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LXR antagonists induce *ABCD2* expression

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ABSTRACT

X-linked adrenoleukodystrophy (X-ALD) is a rare neurodegenerative disorder characterized by the accumulation of very-long-chain fatty acids resulting from a β-oxidation defect. Oxidative stress and inflammation are also key components of the pathogenesis. X-ALD is caused by mutations in the *ABCD1* gene, which encodes for a peroxisomal half ABC transporter predicted to participate in the entry of VLCFA-CoA into the peroxisome, the unique site of their β-oxidation. Two homologous peroxisomal ABC transporters, ABCD2 and ABCD3 have been proven to compensate for ABCD1 deficiency when overexpressed. Pharmacological induction of these target genes could therefore represent an alternative therapy for X-ALD patients. Since LXR activation was shown to repress *ABCD2* expression, we investigated the effects of LXR antagonists in different cell lines. Cells were treated with GSK(17) (a LXR antagonist recently discovered from the GlaxoSmithKline compound collection), 22(S)-hydroxycholesterol (22S-HC, another LXR antagonist) and 22R-HC (an endogenous LXR agonist). We observed up-regulation of *ABCD2*, *ABCD3* and *CTNNB1* (the gene encoding for β-catenin, which was recently demonstrated to induce *ABCD2* expression) in human HepG2 hepatoma cells and in X-ALD skin fibroblasts treated with LXR antagonists. Interestingly, induction in X-ALD fibroblasts was concomitant with a decrease in oxidative stress. Rats treated with 22S-HC showed hepatic induction of the 3 genes of interest. In human, we show by multiple tissue expression array that expression of *ABCD2* appears to be inversely correlated with *NR1H3* (LXRα) expression. Altogether, antagonists of LXR that are currently developed in the context of dyslipidemia may find another indication with X-ALD.

1. Introduction

X-linked adrenoleukodystrophy (X-ALD, OMIM 300100) is the most frequent peroxisomal disorder with an incidence of 1/17,000 births [1,2]. The biochemical hallmark of this neurodegenerative disease is the accumulation of very-long-chain fatty acids (VLCFA, fatty acids with a carbon chain longer than 22 carbons) in plasma and tissues resulting from a β-oxidation defect. Oxidative stress and inflammation are also key components of the pathogenesis of X-ALD. However, the exact link between VLCFA accumulation, demyelination, oxidative stress and inflammation as well as the sequence of events leading to the disease are still unclear. X-ALD is caused by mutations in *ABCD1* gene located in Xq28 [3]. *ABCD1* encodes for a peroxisomal half ABC transporter, which is responsible for the entry of very-long-chain fatty acyl-CoA into the peroxisome, the unique site of their β-oxidation [4]. Two homologous peroxisomal ABC transporters, ABCD2 [5,6] and ABCD3 [7], have been proven to compensate for ABCD1 deficiency when overexpressed in X-ALD fibroblasts [8–11]. Functional redundancy is also recognized *in vivo* since reversion of the adrenomyeloneuropathy-like phenotype has been observed in *Abcd1* null mice overexpressing *Abcd2* in a ubiquitous manner [12]. Several studies have suggested that ABCD2 plays a role not only in the transport of long and very-long-chain saturated fatty acids, but also in the transport of monounsaturated and polyunsaturated fatty acids (PUFA) [13–16]. Actually, the function of ABCD2 appears to be central for lipid homeostasis as suggested by the fact that the *ABCD2* gene is a target of numerous regulation pathways in relation with lipid metabolism. The *ABCD2* gene is up-regulated by cholesterol depletion *via* SREBP [17], by PPARα activators such as fibrates [18], by thyroid hormone and thyromimetics [19–21], by dehydroepiandrosterone [22], by PUFA diets [23], and by inhibitors of histone deacetylases such as 4-

Abbreviations: ACOX, Acyl-CoA oxidase; ABC, ATP binding cassette; DHA, docosahexaenoic acid (C22:6 n−3); H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HC, hydroxycholesterol; HFD, high fat diet; LXR, Liver X receptor; LXRE, LXR response element; MTE, multiple tissue expression; MUFA, monounsaturated fatty acids; PPAR, peroxisome proliferator activated receptor; PUFA, Polyunsaturated Fatty acids; ROS, reactive oxygen species; SREBP, sterol regulatory element binding protein; TcF, T cell factor; T3, triiodothyronine; T4, thyroxine; TR, thyroid hormone receptor; TRE, thyroid hormone response element; VLCFA, very-long-chain fatty acids; X-ALD, X-linked adrenoleukodystrophy

