxidation and to some measureable extent expression of the PPAR-associated genes *ANGPTL4*, *CPT1A* and *CD36*.

3. Discussion

The aim of this study was to prepare new and novel THEFAs and THEFA-containing lipids whose biological properties could be compared against TTA **1**, the parent THEFA. The clinical potential and limitations of TTA **1** are outlined in Section 1, therefore our hope was to identify alternative THEFAs and/or THEFA-containing lipids that might be useful as next generation TTA **1** replacements for use in future potential clinical scenarios.

Of the THEFA and THEFA-containing lipids prepared and studied here, *lyso*-TTA-PC **3**, TTA-MAG **4**, tTTA **9**, and *lyso*-tTTA-PC **11** were all able to increase OA oxidation levels the highest, approximately 4–5-fold above the positive PPARδ control GW501516, and nearly 2-fold over TTA **1**. The efficacies of these compounds were all the more remarkable given that the measured intracellular levels of TTA **1** or tTTA **9** as appropriate in cells post administration were consistently at least 30–40% lower than levels seen post administration of equimolar amounts of TTA **1** alone. These observations are consistent with previous conclusions that THEFA biological effects should be better realized through administration of THEFA-containing lipids in place of free-FA THEFAs (Table 1 and Fig. 2).[13,14] Furthermore, these data also suggest that the introduction of a triple bond into the TTA **1** structure may promote PPAR agonist effects compared with TTA **1** alone. On the other hand, the introduction of a double bond into the TTA **1** structure suggests the opposite given the weaker performance of dTTA-containing lipids under investigation (Table 1 and Fig. 2). Finally the beneficial effects for PPAR agonism of introducing a triple bond were further supported by the apparent increases in mRNA expression of *ANGPTL4*, *CPT1A* and *CD36* induced by administration of either tTTA **9** or *lyso*-tTTA-PC **11** in comparison to effects observed post administration of other THEFA and THEFA-containing lipids (Fig. 3).

Intriguingly, administration of THEFA and THEFA-containing lipids was found to remodel FA distributions in myotubes (Table 1). The competition by THEFAs like TTA **1** was particularly strong towards the SFAs, which were decreased by up to 50% after incubation at the highest concentration. The replacement of MUFA was less dependent on the cellular TTA content. The unsaturated FAs dTTA **6** and tTTA **9** did not compete with the endogenous SFAs, but mainly replaced MUFA, decreasing the MUFA content by up to 20% at the highest concentrations used. Compared to the effect of TTA **1** this seems reasonable since the intracellular levels of the unsaturated THEFAs were markedly lower than those of TTA **1**.

Finally, why might the inclusion of THEFAs into glycerolipid structures result in more pronounced metabolic effects than those effects elicited by treatment with free THEFAs? Certainly, what is

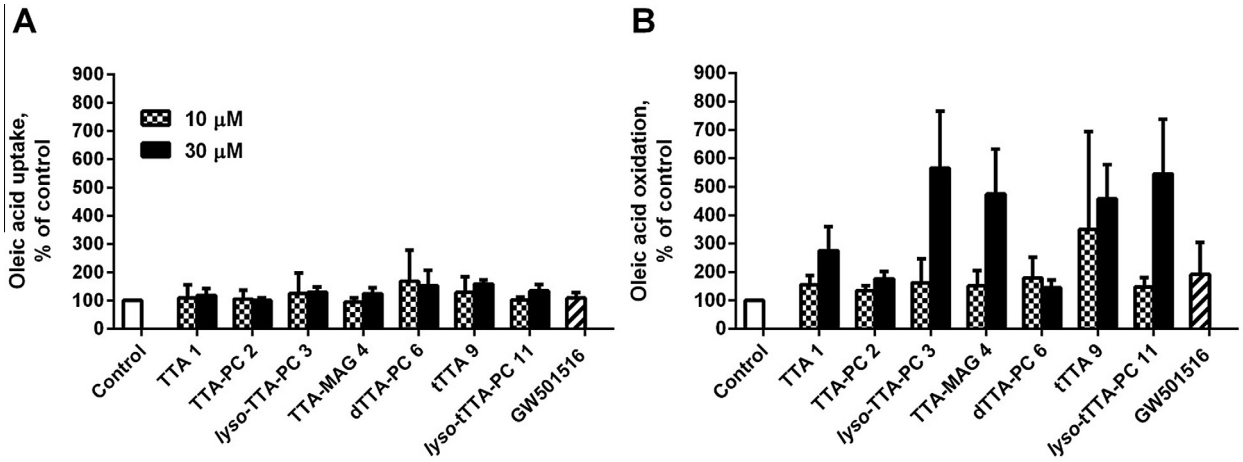


Figure 2. Oleic acid uptake and oxidation. (A) Cellular uptake (CO_2 + CA) and (B) oxidation (CO_2) from [^{14}C]oleic acid (OA) after 4 h incubation of human myotubes treated for 96 h with THEFAs, THEFA-containing lipids or 10 nM GW501516 as indicated. Concentrations of THEFAs and intracellular concentrations of THEFAs are as given in Table 1. For negative control purposes, myotubes were treated with DMSO (0.1%). Values are given as percentage relative to basal uptake or oxidation of OA (100%) in DMSO-treated myotubes. Number of experiments (n) = 8–15. Range control values: OA uptake: 16.4–165.8 nmol/mg protein; OA oxidation: 3.4–46.0 nmol/mg protein.

