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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′-GTGGTATCATTGGTCG TAGCAG-3′, R, 5′-AGCCTTACTCGGAAGCACAG-3′; *hACOX1*: F, 5′-CCTG AGCCTCTGGATCTTCAC-3′, R, 5′-GGTGAGTTCCATGACCCATCTCTG-3′; *hCTNNB1*: F, 5′-ATGTCGAGCGTTTGGCTGAA-3′, R, 5′-TGGTCCTCGTCA TTTAGCAGTT-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′-CGTCCAGCAA TGCGTACTTCG-3′; *rAbcd3*: F, 5′-GGCTGGGCGTGAAATGACTA-3′, R, 5′-GCCATTTGGACCACAAATGA-3′; *r36B4*: F, 5′-CACCTTCCCACTGGCTGA AA-3′, R, 5′-CGCAGCCGCAAATGC-3′; *rCtnnb1*: F, 5′-CTGATAAAGGCAAC TGTTGGA-3′, R, 5′-CCCTGTTCCCGCAAAGG-3′; *rAbcA1*: F, 5′-GTGTTTCT CAGAGCAGTTCTGA-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

plate) were incubated for 30 min at 37 °C with 10 μM H_2DCFDA, trypsinized with 0.25% trypsin/EDTA (Sigma), harvested in 1 mL PBS before flow cytometric analysis. A ROS production positive control was performed by a pre-incubation for 1 h at 37 °C with 50 μM antimycin A. For each sample, 10,000 cells were acquired, and the data were analyzed with FlowJo (Tree Star Inc.) software.

2.6. Fatty acid analyses

Fatty acid contents of X-ALD fibroblasts (ALD-3 or Coriell Institute GM17819 cell lines) under LXR-antagonists treatments were analyzed as pentafluorobenzyl esters by gas chromatography/mass spectrometry in negative chemical ionization mode as previously described [35].

2.7. Human multiple tissue array analyses

A multiple tissue expression (MTE) array containing poly(A)-selected RNA from 61 different adult human tissues was obtained from Clontech. For detection of human *ABCD2* mRNA, a cDNA fragment corresponding to bp 172–1124 of the human *ABCD2* cDNA sequence (accession no. AJ000327) was amplified by PCR reaction from plasmid p115 containing the full-length human *ABCD2* using the following primers: F, 5′-AAATGG ACCAGATCGAGTGCTGC-3′ and R, 5′-CAAAGCTTTAGCAGATCAGAT-3′. For detection of human *NR1H3* (LXRα) mRNA, an 1.4 kb cDNA probe was prepared by digesting plasmid p301 containing the full-length human *NR1H3* cDNA, which was kindly provided by Dr. David Mangelsdorf (UT Southwestern, Texas). For control, a 528-bp glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*) probe was prepared by PCR using 5′-ACCACCATGGAGAAGGCTGG-3′ and 5′-CTCAGTGTAGCCCAGG ATGC-3′ as forward and reverse primers, respectively. The probes were radioactively labeled by random priming using [α-^{32}P] dCTP and hybridized using ExpressHyb solution (BD Bioscience) before autoradiographic exposure to a phosphoimager system (Bio-Rad). Signal intensity of individual dots was quantified using ImageJ software (available from: http://rsbweb.nih.gov/ij/download.html), and the *ABCD2* or *NR1H3* expression relative to that of *GAPDH* was calculated.

2.8. Statistical analysis

Student's *t*-test was used for statistical analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of LXR antagonists on gene expression in HepG2 cells

Since HepG2 hepatoma cells have been used in numerous studies to investigate the effects of LXR activation, this prompted us to analyze the effects of LXR antagonists on gene expression by RT-qPCR in this human cell line. No sign of toxicity was observed at 10 μM for 22S-HC and 22R-HC and at 100 nM for GSK(17) (data not shown). Quantitative real time PCR analysis was performed after 3 days of treatment. Both GSK(17) and 22S-HC treatments significantly up-regulated the *ABCD2* expression level by 1.47- and 1.97-fold, respectively (Fig. 1). As expected, *ABCD2* expression was down-regulated by 22R-HC (0.57-fold). *ABCD3* expression was found significantly increased by 22S-HC treatment but not significantly modified by GSK(17) and 22R-HC (Fig. 1). While *ACOX1* expression was not significantly affected by the treatments, *CTNNB1* (β-catenin) expression followed the same pattern than *ABCD2* (Fig. 1).

