Diabetologia (2007) 50:2171–2180
DOI 10.1007/s00125-007-0760-7

ARTICLE

Liver X receptor antagonist reduces lipid formation and increases glucose metabolism in myotubes from lean, obese and type 2 diabetic individuals

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Received: 24 February 2007 / Accepted: 24 May 2007 / Published online: 28 July 2007

Abstract
Aims/hypothesis Liver X receptors (LXRs) play important roles in lipid and carbohydrate metabolism. The purpose of the present study was to evaluate effects of the endogenous LXR agonist 22-*R*-hydroxycholesterol (22-R-HC) and its stereoisomer 22-*S*-hydroxycholesterol (22-S-HC), in comparison with the synthetic agonist T0901317 on lipid and glucose metabolism in human skeletal muscle cells (myotubes).
Methods Myotubes established from lean and obese control volunteers and from obese type 2 diabetic volunteers were treated with LXR ligands for 4 days. Lipid and glucose metabolisms were studied with labelled precursors, and gene expression was analysed using real-time PCR.
Results Treatment with T0901317 increased lipogenesis (de novo lipid synthesis) and lipid accumulation in myotubes, this increase being more pronounced in myotubes from type 2 diabetic volunteers than from lean volunteers. Furthermore, 22-S-HC efficiently counteracted the T0901317-induced enhancement of lipid formation. Moreover, synthesis of diacylglycerol, cholesteryl ester and free cholesterol from acetate was reduced below baseline by 22-S-HC, whereas glucose uptake and oxidation were increased. Both 22-S-HC and 22-R-HC, in contrast to T0901317, decreased the expression of genes involved in cholesterol synthesis, whereas only 22-R-HC, like T0901317, increased the expression of the gene encoding the reverse cholesterol transporter ATP-binding cassette subfamily A1 (*ABCA1*).
Conclusions/interpretation T0901317-induced lipogenesis and lipid formation was more pronounced in myotubes from type 2 diabetic patients than from lean individuals. 22-S-HC counteracted these effects and reduced de novo lipogenesis below baseline, while glucose uptake and oxidation were increased.

Keywords Glucose metabolism · Human myotubes · Hydroxycholesterols · Lipid metabolism · LXR antagonist · Type 2 diabetes · Liver X receptor

Abbreviations

ChREBP	carbohydrate responsive element-binding protein
FASN	fatty acid synthase
HMGC	hydroxymethylglutaryl-coenzyme A
LXR	liver X receptor
22-R-HC	22-*R*-hydroxycholesterol
22-S-HC	22-*S*-hydroxycholesterol
SREBP	sterol regulatory element-binding protein
SCD1	stearoyl-coenzyme A desaturase 1

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Introduction

Type 2 diabetes is an increasing health problem in Western society and is associated with obesity, insulin resistance and high plasma levels of glucose, triacylglycerol and cholesterols. Liver X receptors (LXRs) are in focus because of

their role in cholesterol, lipid and carbohydrate metabolism [1–4]. Cholesterol metabolites are endogenous ligands for LXR [5–7] and might therefore play an essential role in the pathophysiology of type 2 diabetes and obesity.

LXRs form heterodimers with the 9-*cis* retinoic acid receptor and bind to DNA in this form [8–10]. Two LXR genes have been identified, *LXRα* and *LXRβ* (also known as *NR1H3* and *NR1H2*, respectively), which encode highly preserved isoforms [11–13]. The *LXRβ* isoform is ubiquitously expressed [8], whereas *LXRα* is mainly restricted to tissues known to play an important role in lipid metabolism, such as liver, adipose tissue, macrophages, kidney, skeletal muscle and small intestine [10, 14, 15]. Further, human skeletal muscle cells have higher levels of LXRβ than LXRα [16].

LXRs have profound effects on hepatic lipid metabolism. These effects are mediated in part through the transcriptional regulation of the gene for sterol regulatory element-binding protein-1c (*SREBP-1c*), the master transcriptional regulator of fatty acid and triacylglycerol synthesis [17, 18]. Also, the gene encoding carbohydrate responsive element-binding protein (*ChREBP*, also known as *MLXIPL*), a glucose-sensitive transcription factor that promotes hepatic conversion of excess carbohydrate to lipid, has recently been identified as an LXR target gene [19]. In addition to *SREBP-1c* and *ChREBP*, the genes for other lipogenic enzymes, such as fatty acid synthase (*FASN*), have been reported as direct LXR targets [20]. LXRs are also well known sensors of cholesterol metabolism and control reverse cholesterol transport through regulation of ABC transporters [3, 21–23]. When fed a high-fat, high-cholesterol diet, LXR-null mice are resistant to obesity and demonstrate better glucose tolerance. Interestingly, on the same diet without cholesterol, LXR-null mice gained weight like their wild-type controls, possibly suggesting that LXR regulation of lipid metabolism is integrated through their role in cholesterol homeostasis [24].

The regulation of *LXRα* expression in adipocytes is well defined [25–28], but its role in adipocyte lipid metabolism remains unclear, as studies have reported contradictory results. Synthetic LXR agonists (T0901317 or GW3965) were shown to have no effects on triacylglycerol accumulation in adipocytes [27], while Juvet et al. [26] reported that these compounds were able to increase the size of lipid droplets in 3T3-L1 cells without affecting the expression of *Srebp-1* (also known as *Srebf1*) or the gene for the fatty acid transporter (*Fat*, also known as *Cd36*). Recently, Seo et al. [28] showed that T0901317 increased lipid storage in 3T3-L1 cells and enhanced the expression of *Srebp-1c*, *Fasn* and the genes for adipocyte fatty acid binding protein 2 (*Ap2*, also known as *Fabp4*) and peroxisome proliferator-activated receptor γ (*Pparg*).