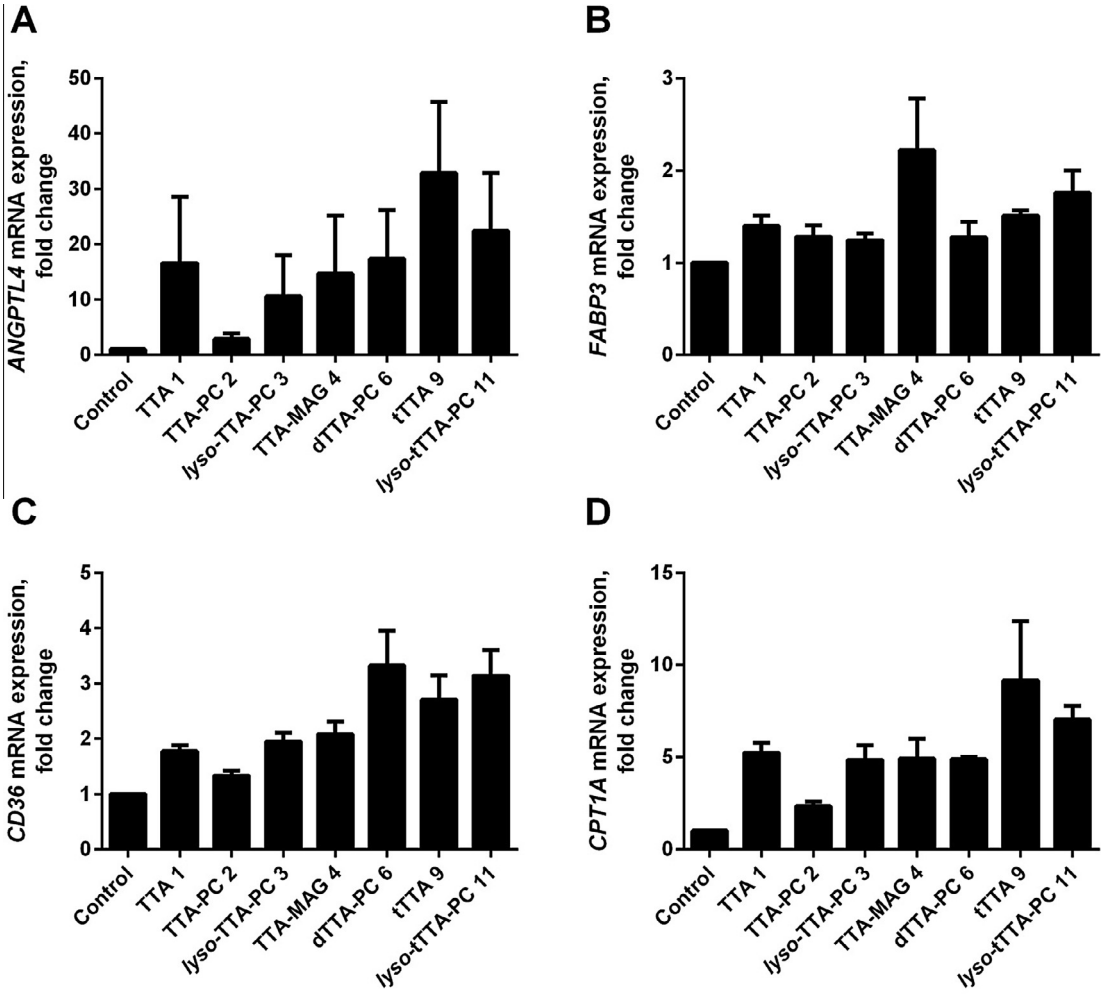


Figure 3. Gene expression. Gene expression of (A) *ANGPTL4*, (B) *FABP3*, (C) *CD36* and (D) *CPT1A* relative to DMSO (0.1%) as control in human myotubes incubated for 96 h with THEFAs and THEFA-containing lipids (30 μM). Number of experiments (n) = 3.

known is that TTA **1** is found endogenously esterified into glycerolipids, triacylglycerols and cholesteryl esters. Therefore, it would be logical to impute that primary PPAR ligands might be glycerolipids in which a THEFA is located in at least one of the ester positions. It is reasonable to assume that when incubating the myotubes with esterified THEFAs, a higher cellular level of the actual PPAR ligand will be achieved compared to incubating the cells with free THEFAs. Consistent with this statement, data have

been produced to show that *lyso*-glycerolipids can be endogenous activators of nuclear receptors (NRs).[22,23] Indeed, four NRs have so far been identified as PL binding proteins: the steroidogenic factor-like NRs liver receptor homolog 1 (LRH-1) and steroidogenic factor 1 (SF-1), PPARα, and ultraspiracle (insect homolog of retinoid X receptor). Additionally, a family of PL transporters that stimulate NR transactivation has been identified and shown to up-regulate the transcriptional activity of both PPARα and hepatocyte nuclear factor 4α.[24,25] Additionally, several groups have also demonstrated that the mammalian NRs SF-1 and LRH-1 interact with phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, and perhaps even phosphatidylinositol-phosphates. Indeed, in one study mass spectrometry experiments identified phosphatidylcholine 16:0/18:1 as one of several lipids bound to PPARα isolated from murine liver tissue.[26] Binding of this PC species was selective for PPARα over PPARδ and PPARγ, and could be inhibited by treatment with a standard PPARα agonist (WY14.643). In a comparable way, studies in Chinese Hamster Ovary cells have been used to demonstrate that PPARγ can be activated by lysophosphatidic acid that is generated intracellularly.[27]

The data obtained in this study regarding THEFAs are thus consistent with the concept of glycerolipids and *lyso*-glycerolipids and similar molecules as endogenous PPAR ligands. Hence, although our current data supports a potential importance for *lyso*-TTA-PC **3** and *lyso*-tTTA-PC **11** as significant lipid mediators of TTA **1** and tTTA **9** effects respectively, THEFA-containing lipids such as these are likely to be transferred and become integrated into other glycerolipid species too, owing to transesterification reactions. Owing to the small number of genes studied other PPAR regulated genes will have to be studied to confirm this conclusion. Further detailed lipidomics type studies would be very helpful to confirm not only the functional importance of THEFA-containing lysophospholipids to PPAR agonism, but also to identify other potentially important PPAR lipid agonists deriving from THEFAs by esterification followed by transesterification, giving rise to next generations of TTA **1** replacements for potential use in clinical scenarios.

4. Experimental section

4.1. Synthetic chemistry

All reactions were carried out under an atmosphere of nitrogen (N_2) or argon, in oven-dried glassware, unless otherwise stated. CH_2Cl_2 was distilled over P_2O_5, and other solvents were bought and pre-dried as required. All chemicals were purchased from Sigma–Aldrich. Flash column chromatography was performed on silica gel 60 (Merck Kieselgel 60 F254 230–240 mesh) according to the method of Still.[28] Thin layer chromatography (TLC) was performed on pre-coated Merck silica gel (0.2 mm, 60 F254) aluminum-backed plates, and visualized with a UV lamp (254 nm) and/or stained with acidic ammonium molybdate, basic potassium manganate ($KMnO_4$), iodine and phosphomolybdic acid. Special chromatography solvent mixtures are: solvent mix A: CH_2Cl_2/MeOH/H_2O (345:90:10 v/v/v); solvent mix B: CH_2Cl_2/MeOH/H_2O (65:25:4 v/v/). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 400. ^{1}H NMR was recorded at 400 MHz and chemical shifts, δ_H are quoted in parts per million (ppm), using residual isotopic solvent as internal reference ($CDCl_3$, δ_H = 7.27 ppm; CD_3OD, δ_H = 3.30 ppm). Data are reported as follows: integration; br = broad; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; coupling constant(s) *J* in Hz to the nearest 0.5 Hz; assignment. Peaks split by the presence of a phosphorus atom are indicated with a superscript p. ^{13}C NMR spectra were recorded at 100 MHz and chemical shifts, δ_C are quoted in parts per million (ppm), using residual isotopic solvent as internal reference ($CDCl_3$, δ_C = 77.00 ppm; CD_3OD, δ_C = 49.05 ppm). Data are reported as follows: C, CH, CH_2, CH_3; d = doublet, q = quadruplet; coupling constant(s) *J* in Hz to the nearest 0.5 Hz; assignment. ^{13}C NMR data were only fully assigned by correlation between ^{1}H and ^{13}C NMR spectral data. Mass spectra were recorded using VG Platform II, VG-070B, Joel SX- 102 or Bruker Esquire 3000 ESI instruments. Mass accuracy is indicated to the nearest 0.1 ppm.