3.2. Effects of LXR antagonists on gene expression in X-ALD fibroblasts

In order to evaluate the effects of LXR ligands in the context of X-ALD, the same experiments were carried out in human skin fibroblasts derived from an X-ALD patient (ALD-3 cell line [9]). As shown in Fig. 2, similar results to those obtained in HepG2 cells were observed. *ABCD2* expression was significantly increased by 1.57-fold with GSK(17) and by 1.68-fold with 22S-HC while it was reduced by 0.60-fold with 22R-HC. *ABCD3* expression was only moderately induced by 22S-HC treatment while *ACOX1* expression was not modified (Fig. 2). The expression level of *CTNNB1* was found weakly induced upon 22S-HC and GSK(17) treatment and remained unchanged with 22R-HC (Fig. 2). These results are in accordance with those obtained with HepG2 cells revealing the specific activation (derepression) of *ABCD2* and *ABCD3* gene expression by LXR antagonists.

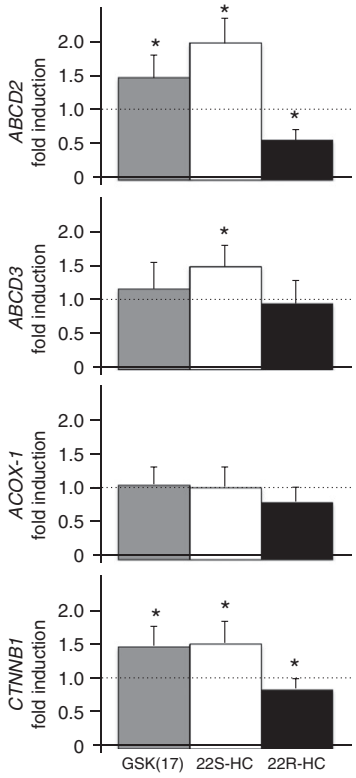

Fig. 1. LXR antagonists (GSK(17) or 22S-HC) induce *ABCD2*, *ABCD3* and *CTNNB1* (β-catenin) gene expression in human HepG2 hepatoma cells treated during 3 days. The mRNA levels of *ABCD2*, *ABCD3*, *ACOX1* and *CTNNB1* were measured using real time RT-qPCR and normalized to 36B4. Data represent the mean ± SD of 4 to 6 experiments and are expressed as fold induction in comparison with the gene expression level in untreated cells taken arbitrary equal to 1. Statistically significant differences from control by student *t* test are indicated by asterisks (*$p < 0.01$).

3.3. Effects of LXR antagonists on oxidative stress and VLCFA content in X-ALD fibroblasts

Oxidative stress has been found to be increased in X-ALD fibroblasts as compared with control fibroblasts [36]. This increase associated with *ABCD1* deficiency is predicted to be caused and amplified by VLCFA accumulation [37]. The observed induction of *ABCD2* and, to a lesser extent, of *ABCD3*, upon treatment with LXR antagonists may therefore compensate for ABCD1 deficiency and improve both the oxidative status and the VLCFA levels. On the contrary, the 22R-HC treatment may amplify oxidative stress and VLCFA accumulation. To test these hypotheses, we treated the ALD-3 and the Coriell X-ALD human skin fibroblasts during 72 h with 10 μM of 22R-HC or 22S-HC or with 100 nM of GSK(17). The fatty acid content was quantified by GC/MS in treated cells and reported to the fatty acid content observed in untreated cells. As shown in Table 1, we did not notice significant effects for the saturated VLCFA. Although results did not reach statistical significance threshold, it is noteworthy that monounsaturated fatty acids (MUFA, C20:1, C22:1 and C24:1) tended to decrease under treatment with