Literature describing the role of LXRs on de novo lipid formation in human skeletal muscle is scarce. Cozzone et al. [29] reported on the basis of gene expression data that LXR agonists may promote increased utilisation of lipids and glucose in muscle cells without affecting insulin action. We have recently reported that: (1) an LXR agonist (T0901317) activates lipogenesis (de novo synthesis of lipids) and lipid accumulation in human myotubes; (2) the endogenous LXR ligand, 22-*R*-hydroxycholesterol (22-R-HC) affects gene expression differently than the synthetic stereoisomer 22-*S*-hydroxycholesterol (22-S-HC); and (3) 22-S-HC reduces synthesis of lipids and represses certain genes involved in lipogenesis and lipid handling by acting like an LXR antagonist [30, 31]. In addition, we have previously shown, using radiolabelled energy substrates, that chronic hyperglycaemia promotes lipogenesis, increases triacylglycerol formation and reduces glucose uptake in human skeletal muscle cells [32].

To date, several forms of treatments (lifestyle and drugs) are available for type 2 diabetes, but none efficiently control or cure the disease. The physiological roles of LXR suggest that it may be an interesting target for drug treatment of type 2 diabetes. Indeed, in a recent study we showed that activation of the LXR pathway in human skeletal muscle may be involved in increased accumulation of intramyotubular lipids in type 2 diabetes [33]. The purpose of the present study was to test whether the newly identified LXR antagonist, 22-S-HC [30], could have beneficial effects on de novo fatty acid synthesis and lipid metabolism, as well as glucose metabolism in myotubes established from lean, obese and obese type 2 diabetic individuals.

Methods

Materials DMEM-Glutamax, FCS, Ultroser G, penicillin–streptomycin–amphotericin B and trypsin–EDTA were obtained from Life Technology (Paisley, UK). Skeletal Muscle Growth Medium Bullet Kit was obtained from Clonetics (BioWittaker, Verviers, Belgium). [1-^{14}C]Palmitic acid (2.0 GBq/mmol), 2-deoxy-D-[^{3}H]glucose (222 GBq/mmol) and D-[U-^{14}C]glucose (4.5 GBq/mmol) were purchased from Dupont NEN Life Science Products (Boston, MA, USA). [1-^{14}C]acetic acid (2.0 GBq/mmol) and D-[1-^{14}C]glucose (2.0 GBq/mmol) were purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Palmitic acid, BSA (essentially fatty acid-free), cytochalasin B and extracellular matrix gel were purchased from Sigma Chemicals (St Louis, MO, USA). Insulin Actrapid was from Novo Nordisk (Bagsvaerd, Denmark). RNeasy Mini kit and RNase-free DNase were purchased from Qiagen Sciences (Oslo, Norway). Primers (directed against *36B4*, the gene for glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*], *GLUT1* [also known as *SLC2A1*], *GLUT4*

[also known as *SLC2A4*] and *ChREBP*) were purchased from Invitrogen (Oslo, Norway). High capacity cDNA archive kit, SYBR Green, TaqMan reverse-transcription reagents kit, TaqMan Universal PCR Master Mix and micro fluidic cards were purchased from Applied Biosystems (Warrington, UK). Protein assay kit was purchased from BioRad (Copenhagen, Denmark). T0901317 was obtained from Cayman Chemicals (Ann Arbor, MI, USA). 22-R-HC and 22-S-HC were purchased from Sigma. A Cholesterol RTU Kit was provided by bioMéreiux (Marcy l'Etoile, France) and the cytotoxicity detection kit (Cytotoxicty plus) was purchased from Roche (Mannheim, Germany). All other chemicals used were of standard commercial high-purity quality.

Human participants A cell-bank of satellite cells was established from muscle biopsy samples of the *M. vastus lateralis* of seven lean and seven obese control volunteers and seven obese type 2 diabetic volunteers, as previously described [33]. Groups were matched with respect to age, but differed by BMI, fasting plasma glucose concentrations, fasting serum insulin levels, hyperinsulinaemic–euglycaemic clamp results and HbA_{1c} (Table 1). Muscle biopsies were obtained in the fasted state by needle biopsy under local anaesthesia. All participants gave written informed consent; the local ethics committee of Funen and Vejle County (Denmark) approved the study.

Cell culture Cell cultures were established from satellite cells [34]. Briefly, muscle tissue was dissected in Ham's F-10 media at 4°C and dissociated by three successive treatments with 0.05% trypsin/EDTA, after which satellite cells were resuspended in Skeletal Muscle Growth Medium (2% FCS) without insulin. The cells were grown on wells coated with extracellular matrix gel. At about 80% confluence, fusion of myoblasts into multinucleated myotubes was achieved by growth for 8 days in DMEM with 2% FCS, physiological concentrations of insulin (25 pmol/l) and glucose (5.5 mmol/l). On day 4, myotubes were exposed to either vehicle (0.1% DMSO), 1 μmol/l T0901317, 1 μmol/l T0901317+10 μmol/l 22-S-HC, 10 μmol/l 22-S-HC or 10 μmol/l 22-R-HC for 4 days. Myotubes were rinsed twice and incubated for 4 h either with labelled palmitic acid, acetate or glucose as described below. Protein content of each sample was determined as described previously [35]. Corresponding RNA extractions were also obtained.