4.1.1. 2-(Tetradecylthio)acetic acid (TTA) 1

Tetradecylbromide **13** (5.36 mL, 18.0 mmol, 1.00 equiv) was added to a solution of thioglycolic acid **14** (1.25 mL, 18.0 mmol, 1.00 equiv) in 25% NaOH in MeOH (6.03 g in 24.0 mL) and the mixture was stirred vigorously at room temperature (rt) for 72 h. After dilution with water, the mixture was acidified to pH 1 with concentrated HCl, and the aqueous phase was extracted with diethyl ether. The ether phase was dried over $MgSO_4$, filtered and concentrated in vacuo to give a crude white solid that was further purified by silica gel (60–120) flash column chromatography, eluting with CH_2Cl_2. Desired compound **1** was obtained as an amorphous solid (3.86 g, 75%), TLC: R_f 0.50 (hexane/EtOAc 3:1 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.27 (s, 2H, Ha), 2.68–2.65 (t, 2H, *J* = 7.5 Hz, Hb), 1.66–1.58 (m, 2H, Hc), 1.41–1.36 (m, 2H, Hd), 1.27 (s, 20H, 10×CH_2), 0.908–0.874 (t, 3H, *J* = 7.0 Hz, CH_3); (EI+) *m*/*z* 289 [M+H]$^+$.

4.1.2. 1,2-Di-[2-(tetradecylthio)acetyl]-*sn*-glycero-3-phosphocholine (TTA-PC) 2

To a solution of TTA **1** (261 mg, 0.905 mmol, 1.00 equiv) in dry DMF (5 mL), and DCC (190 mg, 1.17 mmol, 1.30 equiv), DMAP (135 μL, 0.905 mmol, 1.00 equiv) and L-α-GPC (133 mg, 0.301 mmol, 0.33 equiv; 5 mL) were added. The mixture was stirred vigorously at rt under N_2 for 16 h. A clear yellow solution was obtained. After completion of the reaction the DMF was removed under vacuo, the residue was purified by silica gel (60–120) flash column chromatography, eluting with solvent mix A followed by solvent mix B, to yield TTA-PC **2** (120 mg, 50%) and *lyso*-TTA-PC **3** (32 mg, 20%) as white amorphous solids, TLC: R_f 0.40 [solvent B]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.25 (br, 1H, H2), 4.45–4.41 (dd, 2H, *J* = 12.0 Hz, *J* = 3.0 Hz, H1), 4.28–4.23 (m, 2H, 2×H3), 4.00–3.97 (t, 2H, *J* = 6.0 Hz, H4), 3.85–3.74 (m, 2H, H5), 3.31–3.28 (br, 13H, 3×NCH_3, Ha), 2.63–2.58 (m, 4H, Hb), 1.61–1.54 (m, 4H, Hc), 1.38–1.35 (m, 2H, Hd), 1.26 (s, 40H, 20×CH_2), 0.85–0.82 (t, 6H, *J* = 7.0 Hz, 2×CH_3), ^{13}C (125 MHz; $CDCl_3$) 170.36, 170.12 (C, 2×C=O), 71.47–71.41 (CH, C2), 66.30–66.25 (CH_2, C5), 63.49 (CH_2, C1), 63.33–63.29 (CH_2, C3), 59.45–59.41 (CH_2, C4), 54.37 (3×NCH_3), 33.60, 33.45 (CH_2, 2×Ca), 32.74, 32.66 (CH_2, 2×Cb), 31.90 (CH_2, 2×Cc), 29.66, 29.62, 29.60, 29.35, 29.32, 29.02, 29.00, 28.85, 28.82 (18×CH_2), 22.66 (2×CH_2), 14.09 (2×CH_3); LC–MS, *m*/*z* 798 [M+H]$^+$.

4.1.3. 1-[2-(Tetradecylthio)acetyl]-*sn*-glycero-3-phosphocholine (*lyso*-TTA-PC) 3

^{1}H NMR (400 MHz, $CDCl_3$/MeOD 2:1): δ 4.28–4.11 (m, 3H, 2×H1, H2, 1×H3), 3.95–3.86 (m, 3H, 2×H4, 1×H3), 3.59 (br, 2H, 2×H5), 3.23–3.12 (m, 11H, 2×Ha, 3×NCH_3), 2.60–2.56 (t, 2H, *J* 7.5, 2×Hb), 1.58–1.51 (m, 2H, 2×Hc), 1.34–1.32 (m, 2H, 2×Hd), 1.21 (s, 20H, 10×CH_2), 0.85–0.81 (t, 3H, *J* 7.0, CH_3), ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.60 (C, C=O), 68.55 (CH, C2), 67.00 (CH_2, C5), 66.08 (CH_2, C3), 59.45 (CH_2, C1), 54.52 (3×NCH_3), 40.06, (CH_2), 33.62 (CH_2, C3), 32.69 (CH_2, 2×Cb), 31.89 (CH_2, 2×Cc), 29.68, 29.64, 29.58, 29.46, 29.33, 29.29, 29.14, 29.03, 28.81, (10×CH_2), 22.65 (2×CH_2), 16.30 (CH_3); LC–MS, *m*/*z* 528 [M+H]$^+$.

4.1.4. 2,3-Acetonide-1-[2-(tetradecylthio)acetyl]-*sn*-glycerol 15

HBTU (1.16 g, 3.05 mmol, 3.28 equiv) and DMAP (1.09 g, 8.95 mmol, 9.64 equiv) were added to a solution of TTA **1**

(834.0 mg, 2.89 mmol, 3.11 equiv) and Solketal (85.5 mg, 0.928 mmol, 1.00 equiv) in dry CH_2Cl_2 under argon. The mixture was stirred at rt for 19 h, and then the reaction was quenched with 15 mL of citric acid solution (7%). The aqueous phase was separated from the organic phase and extracted with CH_2Cl_2 (4 × 25 mL). The organic phases were then combined, dried over $MgSO_4$, filtered and concentrated in vacuo to give a white solid crude (2.49 g). The crude solid was further purified by silica gel (60–120) flash column chromatography, eluting with hexane/EtOAc (9:1, 8:2, 6:4 v/v), to yield **15** as a white amorphous powder (297 mg, 80%), TLC: R_f 0.40 (hexane/EtOAc 9:1 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.42–4.36 (m, 1H), 4.35–4.18 (m, 2H), 4.17–4.09 (m, 1H), 3.80–3.76 (m, 1H), 3.27 (s, 2H), 2.67–2.63 (t, 2H), 1.64–1.57 (m, 2H), 1.46 (s, 3H), 1.39 (s, 3H), 1.27 (s, 20H), 0.91–0.88 (t, 3H); LC–MS, *m*/*z* 423 $[M+Na]^+$.