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phenylbutyrate [24,25] or valproic acid [26]. The promoter analysis of the *ABCD2* gene led us to identify a direct repeat motif with a 4 bp spacer as a functional thyroid hormone response element (TRE) [19]. This motif is also known as a response element for the Liver X receptor (LXR), a nuclear receptor for oxysterols [27]. We therefore investigated whether the *ABCD2* promoter could be responsive to LXR ligands and identified LXRα as a negative modulator of *ABCD2* expression [28]. Moreover, this study revealed a complex cross-talk involving key actors of the lipid metabolism (TRs, LXRs and SREBPs) since the TRE/LXRE motif of the *ABCD2* promoter overlaps a SRE motif. LXRα, upon ligand binding, was shown to interfere with SREBP1c-mediated activation of the *Abcd2* promoter. Very recently, β-catenin and TCF-4, which are important components of the Wnt signaling pathway, were described as inducers of the *ABCD2* expression [29]. Interestingly, the Wnt components are known to be repressed by oxysterols [30,31].

Numerous studies have been conducted to identify LXR ligands that could be used to treat dyslipidemia. Screening of the GlaxoSmithKline compound collection resulted in the discovery of an LXR antagonist (GSK1440233A, compound 17) [32]. This synthetic molecule antagonized the expression of LXR target genes in HepG2 and THP1 cells with apparent IC50 values less than 100 nM. GSK(17), as well as 22S-hydroxycholesterol (22S-HC), another antagonist of LXR described to reduce lipogenesis and formation of complex lipids and free cholesterol [33], could therefore be considered in the context of X-ALD.

In this study, we explored the effects of LXR antagonists (GSK(17) or 22S-HC) in human hepatoma cells and human skin fibroblasts from X-ALD patients to see whether these LXR antagonists can induce *ABCD2* expression or other target genes (*ABCD3*, *ACOX1*, which encodes for the key enzyme of the peroxisomal β-oxidation, and *CTNNB1* which encodes for β-catenin). In X-ALD fibroblasts, we also investigated whether the treatments modulate oxidative status and fatty acid content. The results were compared with those obtained from a treatment with 22R-HC, an endogenous LXR agonist. Expression of *Abcd2*, *Abcd3* and *Ctnnb1* genes and other LXR target genes (*Abca1* and *Srebp1*) in liver from control rats or rats treated with 22S-HC was also analyzed. Finally, we investigated and compared the expression level of *ABCD2* and *NR1H3* (encoding for LXRα) genes in human tissues.

2. Materials and methods

2.1. Cell culture and treatments

Human HepG2 (Human hepatoma cell line, ATCC HB-8065), human skin X-ALD fibroblasts (ALD-3 cell line or Coriell Institute GM17819) and WT human skin fibroblasts (Coriell Institute GM03348) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2 in the absence of antibiotics. The ALD-3 cell line [9], which is a non-transformed cell line with a large deletion in the *ABCD1* gene was kindly provided by Dr. A. Pujol, Barcelona, (Spain). Cells at 80% confluence were treated during 3 days with LXR ligands and the medium was changed daily. 22R-HC and 22S-HC (Sigma) were used at 10 μM in ethanol from a stock solution prepared at 10 mM in ethanol. GSK(17) (GSK1440233A) kindly provided by Dr. William J. Zuercher (GlaxoSmithKline), was used at 100 nM in DMSO from a stock solution at 100 μM in DMSO.

2.2. The 22-S-hydroxycholesterol-effect study in Wistar rats

The details of this animal study have been previously published [34]. In summary, male Wistar rats were fed *ad libitum* a regular maintenance diet (Special Diets Services, Witham, Essex, UK) for 5 days after arrival to our animal facilities. Then a feeding regimen was adopted using high-fat diet (HFD) for 21 days. The rats were about 9 weeks old at the start of the experimental feeding. Wistar rats were randomly divided into two groups with 6 animals each, receiving HFD ± 22S-HC (30 mg/kg/day) for 21 days. There were 3 animals in each cage and they had free access to tap water. The experimental protocol (Id: 823) was approved by the National Animal Research Authority. Immediately after termination of the animals, tissues were collected.