Table 1 Clinical characteristics of the study participants

	Participants		
	Lean	Obese	Obese type 2 diabetic
Age (years)	51±1	48±2	49±2
BMI (kg/m^2)	24±1	37±2[a]	35±1[a]
Fasting plasma glucose (mmol/l)	5.7±0.2	5.9±0.1	9.9±0.9[b]
Fasting serum insulin (pmol/l)	24±7	37±7	92±10[b]
HbA_{1c} (%)	5.6±0.1	5.5±0.1	7.9±0.6[b]
Glucose infusion rate (mg min^{-1} m^{-2})	406±21	224±28[a]	100±23[b]

Values are means±SEM (n=7).
[a] $p<0.05$ vs lean.
[b] $p<0.05$ vs lean and obese.

Cytotoxicity A cytotoxicity detection kit (Roche) was used according to the suppliers protocol to measure total and extra-cellular lactate dehydrogenase, which is a marker for cellular lysis.

Lipid distribution from labelled acetate To study lipid distribution from labelled acetate, myotubes were exposed to DMEM supplemented with 0.24 mmol/l BSA, 1.0 mmol/l L-carnitine, 25 pmol/l insulin, 10 mmol/l HEPES, [1-^{14}C] acetate (74 kBq/ml, 0.1 mmol/l) and 25 pmol/l insulin for 4 h. Myotubes were placed on ice, washed with PBS (1 ml), harvested into a tube with two additions of 125 μl distilled water and frozen at −20°C. Cells were later assayed for protein [35] and cellular lipids were extracted [36]. Briefly, homogenised cell fractions were extracted, lipids separated by thin-layer chromatography and radioactivity quantified by liquid scintillation. Another non-polar solvent mixture of hexane:ether:acetic acid (50:50:1) followed by hexane was used to separate free cholesterol from diacylglycerol.

Total cholesterol Myotubes were harvested in 1 ml of distilled H_2O and the concentration of total cholesterol was measured according to the suppliers protocol (bioMéreiux).

Cell-associated and oxidised palmitic acid Human myotubes were grown and differentiated in 96-well CellBIND tissue culture plates from Costar (Corning, NY, USA). Cells were exposed for 4 h to Dulbecco's phosphate-buffered saline with 0.49 mmol/l Mg^{2+} and 0.90 mmol/l Ca^{2+} supplemented with [1-^{14}C]palmitic acid (37 kBq/ml, 0.1 mmol/l), 10 mmol/l HEPES, 5 mmol/l glucose, 25 pmol/l insulin and 1 mmol/l L-carnitine and adjusted to pH 7.2. Radioactive $^{14}CO_2$ was trapped in a filter plate for 4 h before myotubes were placed on ice, washed twice with PBS (150 μl/well) and 200 μl 0.05 mol/l NaOH added. Lysed cell fractions (50 μl) and filter plates were quantified by liquid scintillation (Wallac MicroBeta Trilux, Perkin-Elmer, Oslo, Norway), as previously described [37]. Cells were later assayed for protein.

RNA isolation and micro fluidic cards Myotubes were washed and pelleted before total RNA was isolated by RNeasy Mini kit according to the supplier protocol (Qiagen). Samples were additionally incubated with RNase-free DNase for a minimum of 15 min. Equal amounts of RNA obtained from myotubes established from three individuals from each donor group were pooled and reverse-transcribed with the cDNA archive kit. Each sample (100 ng cDNA) was added to the slot of a micro fluidic card giving a total cDNA amount per well of about 2 ng. The samples were run once on two separate micro fluidic cards according to the suppliers protocol (Applied Biosystems). A micro fluidic card was designed to analyse 93 genes involved in lipid metabolism. *GAPDH* was used as an internal control and a fold change of ≥2 was considered an increase or a decrease in expression level.

Glucose uptake To study basal and insulin-mediated glucose uptake, cultures were exposed for 4 h to DMEM supplemented with 0.24 mmol/l BSA, 2-deoxy-D-[^{3}H]glucose (7.4 kBq/well) and 25 pmol/l or 1 μmol/l insulin. Cells were solubilised by addition of 500 μl 0.1 mol/l NaOH. An aliquot (50 μl) was removed for protein determination [35] and 300 μl was counted by liquid scintillation [38].

Glucose oxidation To study basal and insulin-mediated glucose oxidation, cultures were incubated for 4 h with DMEM supplemented with 0.24 mmol/l BSA, D-[1-^{14}C]glucose (74 kBq/ml, 5.5 mmol/l) and 25 pmol/l or 1 μmol/l insulin, as previously described [33].

Analysis of gene expression by RT-PCR RNA was isolated as described above. Total RNA (1 μg/μl) from seven lean donors was reverse-transcribed with hexamer primers using a thermal cycler (9600; Perkin-Elmer) (25°C for 10 min, 37°C for 1 h, 99°C for 5 min) and a TaqMan reverse-transcription reagents kit. Real-time PCR was performed using a detection system (ABI PRISM 7000; Applied Biosystems, Oslo, Norway). DNA expression was determined by SYBR Green and primers, using PrimerExpress (Applied Biosystems), as follows: *36B4* (Acc. no. M17885) Forward: CCATTCTATCATCAACGGGTACAA, Reverse: AGCAAGTGGGAAGGTGTAATCC); *GAPDH* (Acc. no. J04038/M33197) Forward: TGCACCACCAACTGCT TAGC, Reverse: GGCATGGACTGTGGTCATGAG); *GLUT1* (Acc. no. K03195) Forward: CAGCAGCCC TAAGGATCTCTCA, Reverse: CCGGCTCGGCTGA CATC); *GLUT4* (Acc. no. M20747) Forward: GCTACCTCTACATCATCCAGAATCTC, Reverse: CCA GAAACATCGGCCCA); *ChREBP* (Acc. no. NM_032951) Forward: CGGCATTGAGCTCCTCAATC, Reverse: GCA GAAGACAGCTGAGTACATCCTTA. Each target gene was quantified in triplicate, this being carried out in a 25 μl reaction volume according to the supplier's protocol. All assays were run for 40 cycles (95°C for 12 s followed by 60°C for 60 s). The transcription levels were normalised to the housekeeping control genes *36B4* and *GAPDH*.