4.1.5. 1-[2-(Tetradecylthio)acetyl]-*sn*-glycerol (TTA-MAG) 4

To a solution of **15** (2.59 g, 5.39 mmol) in MeOH (15 mL) at 0 °C, TFA (1 mL) was added. The reaction was stirred at rt for 30 min before being neutralized with concentrated ammonium hydroxide. The solvent was removed under reduced pressure to obtain a gum-like residue. This residue was purified by silica gel (60–120) column chromatography, eluting with EtOAc/hexane (6:4 v/v), to yield **4** was obtained as a white solid (1.1 g, 55%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.42–4.36 (m, 1H), 4.35–4.18 (m, 2H), 4.17–4.09 (m, 1H), 3.80–3.76 (m, 1H), 3.27 (s, 2H), 2.67–2.63 (t, 2H), 1.64–1.57 (m, 2H), 1.46 (s, 3H), 1.39 (s, 3H), 1.27 (s, 20H), 0.91–0.88 (t, 3H); LC–MS, *m*/*z* 384 $[M+Na]^+$.

4.1.6. 7-(2-Tetrahydropyranyloxy)heptane-1-ol 17

To 4.00 g of 1,7-heptanediol **16** in 30 mL of CH_2Cl_2, DHP (35.85 mmol) and catalytic amount of PTSA were added dropwise. The reaction was stirred for 24 h at rt. After completion of the reaction the organic layer was washed with water (2 × 50 mL), $NaHCO_3$ (3 × 50 mL), dried over $MgSO_4$, filtered, and evaporated in vacuo. The resulting crude oil (4.5 g) was purified by silica gel (60–120) column chromatography, eluting with EtOAc/hexane (7:3 v/v) to yield **17** as an off-white liquid (5.18 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.42–4.36 (m, 2H), 4.58–4.59 (m, 2H), 3.80–3.91 (m, 3H), 3.723–3.36 (m, 1H), 3.37–3.40 (m, 2H), 3.18–3.20 (t, 2H), 178–1.85 (m, 2H), 1.50–1.61 (m, 4H), 1.39–1.41 (m, 6H); ESI, *m*/*z* 217 $[M+H]^+$.

4.1.7. 1-Iodo-7-(2-tetrahydropyranyloxy)heptane 18

To a solution containing triphenylphosphine (PPh_3; 20.1 g, 76.3 mmol), imidazole (10.34 g, 152 mmol) and **17** (10.67, 50.8 mmol) in 140 mL of THF, iodine (19.36 g, 76.3 mmol) was added at -10 °C. The solution was allowed to warm up to rt and then stirred for 30 min. The reaction mixture was diluted with diethyl ether, washed successively with a saturated solution of sodium thiosulfate (100 mL) and a saturated solution of $NaHCO_3$ (100 mL), dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was stirred with petroleum ether to precipitate triphenylphosphine oxide, then filtered and concentrated under reduced pressure to give a crude oily liquid (crude weight 28.5 g). This oily liquid that was further purified by silica gel (60–120) column chromatography, eluting with EtOAc/hexane (3:97 v/v), to yield **18** as a clear oil (12.4 g, 75%), TLC: R_f 0.43 (hexane/EtOAc 9:1 v/v). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.6 (m, 2H), (m, 2H), 3.85–3.90 (m, 2H), 3.49–3.57 (m, 1H), 37–3.41 (m, 2H), 3.32 (t, 2H), 1.53–1.60 (t, 2H), 1.39–1.41 (m, 4H), 1.36–1.38 (m, 6H); ESI, *m*/*z* 327 $[M+H]^+$.

4.1.8. (7-[2-Tetrahydropyranyloxy]heptyl)triphenyl phosphonium iodide 19

A solution containing triphenylphosphine (PPh_3; 19.3 g, 73.6 mmol), **18** (12 g, 36.7 mmol) and calcium carbonate (2 g) in 100 mL of acetonitrile was heated at 85 °C for 22 h. Then the reaction mixture was filtered and concentrated under reduced pressure to obtain a gum-like residue that was dissolved in a minimum amount of CH_2Cl_2. Addition of diethyl ether resulted in a precipitate that was collected by filtration then dried under vacuum (0.05 mmHg) at rt to give phosphonium salt **19** as a gum (19.9 g, 92.6%).

4.1.9. 2-Tetradec-(7*Z*)-enyloxy-tetrahydropyran 20

To a solution of **19** (12.91 g, 31.97 mmol) in dry THF (60 mL), $KOBu^t$ (2.98 g, 26.62 mmol) was added at −5 °C and stirred for 3 h at the same temperature. A solution of heptaldehyde (4.7 g, 10.65 mmol) in THF (15 mL) was added dropwise and stirred at rt for 5 h. Saturated aqueous NH_4Cl solution (30 mL) was added and extracted with EtOAc (3 × 40 mL). The organic layer was washed with water (3 × 50 mL), brine (3 × 50 mL), dried over $MgSO_4$, filtered and evaporated under reduced pressure to give a liquid (crude weight 19 g). This liquid was purified further by silica gel (60–120) column chromatography, eluting with EtOAc/hexane (1:9 v/v) to yield **20** as an oil (4.74, 50%), TLC: R_f 0.65 (EtOAc/hexane 1:9 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32–5.38 (m, 2H), 4.58–4.60 (m, 2H), 3.86–3.89 (m, 1H), 3.50–3.53 (m, 2H), 3.40–3.49 (m, 2H), 2.01 (m, 4H), 1.71–1.83 (m, 6H), 1.44–1.59 (t, 8H), 1.21–1.31 (m, 6H), 0.73–0.85 (m, 3H); ESI, *m*/*z* 297 $[M+H]^+$.

4.1.10. 1-Bromo-tetradec-7-ene 21

To a stirred solution of dibromo triphenylphosphine (PPh_3Br_2; 5.87 g, 13.9 mmol) and PPh_3 (1.41 g, 5.36 mmol) in anhydrous CH_2Cl_2 (150 mL) at 0 °C under N_2, **20** (3.61 g, 10.7 mmol) was added. After 15 min, excess PPh_3Br_2 was quenched with 10% K_2CO_3 solution (100 mL). The organic phase was separated, and the aqueous phase was re-extracted with CH_2Cl_2 (3 × 150 mL). The combined organic layers were washed with water (150 mL), brine (150 mL), dried over $MgSO_4$, filtered, concentrated and obtained as light yellow oily liquid (crude weight 6.5 g). This oily liquid was purified by silica gel (60–120) column chromatography, eluting with EtOAc/hexane (1:9 v/v), to afford **21** as a pale yellow oil (2.64 g, 90%), TLC: R_f 0.68 (hexane/EtOAc 9:2 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.34–5.42 (m, 2H), 3.35–3.45 (t, 2H), 2.06–2.29 (m, 4H), 1.76–1.90 (m, 4H), 1.37–1.50 (m, 4H), 1.20–1.33 (m, 8H), 0.88–1.01 (m, 3H); ESI, *m*/*z* 275 $[M+H]^+$.