2.3. RT-qPCR analysis from treated cells

Cells were harvested with 0.25% trypsin/EDTA (Sigma) and washed twice with PBS. Total RNA was isolated and treated with DNase to discard genomic DNA using the RNeasy Mini kit (Qiagen) following manufacturer's instruction. After quantification and verification of their quality by gel electrophoresis, 1 μg of RNA was reverse transcribed to generate cDNA using the "iScript cDNA synthesis kit" (Bio-Rad). These cDNA were further analyzed by quantitative PCR using the SYBR Green real-time PCR technology and a StepOne Plus system (Applied Biosystems). PCR reactions were carried out in duplicate in a final volume of 25 μl containing 12.5 μl of GoTaq qPCR Mastermix (Promega), 300 nM of forward and reverse primers and 5 μl of diluted cDNA (dilution 1/20 for *Abcd2* gene or dilution 1/40 for the other genes). The PCR enzyme (*Taq* DNA polymerase) was heat-activated at 95 °C for 2 min, and the DNA was amplified for 40 cycles at 95 °C for 15 s and 60 °C for 1 min, followed by a melting curve analysis to control the absence of non-specific products. For each transcript, the amplification efficiency was determined by the slope of the standard curve generated from 2-fold serial dilutions of cDNA. Quantification of gene expression was performed using Cycle to threshold (Ct) values and normalized by the *36B4* gene. Primers for human (h) genes were chosen using the Primer Express software program (Applied Biosystems) and purchased from Eurogentec: *hABCD2*: F, 5′-GAACTGCTGTCATTCAAGAATCTG-3′, R, 5′-TGCCAATGTGTCACTGAGAGG-3′; *hABCD3*: F, 5′-GTGGTATCATTGGTCGTAGCAG-3′, R, 5′-AGCCTTACTCGGAAGCACAG-3′; *hACOX1*: F, 5′-CCTGAGCCTCTGGATCTTCAC-3′, R, 5′-GGTGAGTTCCATGACCCATCTCTG-3′; *hCTNNB1*: F, 5′-ATGTCGAGCGTTTGGCTGAA-3′, R, 5′-TGGTCCTCGTCATTTAGCAGTT-3′; *h36B4*: F, 5′-CTCCTTTGGGCTGGTCATCC-3′, R, 5′-CAGACAGACACTGGCAACATTG-3′.

2.4. RT-qPCR analysis from treated rats

Liver was collected from each rat and stored at −70 °C. The tissues were homogenized and total RNA were isolated and reversely transcribed as previously described [34]. Real time qPCR was performed using an ABI PRISM® 7000 Detection System. DNA expression was determined using the SYBR Green real-time PCR technology. Primers (*rAbcd2*: F, 5′-CAGCGTCCACCTCTACCACATAG-3′, R, 5′-CGTCCAGCAATGCGTACTTCG-3′; *rAbcd3*: F, 5′-GGCTGGGCGTGAAATGACTA-3′, R, 5′-GCCATTTGGACCACAAATGA-3′; *r36B4*: F, 5′-CACCTTCCCACTGGCTGAAA-3′, R, 5′-CGCAGCCGCAAATGC-3′; *rCtnnb1*: F, 5′-CTGATAAAGGCAACTGTTGGA-3′, R, 5′-CCCTGTTCCCGCAAAGG-3′; *rAbcA1*: F, 5′-GTGTTTCTCAGAGCAGTTCTGA-3′, R, 5′-CGTGCCCAATGTCCTCCA-3′; *rSrebp1*: F, 5′-GGAGCCATGGATTGCACATT-3′, R, 5′-CCTGTCTCACCCCCAGCATA-3′) were designed using Primer Express® (Applied Biosystems) and purchased from Invitrogen. Each target gene was quantified in duplicates and 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 normalized to the housekeeping control gene *36B4*.

2.5. Oxidative stress assessed by flow cytometry

The whole intracellular ROS production was analyzed using the 2′,7′-dichlorodihydrofluorescein diacetate probe (H_2DCFDA). This non-fluorescent compound passively diffuses into cells where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell and can be monitored by flow cytometry. Treated X-ALD fibroblasts or WT fibroblasts as reference (confluence 60% in 6-well