Statistical analysis Data in text, tables and figures are given as means (±SEM) and all cell culture experiments were run in triplicate. Comparisons of different treatments and between groups were evaluated by paired and unpaired Student's *t* tests, respectively. A value of $p<0.05$ was considered significant.

Results

Cell cultures Myotubes showed no difference in morphological appearance after treatments with either T0901317, T0901317+22-S-HC, 22-S-HC or 22-R-HC for 4 days. Neither of the treatments reduced cellular protein levels (data not shown) or leakage of lactate dehydrogenase ($p>0.4$). Concentration of total cholesterol in myotubes was unchanged by T0901317 and both 22-HCs after 4 days (range 204±34 to 266±27 nmol/mg cell protein) ($p>0.15$).

Incorporation of radiolabelled acetate into fatty acids and complex lipids Myotubes were treated either with T0901317, T0901317+22-S-HC, 22-S-HC or 22-R-HC for 4 days before they were incubated with [1-^{14}C]acetate for 4 h. The synthetic LXR agonist, T0901317, increased total lipogenesis by two- to threefold (Fig. 1a). This effect was more pronounced in myotubes from obese type 2 diabetic patients than in cells from lean individuals (Fig. 1a), while 22-S-HC reduced lipogenesis 1.5- to 2.5-fold when compared with baseline (DMSO 0.1%) and completely abolished the effect of T0901317. Total lipogenesis was also reduced by 22-R-HC in myotubes from lean and obese individuals (Fig. 1a). In addition, T0901317 increased incorporation of acetate into NEFA by three- to fivefold, this increase being significantly higher in type 2 diabetic cells than in myotubes from lean volunteers (Fig. 1b). The synthetic LXR antagonist, 22-S-HC, reduced NEFA levels in myotubes from lean volunteers by 30% when compared with baseline and abolished almost completely the effect of T0901317 (Fig. 1b). Diacylglycerol levels were increased about twofold after treatment with T0901317 in cells from obese and type 2 diabetic volunteers (Fig. 1c). A combination of T0901317 and 22-S-HC reduced diacylglycerol levels by twofold compared with baseline, while 22-S-HC reduced diacylglycerol levels by threefold (Fig. 1c). Cellular triacylglycerol levels were also increased by 3.5- to 5.5-fold after exposure to T0901317, an effect that was markedly reduced when T0901317 was combined with