4.1.11. 2-(Tetradec-[7-enyl]thio)acetic acid (dTTA) 5

Compound **21** (5.36 mL, 18.0 mmol, 1.00 equiv) was added to a solution of thioglycolic acid (1.25 mL, 18.0 mmol, 1.00 equiv) in 25% NaOH in MeOH (6.03 g in 24 mL) and the mixture was stirred vigorously at rt for 72 h. After dilution with water, the mixture was acidified to pH 1 with concentrated HCl, and the aqueous phase was extracted with diethyl ether. The ether phase was dried over $MgSO_4$, filtered and concentrated in vacuo to obtain a white solid, that was purified by silica gel (60–120) flash column chromatography, eluting with CH_2Cl_2, to yield **5** as an amorphous solid compound (3.36 g, 65%), TLC: R_f 0.50 (EtOAc/hexane 1:3 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35–5.40 (m, 2H), 3.38 (s, 2H), 2.67 (t, 2H), 2.03–2.10 (m, 4H), 1.60–1.64 (m, 8H), 1.17–1.38 (m, 8H), 0.73–0.89 (t, 3H); ^{13}C (100 MHz, $CDCl_3$), 176.87 (C, C═O), 130.08, 129.56 (HC═CH), 77.00, 33.43 (CH_2, Ca), 32.73 (CH_2, Cb), 31.75 (CH_2, Cc), 29.69, 29.54, 28.95, 28.81, 28.77, 28.59, 27.19, 27.06, 22.63 (9×CH_2), 14.08 (CH_3); LC–MS, *m*/*z* 285 $[M+H]^+$.

4.1.12. 1,2-Di-[2-(tetradec-[7-enyl]thio)acetyl]-*sn*-glycero-3-phosphocholine (dTTA-PC) 6

To a solution of **5** (261 mg, 0.905 mmol, 1.00 equiv) in CH_2Cl_2 (5 mL), DCC (190 mg, 1.17 mmol, 1.30 equiv), DMAP (135 μL, 0.905 mmol, 1.00 equiv) and L-α-GPC (133 mg, 0.301 mmol, 0.33 equiv; 5 mL) were added. The mixture was stirred vigorously

at rt under N_2 for 16 h. A clear yellow solution was obtained. After completion of the reaction the DMF was removed under vacuo and the residue purified by silica gel (60–120) flash column chromatography, eluting with solvent mix A followed by solvent mix B, to yield **6** (119 mg, 50%) and *lyso*-dTTA-PC **7** (55 mg, 35%) as white amorphous solids, R_f 0.40 (solvent B). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32–5.40 (m, 4H), 4.39–4.45 (m, 4H), 4.05 (m, 2H), 3.89 (m, 2H), 3.42 (s, 4H), 3.25 (t, 8H), 2.61–2.65 (m, 10H), 2.03–2.04 (m, 12H), 1.57 (m, 4H), 1.30–1.36 (m, 20H), 0.90 (t, 6H), ^{13}C (100 MHz, $CDCl_3$) δ 170.13, 169.88 (c, C═O), 130.37, 129.89, 129.38 (2×HC═CH), 71.38–71.31 (CH, C2), 66.18–66.13 (CH_2, C5), 63.36 (CH_2, C1), 63.08 (CH_2, C3), 59.24 (CH_2, C4), 54.27 (N 3×CH_3), 33.42, 33.26 (CH_2, 2×Ca), 32.53, 32.47 (CH_2, 2×Cb), 32.35 (CH_2, 2×Cc), 31.59, 29.53, 29.47, 28.79, 28.76, 28.54, 28.52, 27.56, (18×CH_2), 22.65 (2×CH_2), 16.10 (2×CH_3); LC–MS, *m*/*z* 794 $[M+H]^+$.

4.1.13. 1-[2-(Tetradec-[7-enyl]thio)acetyl]-*sn*-glycero-3-phosphocholine (*lyso*-dTTA-PC) 7

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.27–5.35 (m, 2H), 4.35 (m, 2H), 4.15 (m, 1H), 3.85–3.98 (m, 2H), 3.37 (s, 2H), 3.12–3.23 (m, 6H), 2.60 (t, 2H), 2.02–2.11 (m, 8H), 1.55 (m, 2H), 1.29–1.37 (m, 20H), 0.09 (3H, t); LC–MS, *m*/*z* 526 $[M+H]^+$

4.1.14. 2,3-Acetonide-1-[2-(tetradec-[7-enyl]thio)acetyl]-*sn*-glycerol 22

HBTU (1.16 g, 3.05 mmol, 3.28 equiv) and DMAP (1.09 g, 8.95 mmol, 9.64 equiv) were added to a solution of dTTA **5** (834.0 mg, 2.89 mmol, 3.11 equiv) and Solketal (85.5 mg, 0.928 mmol, 1.00 equiv) in dry CH_2Cl_2 under argon. The mixture was stirred at rt for 19 h before reaction was quenched with 15 mL of citric acid solution (7%). The aqueous phase was separated from the organic phase and extracted with CH_2Cl_2 (4 × 25 mL). The organic phases were combined, dried over $MgSO_4$, filtered and concentrated in vacuo to obtain as a crude white solid (2.49 g). The solid was purified by silica gel (60–120) flash column chromatography, eluted with hexane/EtOAc (9:1, 8:2, 6:4 v/v, EtOAc), to yield **22** as a white amorphous powder (308 mg, 83%), TLC: R_f 0.40 (hexane/EtOAc 9:3 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.36–5.40 (m, 2H), 4.26 (m, 2H), 4.09 (m, 1H), 4.30 (m, 2H), 3.32 (s, 2H), 2.64–2.68 (t, 2H), 2.01–2.04 (m, 4H), 1.55–1.60 (m, 4H), 1.30–1.46 (m, 18H), 0.91–0.92 (t, 3H); ESI-MS, *m*/*z* 400 $[M]^+$.

4.1.15. 1-[2-(Tetradec-[7-enyl]thio)acetyl]-*sn*-glycerol (dTTA-MAG) 8

To a solution of compound **22** (2.59 g, 5.39 mmol) in MeOH (15 mL) at 0 °C, TFA (1 mL) was added. The reaction was stirred at rt for 30 min. After completion of the reaction it was neutralized with concentrated ammonium hydroxide and the solvent removed under reduced pressure. The resulting crude solid was purified by silica gel (60–120) column chromatography, using EtOAc/hexane (4:6 v/v), to obtain **8** as a white solid (0.97 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.34–5.39 (m, 2H), 4.24–4.27 (m, 2H), 3.98 (m, 1H), 3.73 (m, 2H), 3.64 (m, 2H), 3.32 (s, 2H), 2.64 (t, 2H), 2.23 (m, 2H), 2.03 (m, 4H), 1.62–1.64 (m, 2H), 1.30–1.48 (m, 12H), 0.89–0.92 (t, 3H), ^{13}C (100 MHz, $CDCl_3$), 170.88 (C═O), 130.08, 129.54 (HC═CH), 69.98, 66.00, 63.22, 62.00, 33.46, 32.71, 32.56, 32.44, 31.73, 29.68, 29.54, 29.41, 28.94, 28.87, 28.78, 28.60, 27.19, 27.06, 22.61(10×CH_2), 16.10 (CH_3); ESI^+, *m*/*z* 360 $[M+H]^+$.

4.1.16. 2-(6-Bromohexyl-1-oxy)tetrahydropyran 24

To a stirred solution of 6-bromohexyl-1-ol **23** (2.0 g, 9.61 mmol) in anhydrous CH_2Cl_2 (40 mL) at 0 °C, under N_2, DHP (1.051 mL, 11.53 mmol) and PTSA (603 mg, 2.40 mmol) were added. The reaction mixture was allowed to stir for 16 h in the water bath, under N_2 atmosphere, by which time the solution had turned slightly brown in color. After completion, the reaction mixture was neutralized with aqueous $NaHCO_3$ (50 mL), the organic phase was collected and the aqueous phase re-extracted with CH_2Cl_2 (2 × 30 mL). Organic fractions were combined, washed with water (30 mL), brine (30 mL) over $MgSO_4$, filtered and concentrated to a crude residue. This crude residue was purified by silica gel (60–120) flash column chromatography, eluting with hexane/EtOAc (9:0.5 v/v), to furnish **24** as a bright yellow oil (2.5 g, 95%): R_f 0.60 (hexane/EtOAc, 9:1 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.31–1.62 (m, 10H 5×CH_2), 1.66–1.71 (m, 1H CH_2), 1.88–2.0 (m, 3H), 3.37–3.48 (m, 3H), 3.50–3.54 (m, 1H), 3.72–3.75 (m, 1H, CHO), 3.85–3.87 (m, 1H, CHO), 4.57–4.59 (t, 1H, CHO); ESI^+, *m*/*z* 264 $[M+H]^+$.

4.1.17. 2-(6-Iodohexyl-1-oxy)tetrahydropyran 25

NaI (8.59 g, 57.3 mmol) was dissolved in a solution of **24** (8.40 g, 28.6 mmol) in acetone (160 mL). The stirred, slightly cloudy solution was then placed under reflux under N_2 for 15 min, during which period a slide precipitated out of the solution. The reaction mixture was filtered and concentrated and the residue was dissolved in water (150 mL) and CH_2Cl_2 (200 mL). Then, the organic layer was separated, washed with water (100 mL), brine over $MgSO_4$, filtered, and concentrated to afford crude product **25** as bright non-viscous oil (12.5 g). This residue was purified by silica gel (60–120) column chromatography, eluting with hexane/EtOAc (13:1 v/v), to obtain **25** as a pale yellow, non-viscous oil (7.68 g, 89%), TLC: R_f 0.62 [hexane/EtOAc, 1:1 v/v]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.36–1.39 (m, 4H, 2×CH_2), 1.40–1.60 (m, 6H, 3×CH_2), 1.65–1.78 (m, 1H), 1.79–189 (m, 3H), 3.19–3.22 (t, 2H, CH_2I), 3.37–3.41 (m, 1H, CH_2O), 3.49–3.53 (m, 1H, CHO), 3.72–3.76 (m, 1H, CH_2O), 4.58–4.59 (m, 1H, OCHO); ESI^+, *m*/*z* 302 $[M]^+$.

4.1.18. 2-(Hexadec-7-ynyl-1-oxy)tetrahydropyran 26

To a stirred solution of 1-octyne (2.4 mL, 13.5 mmol) in anhydrous THF (23 mL) at 0 °C under N_2 atmosphere, *n*-BuLi (2.17 M in hexane; 8.71 mL, 18.9 mmol) was added dropwise. After 15 min the solution became orange-red in color. HMPA (23 mL) was added to the solution, turning the reaction mixture blood red in color. After 15 min **25** (5.50 g, 16.2 mmol) was added with anhydrous THF (11 mL), turning the solution yellow-orange. Within 1.5 h the solution turned darker in color. The reaction mixture was allowed to stir at rt for a further 48 h and then poured onto an ice/water mixture (150 mL). Crude **26** was extracted with diethyl ether (3 × 150 mL). The combined organic layer was washed with water (75 mL), brine (75 mL), dried over $MgSO_4$, filtered, and concentrated. The crude residue (5.6 g) was purified by silica gel (60–120) flash column chromatography, eluting with hexane/EtOAc (2:1 v/v), to yield **26** as a pale-yellow oil (2.98 g, 72%), TLC: R_f 0.58 (hexane/EtOAc, 2:1 v/v). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.89–0.93 (t, 3H, CH_3), 1.27–1.82 (m, 20H, 10×CH_2), 1.84–1.86 (m, 1H), 1.87–1.90 (m, 1H), 2.13–2.18 (m, 4H, $H_2CC{\equiv}CCH_2$), 3.38–3.55 (m, 2H, CHO), 3.86–3.88 (m, 1H, CHO), 3.89–3.92 (m, 1H, CHO), 4.59–4.61 (t, 1H, OCHO); ESI^+, *m*/*z* 295 $[M+H]^+$.

4.1.19. 1-Bromohexadec-7-yne 27

To a stirred solution of PPh_3Br_2 (5.87 g, 13.9 mmol) and PPh_3 (1.41 g, 5.36 mmol) in anhydrous CH_2Cl_2 (150 mL) at 0 °C under N_2, **26** was added (3.61 g, 10.7 mmol). After 15 min, excess PPh_3Br_2 was quenched with 10% K_2CO_3 solution. The organic phase was separated, and the aqueous phase was re-extracted with CH_2Cl_2 (3 × 150 mL). The combined organic layers were washed with water (150 mL), brine (150 mL), dried ($MgSO_4$), filtered, and concentrated. Crude (4.65 g) **27** was purified by silica gel

(60–120) column chromatography, eluting with hexane/EtOAc 9:1 (v/v), to furnish the title compound **27** as a pure pale-yellow oil (2.62 g, 90%), TLC: R_f 0.61 [hexane/EtOAc, 9:2 v/v]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90–0.93 (t, 3H, CH_3), 1.25–1.46 (m, 14 H, 7×CH_2), 1.86–1.93 (quart, 2H, CH_2CH_2Br), 2.14–2.20 (m, 4H, $H_2CC{\equiv}CCH_2$) 3.42–3.45 (t, 2H, *J* 6.9, CH_2Br), (ESI$^+$) *m*/*z* 272 [M]$^+$.