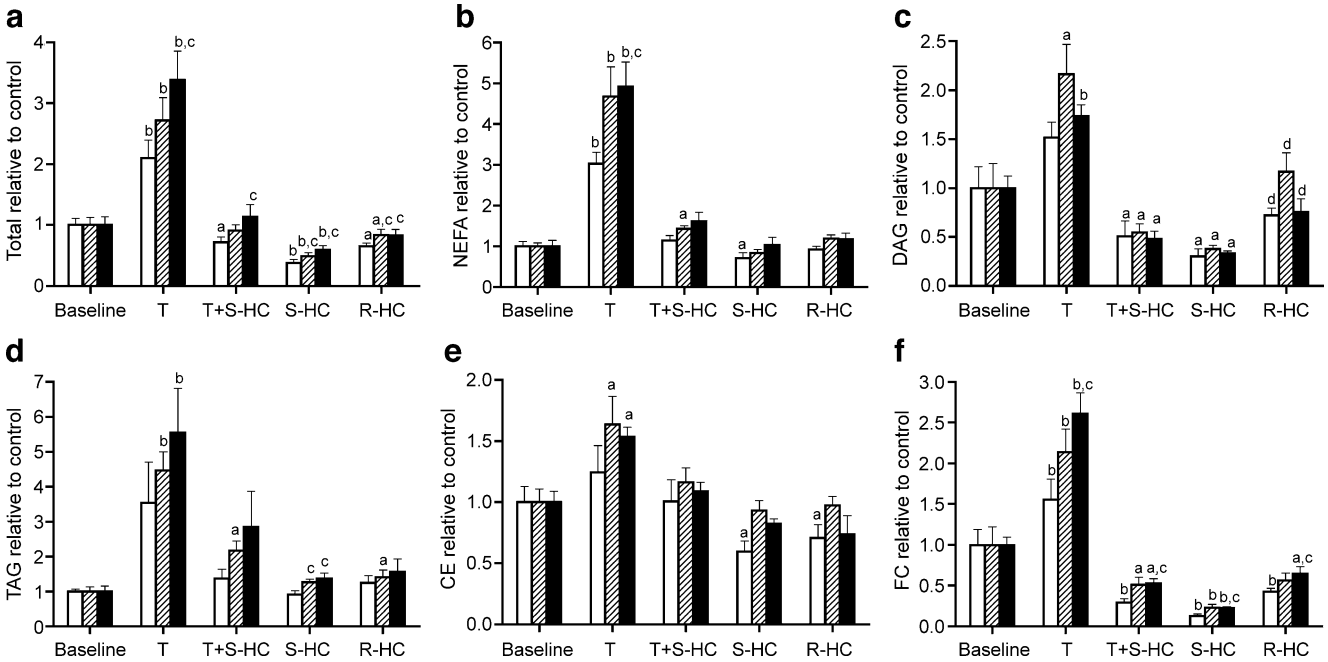


Fig. 1 Synthesis of cellular lipids from acetate after treatment with LXR modulators. Differentiated myotubes were treated for 4 days with 1 μmol/l T0901317 (T), 1 μmol/l T+10 μmol/l 22-S-HC (T+S-HC), 10 μmol/l 22-S-HC or 10 μmol/l 22-R-HC (R-HC) and then incubated with [1-^{14}C]acetic acid (74 kBq/ml, 0.1 mmol/l) for 4 h. **a** Total lipogenesis (range for baseline 0.4–1.4 nmol/mg cell protein; **b** NEFA; **c** diacylglycerol (DAG); **d** triacylglycerol (TAG); **e** cholesteryl ester (CE); and **f** free cholesterol (FC). Results are means±SEM (n=5–7). [a]p<0.05 vs baseline, [b]p<0.05 vs all other treatments, [c]p<0.05 vs lean myotubes, [d]p<0.05 vs 22-S-HC. Open bars, lean; hatched bars, obese; closed bars, type 2 diabetes

22-S-HC (Fig. 1d). Triacylglycerol levels were not altered by treatment with 22-S-HC. However, after 22-S-HC treatment triacylglycerol formation from acetate was significantly higher in myotubes from obese and type 2 diabetic volunteers than in myotubes from lean volunteers (Fig. 1d). Cholesteryl esters were increased by 50% after treatment of myotubes from obese and type 2 diabetic participants with T0901317; in myotubes from lean participants only cholesteryl esters decreased after exposure to 22-S-HC and 22-R-HC (Fig. 1e). Levels of free cholesterol were 1.5- to 2.5-fold increased after exposure to T0901317 and were significantly higher in type 2 diabetic cells than in myotubes from lean volunteers. After exposure to oxysterols, free cholesterol was reduced two- to eightfold, but significantly less in myotubes from type 2 diabetic than in those from lean volunteers (Fig. 1f).

Palmitic acid uptake and oxidation to carbon dioxide Cell-associated palmitic acid was increased in all donor groups after T0901317 treatment, but tended to increase more in myotubes from obese and type 2 diabetic volunteers (Fig. 2a). The T0901317-induced increase was 50% for

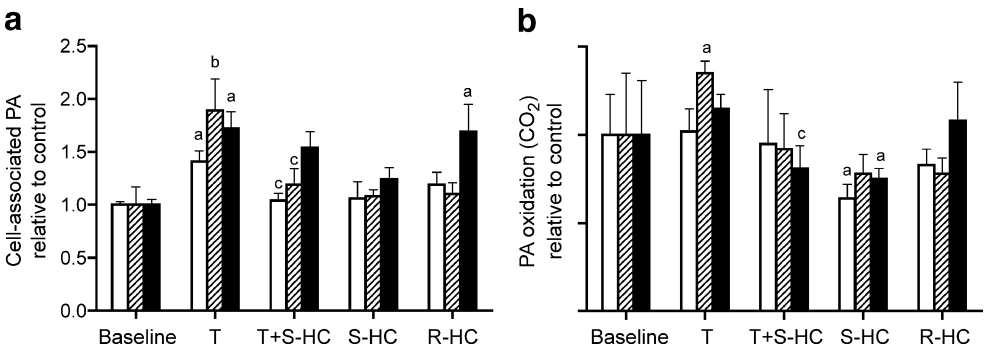


Fig. 2 Cell-associated and oxidised palmitate after treatment with LXR modulators. Human myotubes were grown and differentiated in 96-well CellBIND tissue culture plates, treated as described in Fig. 1 and then exposed for 4 h to [1-^{14}C]palmitic acid (37 kBq/ml, 0.1 mmol/l) to measure cell-associated and oxidised palmitate. **a** Range for baseline 38.0–86.5 nmol/mg cell protein; **b** range for baseline 0.6–5.6 nmol/mg cell protein. CO_2 was trapped on 96 well filter plates for 4 h. Results are presented as means±SEM (n=5–6). [a]p<0.05 vs baseline, [b]p<0.05 vs all other treatments, [c]p<0.05 vs T0901317. Open bars, lean; hatched bars, obese; closed bars, type 2 diabetes

lean and nearly twofold for obese and type 2 diabetic myotubes, while the combination of 22-S-HC and T0901317 significantly reduced the effect of T0901317 for lean and obese cells. In addition, cell-associated palmitic acid was not influenced after treatment with 22-S-HC or 22-R-HC (except for type 2 diabetic cells) and there were no differences between cells from lean, obese and type 2 diabetic participants (Fig. 2a). In obese myotubes, palmitic acid oxidation to CO_2 was increased 40% after T0901317 treatment and reduced ~30% against baseline, for lean and type 2 diabetic cells, by exposure to 22-S-HC. When compared with T0901317, T0901317+22-S-HC reduced palmitic acid oxidation only in cells of type 2 diabetic origin (Fig. 2b).