4.1.20. 2-(Tetradec-[7-ynyl]thio)acetic acid (tTTA) 9

Compound **27** (5.36 mL, 18.0 mmol, 1.00 equiv) was added to a solution of thioglycolic acid (1.25 mL, 18.0 mmol, 1.00 equiv) in 25% NaOH in MeOH (6.03 g in 24 mL) and the mixture was stirred vigorously at rt for 72 h. After dilution with water the mixture was acidified to pH 1 with concentrated HCl and the aqueous phase was extracted with diethyl ether. The ether phase was dried over $MgSO_4$, filtered, and concentrated in vacuo to obtain a white solid, that was purified by silica gel (60–120) flash column chromatography, eluting with CH_2Cl_2, to yield tTTA **9** as an amorphous solid (4.36 g, 85%), TLC: R_f 0.50 (hexane/EtOAc 3:1 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89–0.96 (t, 3H, CH_3), 1.25–1.42 (m, 14H, 7×CH_2), 1.61–1.68 (m, 2H, CH_2), 2.14–2.19 (m, 4H, $H_2CC{\equiv}CCH_2$) 2.67–2.70 (t, 2H, CH_2-S), 3.28 (s, 2H, S—CH_2COOH), ^{13}C (500 MHz, $CDCl_3$), 176.53 (C═O), 80.42, 79.91 (HC≡CH), 33.44, 32.72, 31.34, 29.09, 28.91, 28.77, 28.51, 28.31, 28.22, 22.54, 18.72, 18.64 (9×CH_2) 16.05 (CH_3); ESI-MS, *m*/*z* 283 [M−H]$^+$.

4.1.21. 1,2-Di-[2-(tetradec-[7-ynyl]thio)acetyl]-*sn*-glycero-3-phosphocholine (tTTA-PC) 10

To a solution of **9** (261 mg, 0.905 mmol, 1.00 equiv) in DMF (5 mL) and DCC (190 mg, 1.17 mmol, 1.30 equiv), DMAP (135 μL, 0.905 mmol, 1.0 equiv) and L-α-GPC (133 mg, 0.301 mmol, 0.33 equiv; 5 mL) were added. The mixture was stirred vigorously at rt under N_2 atmosphere for 16 h. A clear yellow solution was obtained. After completion, DMF was removed in vacuo and the residue purified by silica gel (60–120) flash column chromatography, eluting with solvent mix A followed by solvent mix B, to yield **10** (143 mg, 60%) and *lyso*-tTTA-PC **11** (32 mg, 20%) as white amorphous solids, TLC: R_f 0.40 (Solvent A, Solvent B), ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.73–0.90 (t, 6H, 2×CH_3), 1.24–1.61 (m, 32H, 16×CH_2), 2.01–2.15 (t, 8H, 2×$H_2CC{\equiv}CCH_2$), 2.60–2.64 (m, 4H, 2×CH_2—S), 3.20–3.30 (s, 4H, 2×S—CH_2—COOH), 3.76 (t, 2H, CH_2O), 3.84 (t, 2H, CH_2—N), 4.24–4.46 (m, 4H, 2×CH_2O), 5.26–5.32 (m, 1H, CHO), ^{13}C (125 MHz, $CDCl_3$), 170.27, 170.02 (C═O), 80.34, 79.88 (HC≡CH), 71.48–71.42, (CH, C2), 66.40–66.35 (CH_2, C5), 63.47 (CH_2, C1), 63.24 (CH_2, C3), 59.42 (CH_2, C4), 54.50 (N 3×CH_3), 33.57, 33.42 (CH_2, 2×Ca), 32.65, 32.58 (CH_2, 2×Cb), 31.32 (CH_2, 2×Cc), 29.63, 29.08, 29.05, 29.00, 28.99, 28.94, 28.84, 28.50, 28.42, 28.32, 28.26 (18×CH_2), 22.52, 18.71 (2×CH_2), 14.05, 14.01 (2×CH_3); EI$^+$, *m*/*z* 790 [M]$^+$.

4.1.22. 1-[2-(Tetradec-[7-ynyl]thio)acetyl]-*sn*-glycero-3-phosphocholine (*lyso*-tTTA-PC) 11

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.8–0.93 (t, 3H, CH_3), 1.2–1.6 (m, 16H, 8×CH_2), 2.13–2.15 (t, 4H, $H_2CC{\equiv}CCH_2$), 2.61–2.64 (m, 2H, —S—CH_2), 3.25 (s, 2H, S—CH_2—COOH), 3.30 (s, 9 H, $(CH_3)_3$—N), 3.66–3.76 (m, 2 H, CH_2—N), 3.82 (m, 2H, CH_2O), 3.98 (m, 2H, CH_2O), 4.16 (br, 2H, CH_2O), 4.99 (br, 1H, —CH—OH), ^{13}C ($CDCl_3$, 125 MHz), 170.51 (C═O), 80.28, 79.85 (HC≡CH), 68.49, 68.45 (CH, C2), 66.87–66.06 (CH_2, C5), 59.38 (CH_2, C1), 57.89 (CH_2, C3), 54.24 (N, 3×CH_3), 39. 82, (CH_2, C4), 33.55, 32.55 (CH_2, Ca), 32.49 (CH_2, Cb), 31.49 (CH_2, Cc), 31.27, 29.58, 29.04, 28.96, 28.96, 28.84, 28.51, 28.45, 28.38, 28.24, 28.26 (9×CH_2), 22.50, 18.90 (CH_2), 16.05, (CH_3); ESI-MS, *m*/*z* 524 [M+H]$^+$.

4.1.23. 2,3-Acetonide-1-[2-(tetradec-[7-ynyl]thio)acetyl]-*sn*-glycerol 28

HBTU (1.16 g, 3.05 mmol, 3.28 equiv) and DMAP (1.09 g, 8.95 mmol, 9.64 equiv) were added to a solution of **9** (834.0 mg, 2.89 mmol, 3.11 equiv) and Solketal (85.5 mg, 0.928 mmol, 1.00 equiv) in dry CH_2Cl_2 under argon. The mixture was stirred at rt for 19 h before the reaction was quenched with 15 mL of citric acid solution (7%). The aqueous phase was separated from the organic phase and extracted with CH_2Cl_2 (4 × 25 mL). The organic phases were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo to give a white solid (2.49 g). The solid was purified by silica gel (60–120) flash column chromatography, eluting with hexane/EtOAc (9:1, 8:2, 6:4 v/v, EtOAc), to yield **28** as a white amorphous powder (298 mg, 81%), TLC: R_f 0.40 (hexane/EtOAc 9:1 v/v), ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89–0.93 (t, 3H, CH_3), 1.25–1.43 (m, 20H, 2×CH_3, 7×CH_2), 1.59–1.64 (m, 2H, CH_2), 2.13–2.20 (m, 4H, $H_2CC{\equiv}CCH_2$), 2.64–2.68 (t, 2H, CH_2—S), 3.27 (s, 2H, SCH_2—COOH), 3.76–3.80 (m, 1H, CHO), 4.09–4.20 (m, 3H, CH_2O), 4.35–4.38 (quint, 1H, OCHO), 4.27 (s, 20H,), 0.91–0.88 (t, 3H,), *m*/*z* 398 [M]$^+$.