Expression of genes involved in lipid formation and cholesterol homeostasis Pooled RNA samples from myotubes of lean, obese and type 2 diabetic individuals treated with T0901317, T0901317+22-S-HC, 22-S-HC and 22-R-HC were analysed on real-time micro fluidic cards. Since there were no clear differences between the groups in gene expression from pooled RNA, only data from lean myotubes are presented. Expression of *LXRα* and *SREBP-1* was increased several times after chronic T0901317 treatment (Fig. 3a). The T0901317-induced elevation of *LXRα* and *SREBP-1* expression was reduced upon treatment with T0901317+22-S-HC, while 22-S-HC alone did not alter expression. Expression of *LXRα* and *SREBP-1* were moderately increased by 22-R-HC (Fig. 3a). The expression levels of genes directly involved in lipogenesis and lipid formation, e.g. acetyl-coenzyme A carboxylase (*ACC1*, also known as *ACACA*), *FASN,* stearoyl-coenzyme A desaturase (*SCD1*) and acyl-coenzyme A synthetase long chain family member 1 (*ACSL1*), were all increased by T0901317 treatment (Fig. 3b). Interestingly, this effect was abolished when T0901317 was combined with 22-S-HC (Fig. 3b). The synthetic LXR ligand, 22-S-HC, reduced expression levels of *SCD1* and *FASN* (Fig. 3b) by sevenfold below baseline, while 22-R-HC did not influence the expression of any of these genes (Fig. 3b). None of the treatments had effect on acetyl-coenzyme A carboxylase 2 (*ACC2*) expression (data not shown). Genes involved in cholesterol homeostasis were also influenced by LXR-ligands (Fig. 3c). Expression of the ATP-binding cassette subfamily A1 (*ABCA1*) was increased 20-fold and 6-fold after exposure to T0901317 and 22-R-HC, respectively. Importantly, 22-S-HC alone or in combination with T0901317 did not alter the expression of this gene (Fig. 3c). In addition, expression of hydroxymethylglutaryl-coenzyme A (HMGC) synthase 1 (*HMGCS1*) and HMGC reductase (*HMGCR*) was not regulated by T0901317, but decreased six- to sevenfold by 22-S-HC and by 2.5- to 3.5-fold by 22-R-HC (Fig. 3c).

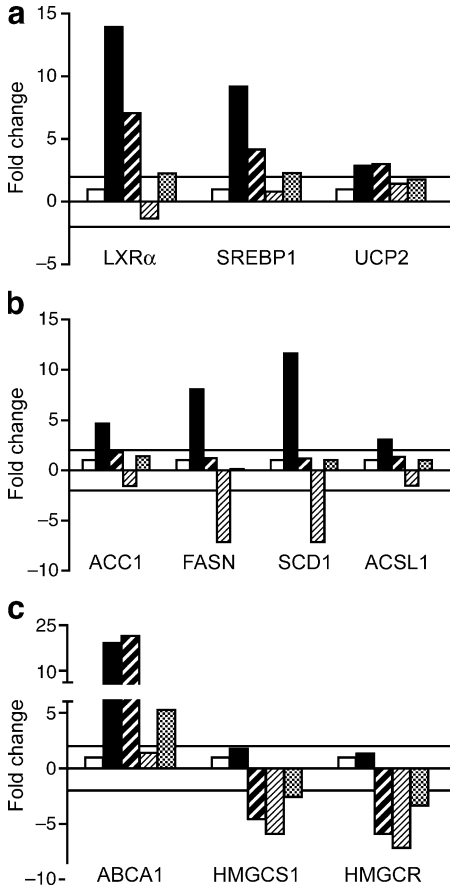
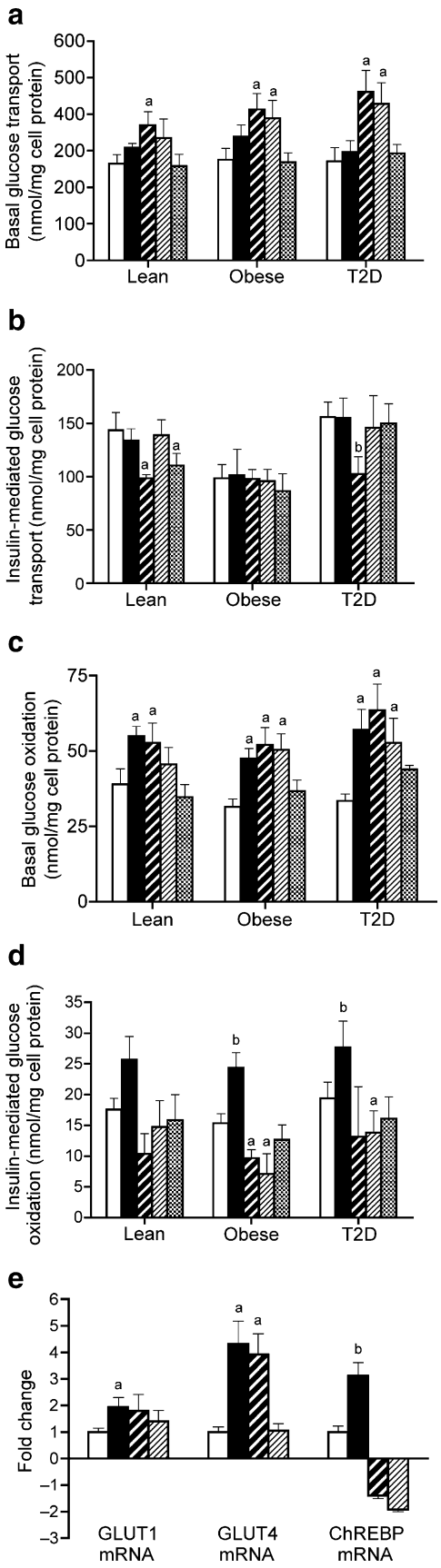


Fig. 3 Expression of genes involved in lipogenesis and cholesterol homeostasis. Differentiated myotubes were treated with 1 μmol/l T0901317 (closed bars), 1 μmol/l T0901317+10 μmol/l 22-S-HC (dark hatched bars), 10 μmol/l 22-S-HC (light hatched bars) and 10 μmol/l 22-R-HC (checkered bars) for 4 days. Total RNA was extracted, reverse-transcribed and analysed on micro fluidic cards. **a** Expression of LXRα, *SREBP-1* and the gene encoding uncoupling protein 2 (*UCP2*). **b** Expression of genes involved in lipid metabolism. **c** Expression of genes involved in cholesterol metabolism. Results are normalised to levels of *GAPDH*, and a fold change ≥2 from baseline (open bars) was considered to be an increase or a decrease in the level of expression (n=3 for each treatment, pooled RNA)

Glucose transport and oxidation T0901317 slightly increased basal glucose transport as reported earlier. This increase, however, was only significant when data from all three donor-groups were pooled (data not shown). Treatment with 22-S-HC increased glucose uptake by 30%, 40% and 60% in myotubes from lean, obese and type 2 diabetic individuals, respectively (Fig. 4a). Moreover, 22-S-HC increased glucose transport equally in combination with T0901317 (Fig. 4a). The insulin-stimulated glucose uptake was in general unchanged after treatment with T0901317, T0901317+22-S-HC, 22-S-HC or 22-R-HC in all groups (Fig. 4b). To further evaluate intracellular glucose metabolism after chronic treatment with LXR ligands, glucose oxidation assessed by CO_2 trapping was studied. T0901317 increased basal glucose oxidation in myotubes from lean,



◀ **Fig. 4** Glucose transport and oxidation in myotubes after chronic treatment with LXR modulators. Differentiated myotubes were treated as described in Fig. 1 and incubated with 2-deoxy-D-[^{3}H]glucose or D-[1-^{14}C]glucose with or without 1 μmol/l insulin for 4 h or harvested for total RNA extraction, reverse-transcribed and analysed by real-time PCR. **a** Basal glucose transport; **b** insulin-stimulated minus basal glucose transport; **c** basal glucose oxidation (CO_2); **d** insulin-stimulated minus basal glucose oxidation; and **e** *GLUT1*, *GLUT4* and *ChREBP* mRNA levels. Results are normalised to levels of *36B4*. Means±SEM (n=6–7). $^{a}p<0.05$ vs baseline (open bars), $^{b}p<0.05$ vs all other treatments.T2D, type 2 diabetes. Closed bars, T0901317; dark hatched bars, T0901317+22-S-HC; light hatched bars, 22-S-HC; checkered bars, 22-R-HC

obese and type 2 diabetic individuals by 40%, 50% and 70%, respectively (Fig. 4c). All treatments with 22-S-HC alone or in combination with T0901317, except for 22-S-HC in lean myotubes, resulted in a significant increase (35–90%) in glucose oxidation (Fig. 4c). The insulin-stimulated glucose oxidation increased for T0901317 when compared with baseline (Fig. 4d).

Expression of genes involved in glucose metabolism Myotubes from lean volunteers were treated with T0901317, T0901317+22-S-HC and 22-S-HC, before genes involved in glucose metabolism were analysed by real-time PCR. The expression levels of *GLUT1* (Fig. 4e) were significantly increased only after T0901317. *GLUT4* was increased fourfold after treatment with both T0901317 and T0901317 +22-S-HC, while 22-S-HC did not alter *GLUT4* mRNA expression (Fig. 4e). Importantly, *ChREBP* was increased 3.5-fold by T0901317, but this effect was completely abolished when combined with 22-S-HC (Fig. 4e). Exposure to 22-S-HC tended to reduce mRNA expression of *ChREBP* (Fig. 4e).

Discussion

The present study shows that 22-S-HC, a possible LXR antagonist, (1) repressed lipogenesis; (2) decreased formation of complex lipids and free cholesterol below baseline; and (3) increased glucose transport and utilisation equally in myotubes from lean, obese and type 2 diabetic volunteers, without interfering with expression of the *ABCA1* transporter gene or concentration of total cholesterol. Also, the synthetic LXR agonist T0901317 increased both the expression of lipogenic genes and lipogenesis in myotubes from lean, obese and type 2 diabetic individuals, while 22-S-HC markedly suppressed these effects. Importantly, the T0901317-induced increase in lipogenesis was more pronounced in myotubes from type 2 diabetic patients than in cells from lean individuals.