4.1.24. 1-[2-(Tetradec-[7-ynyl]thio)acetyl]-*sn*-glycerol (tTTA-MAG) 12

To a solution of compound **28** (2.59 g, 5.39 mmol) in MeOH (15 mL) at 0 °C, TFA (1 mL) was added. The reaction was stirred at rt for 15 min before neutralized with concentrated NH_4OH and the solvent was removed on reduced pressure. The crude product was purified by silica gel (60–120) column chromatography, eluting with EtOAc/hexane (6:4 v/v), to obtain **12** as a white solid (1.16 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89–0.93 (t, 3H, CH_3), 1.25–1.43 (m, 14H, 7×CH_2), 1.50–1.65 (m, 2H), 1.99 (br, 2H), 2.14–2.16 (m, 4H, $H_2CC{\equiv}CCH_2$), 2.64–2.68 (t, 2H, CH_2—S), 3.25–3.28 (s, 2H, S—CH_2—COOH) 3.64–3.82 (m, 2H, CHO), 3.97–4.0 (m, 1H, CHO), 4.21–4.32 (m, 2H, CH_2—OH), ^{13}C (125 MHz, $CDCl_3$), 170.85 (C═O), 80.42, 79.91 (HC≡CH), 69.99 (CH, C2), 66.01 (CH_2, C5), 63.23 (CH_2, C1), 63.05 (CH_2, C3), 33.42, 32.68, 31.32, 29.08, 28.91, 28.82, 28.50, 28.46, 28.31, 28.25, 28.22, (10×CH_2), 22.53, 18.71 (CH_2), 16.10, (CH_3); ESI-MS, *m*/*z* 359 [M+H]$^+$.

4.2. Human skeletal muscle cell cultures

Human skeletal muscle cells (myotubes) grown from satellite cells were isolated as previously described[16] from *musculus obliquus internus abdominis* from healthy donors, aged 48 ± 3 years and body mass index 24.5 ± 0.8 kg/m^2. The biopsies were obtained with informed consent and approved by the National Committee for Research Ethics (Oslo, Norway). The satellite cells were cultured in DMEM-Glutamax™ (5.5 mM glucose, Gibco, Life Technologies, Paisley, UK) with supplements during proliferation and differentiation into myotubes as previously described.[29] Experiments were performed after 7 days of differentiation, and the cells were exposed to DMSO (0.1%, Sigma Aldrich), different concentrations of THEFAs, THEFA-containing lipids or GW501516 for 96 h before initiation of experiments.

4.3. Fatty acid analysis

Human myotubes were cultured in 25 cm^2 Nunc™ Cell Culture Treated EasYFlasks™ (Thermo Scientific, Roskilde, Denmark) and the cells were treated with DMSO (0.1%), THEFAs or THEFA-containing lipids (10 μM or 30 μM) in culture media for 96 h. The cells were then washed with DPBS (with Ca^{2+} and Mg^{2+}), harvested in cold DPBS (0.5 mL) and then stored at −80 °C. Protein content was measured, lipids from washed myotubes were extracted and

methyl esters were obtained by heating of lipids with methanol at 90 °C for one hour as previously described.[30,31] Sulfuric acid was used as a catalyst. After extraction into an organic solvent, FA methyl esters were analyzed by gas–liquid chromatography. Gas chromatograph GC 8000 TOP (Finnigan, USA) was equipped with a programmed temperature vaporization injector, flame-ionization detector, AS 800 autosampler, and a fused silica capillary column coated with dimethylpolysiloxane stationary phase, DB1-ms (J&W Scientific, USA). Hydrogen was used as carrier gas. Column temperature was programmed from 110 to 310 °C with a gradient of 2.5 °C/min. GC signal was acquired and evaluated with Chromeleon software (Dionex, USA). Peaks were identified by means of known FA standards and by means of mass spectra, obtained by GC/MS analysis (GCQ, Finnigan, USA) on the same column. Internal standard C21:0 was used for quantitation after calibration with known mixtures of FA standards.

4.4. Substrate oxidation assay

Human myotubes were cultured on 96-well CellBIND® microplates (Corning Life-Sciences, Schiphol-Rijk, The Netherlands), and the cells were treated for 96 h with DMSO (0.1%), THEFAs or THEFA-containing lipids (10 μM or 30 μM), or the PPARδ agonist GW501516 (10 nM, gift from Trond Vidar Hansen, School of Pharmacy, University of Oslo) in culture media. [1-^{14}C]oleic acid (OA, 0.5 μCi/mL, 100 μM, PerkinElmer, Boston, MA, US) in Dulbecco's Phosphate Buffered Saline (DPBS, with Mg^{2+} and Ca^{2+}, Gibco, Life Technologies, Paisley, UK) with HEPES (10 mM) and L-carnitine (1 mM) was given during 4 h incubation for CO_2 oxidation assessment. A 96-well UniFilter-96® GF/B micro plate (PerkinElmer, Shelton, CT, US) was mounted on top of the CellBIND® plate as previously described[32] and the cells were incubated at 37 °C for 4 h. The [^{14}C]OA trapped in the filter was counted by liquid scintillation, and the result reflects CO_2 production. The remaining cell-associated radioactivity (CA) was also assessed by liquid scintillation, and the sum of CO_2 and CA was considered as total substrate uptake. After completion of the substrate oxidation assay, protein content in each well was measured according to Bradford.[33]

4.5. RNA-isolation and qPCR

Human myotubes were cultured in Nunc™ Cell Culture Treated EasYFlasks™ (25 cm^2, Thermo Scientific) and the cells were treated with DMSO (0.1%), THEFAs or THEFA-containing lipids (10 μM or 30 μM) in culture media for 96 h. The myotubes were washed with cold DPBS and centrifuged to a pellet before total RNA was isolated. Total RNA was purified using 1 mL TRIzol® reagent (Ambion™, Life Technologies, Carlsbad, CA, USA) combined with the RNeasy Mini Kit from QIAGEN (Hilden, Germany). The quantity of RNA was assessed spectrophotometrically using NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE, USA). The purity of RNA was measured using Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). The quality limit for further use of RNA was set to a RNA Integrity Number $\geqslant 7$ (out of 10). cDNA was obtained using the High Capacity Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, USA). Real-time PCR was performed on an ABI prism 7900 H sequence detection system (Applied Biosystems) using 384-well multiply PCR plates (Sarstedt Inc., Newton, USA), with gene specific primers from Applied Biosystems. Three different reference genes were included: 18*s* (Kit-FAM-TAMRA) from Eurogentec, Belgium, glyceraldehyde-3-phosphate dehydrogenase (Taqman Rodent *GAPDH*) and ribosomal protein, large, P0 (*RPLP0*) from Applied Biosystems. The NormFinder software was used to evaluate the reference genes and data normalized against all three reference genes are presented.[34]

Authors contribution

J.L.	Conducted biological assays, interpretation of data, wrote the manuscript
C.S.	Conducted biological assays, interpretation of data
P.B.	Conducted fatty acid analysis
H.T.	Supervised assays, interpretation of data
R.	Conducted synthetic work
M.W.	Conducted synthetic work
A.K.	Conducted synthetic work
A.C.R.	Supervised biological assays, interpretation of data
R.K.B.	Designed and supervised the study
A.D.M.	Designed and supervised chemical synthesis, wrote the manuscript
J.S.	Designed and supervised the study, interpretation of data, wrote the manuscript

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