Activators of LXRs are potent stimulators of fatty acid and triacylglycerol synthesis [1, 26]. This effect is mediated to a large degree by LXR-dependent expression of *SREBP-1c* and its downstream targets, *FASN*, *ACC* and *SCD1* [18, 39]. Chisholm et al. [40] have demonstrated that administration of T0901317 to male db/db mice for 12 days resulted in a more severe hepatic triacylglycerol accumulation than observed in non-diabetic mice. Recently, it has been shown in human myotubes that LXR activation promotes lipogenesis [30] and triacylglycerol accumulation [29, 33]. These studies confirmed that activation of LXR increases lipogenesis and lipid formation from acetate in human myotubes and that myotubes established from type 2 diabetic individuals are more responsive than myotubes from lean donors. In contrast, we have shown that 22-S-HC reduced de novo lipogenesis in myotubes from all three donor groups and efficiently counteracted the effects of T0901317 on enhancing lipogenesis and lipid formation. These functional isotope data correlated with the increased expression levels of genes involved in lipogenesis after treatment with T0901317 and with reduced expression after exposure to 22-S-HC [30]. Several in vivo studies have reported that insulin sensitivity can be restored after depletion of accumulated lipids in rat skeletal muscle, supporting a direct link between intramyocellular lipid accumulation and insulin resistance [41, 42]. Previously, we have shown in human myotubes from lean and type 2 diabetic volunteers that LXR activation by chronic agonist treatment can increase uptake of fatty acids (palmitate) and incorporation into diacylglycerol and triacylglycerol as well as increasing fatty acid oxidation [33]. The present study confirmed that LXR activation leads to an increased uptake of fatty acids in myotubes from all three donor groups and also showed that 22-S-HC can counteract these effects. Therefore, the possibility of reducing accumulation of intramyocellular lipids by an LXR antagonist could have a great therapeutic potential, although the mechanism linking intramyocellular lipid to insulin resistance is still not fully elucidated [43].

Effects of LXR ligands on cholesterol turnover in cells have not been comprehensively examined. Aravindhan et al. [44] recently showed in HepG2 cells that cellular cholesterol fluxes were altered by LXR activation and that the increase in cholesterol synthesis did not compensate for the increased cellular cholesterol efflux, resulting in a net cellular cholesterol loss. We have previously demonstrated that LXR activation increased levels of cholesterol esters from labelled palmitic acid in human myotubes [33]. The present study shows that levels of free cholesterol and cholesterol esters from labelled acetate and palmitic acid increased after T0901317 treatment, but were markedly reduced by both 22-HC isoforms. Furthermore, Peet et al. [45] have reported that LXR$\alpha^{-/-}$ knockout mice have impaired expression of *HMGCS* and *HMGCR* and that expression and degradation of both are controlled by sterols through their sterol sensing domain and SREBP2 (reviewed in [46]). We observed decreased mRNA levels of *HMGCS* and *HMGCR* after exposure to both 22-HC isomers. Interestingly, unlike 22-R-HC, 22-S-HC decreased expression of *HMGCS* and *HMGCR* without altering the expression of *ABCA1*. In contrast to observations in genes involved in lipogenesis, 22-S-HC was unable to counteract the T0901317-induced expression of *ABCA1* and *GLUT4*. This suggests that LXR-dependent regulation of lipogenesis versus reverse cholesterol and glucose transport may involve different regulatory pathways.

Surprisingly, 22-S-HC seemed to increase basal glucose transport and glucose oxidation. This could not be explained by increased expression of glucose transporters since expression of *GLUT1* and *GLUT4* was unaltered by 22-S-HC. In line with previous observations [33], the expression of glucose transporters was induced by T0901317, but resulted in a marginal increase in glucose uptake. Thus, the mechanisms by which 22-S-HC elevates basal glucose transport is at present unknown. However, increased glucose uptake and oxidation can be secondary effects to reduced lipogenesis and lipid handling, resulting in an increased capacity of mitochondria to oxidise glucose and possibly demand a higher glucose uptake [47]. In line with this, Shigematsu et al. [48] have shown that depletion of plasma membrane cholesterol resulted in an insulin-independent increase in membrane-localised GLUT4, which was reversible by cholesterol replenishment. We observed that 22-S-HC reduced synthesis of free cholesterol in myotubes and increased transport of glucose suggesting a possible connection. However, concentrations of total cell cholesterol were unchanged and 22-R-HC also reduced synthesis of free cholesterol without increasing glucose transport. In addition, two research groups have recently demonstrated in *ob/ob* mice deficient in ChREBP or with liver-specific inhibition of this protein that these animals have reduced plasma glucose concentrations without a reduction in plasma insulin levels as well as reduced lipogenesis due to a decrease in *Scd1* and *Acc1* mRNA levels and activity [49, 50]. In line with this, we observed that exposure to 22-S-HC abolished T0901317-induced expression of *ChREBP*, reduced basal mRNA levels, increased basal glucose transport and reduced lipogenesis. This implies that the observed effects of 22-S-HC on glucose transport and lipogenesis in human skeletal muscle could be mediated through reduced expression of *ChREBP*.

In summary, this study shows that treatment with an LXR agonist (T0901317) increased lipogenesis and lipid formation more extensively in myotubes from type 2 diabetic patients than in myotubes from lean individuals. Further, 22-S-HC counteracted the effects of T0901317 on

lipid metabolism and reduced de novo lipogenesis below baseline, while at the same time glucose uptake and oxidation were increased. Importantly, 22-S-HC also decreased the expression of genes involved in cholesterol synthesis without altering the expression of the reverse cholesterol transporter *ABCA1*. On the basis of the observed metabolic effects on myotubes in vitro, an LXR antagonist could have therapeutic potential in the treatment of obesity and type 2 diabetes. However, further research is required to establish whether 22-S-HC itself or derivatives might have similar effects in vivo.

Acknowledgements We thank I. Lynfort, M.-A. Baltzersen and B. Andersen for the excellent technical assistance. The Norwegian Research Council, The Norwegian Pharmaceutical Society, Norwegian Diabetes Foundation, Freia Chokoladefabrik Medical Foundation, Nansen Foundation, Danish Medical Research Council, the Danish Diabetes Association and the Novo-Nordisk Foundation are thanked for the financial support.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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