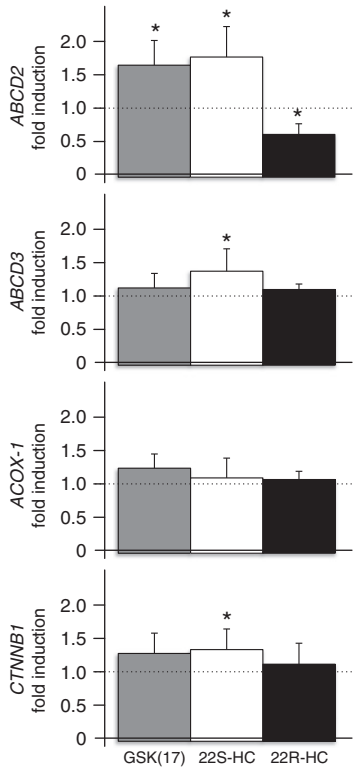

Fig. 2. LXR antagonists (GSK(17) or 22S-HC) induce *ABCD2*, *ABCD3* and *CTNNB1* (β-catenin) gene expression in human X-ALD fibroblasts treated during 3 days. The mRNA levels of *ABCD2*, *ABCD3*, *ACOX1* and *CTNBB1* were measured using real time RT-qPCR and normalized to 36B4. Data represent the mean ± SD of 4 to 6 experiments and are expressed as fold induction in comparison with the gene expression level in untreated cells taken arbitrary equal to 1. Statistically significant differences from control by student *t* test are indicated by asterisks (*$p < 0.01$).

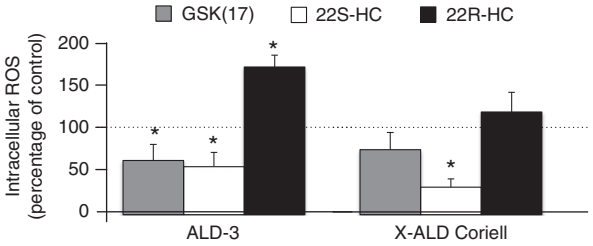

Fig. 3. LXR antagonists (GSK(17) or 22S-HC) decrease oxidative stress in human X-ALD fibroblasts treated during 3 days. The intracellular ROS production was analyzed using the H_2DCFDA probe as described in Materials and methods. Data represent the mean ± SD of 3–5 experiments and are expressed as percentage of control in comparison with the ROS production level in untreated cells taken arbitrary equal to 100. Statistically significant differences from control by student *t* test are indicated by asterisks (*$p < 0.01$).

LXR antagonists while their levels increased under treatment with 22R-HC (Table 1). The level of C24:6 n − 3, the immediate precursor of DHA (C22:6 n − 3), was shown to be reduced in cells treated by LXR antagonists (the decrease was only significant in Coriell cells treated with 22S-HC). The oxidative status analyzed by flow cytometry assay using the H_2DCFDA probe displayed significant modifications. In ALD-3 cells, both LXR antagonists triggered a significant decrease of ROS as compared with control cells, while 22R-HC treatment yield a 1.68-fold increase (Fig. 3). Similar results were obtained with the Coriell X-ALD cell line even if significant results were observed only for the 22S-HC treatment (Fig. 3). Thus, the use of LXR antagonists may participate to lower ROS production and oxidative stress.

3.4. Effects of dietary 22S-HC on hepatic expression

The expression levels of carnitine palmitoyl transferase 2 and uncoupling protein 3 have been shown to be induced in liver of rats fed a normal or high fat diet enriched in 22S-HC (30 mg/kg/day for 3 weeks) [34]. In order to study whether 22S-HC could induce hepatic expression of *Abcd2*, *Abcd3* or *Ctnnb1 in vivo*, cDNA samples from these rats were analyzed by RT-qPCR. In concordance with the results from the *in vitro* cell assays (in X-ALD fibroblasts and HepG2 cells), we observed a moderate but significant increase in expression of *Abcd3* (1.51-fold) and *Ctnnb1* (1.39-fold) after exposure to 22S-HC (Fig. 4). Although at the limit of significance ($p = 0.07$), *Abcd2* hepatic expression was found considerably increased (9.66-fold). The expression levels of *Abca1* and *Srebp1*, two known LXR target genes, were also analyzed and found unchanged by the treatment (data not shown).

3.5. Compared tissue expression of NR1H3 (LXRα) and ABCD2 in human

We have shown that treatments with LXR antagonists trigger induction of *Abcd2* and *Abcd3* genes *in vitro* in human cells and *in vivo* in rat liver. Based on these findings, we investigated and compared the expression level of *ABCD2* and *NR1H3* (LXRα) genes in a large variety of human tissues using the multiple tissue expression (MTE) array containing poly(A)-selected RNA from 61 different tissues of human origin (Clontech). Dot blot quantification in tissues with the *NR1H3* highest

Table 1
Relative effect of GSK(17), 22S-HC or 22R-HC on the fatty acid content in X-ALD fibroblasts (Coriell Institute GM17819 or ALD-3 cell lines). The fatty acid content was quantified by GC/MS in treated cells and reported to the fatty acid content observed in untreated cells. The means of the effects and standard deviations of 3 independent experiments are reported in the table. Statistically significant differences from control by student *t* test are indicated by asterisks (*$p < 0.05$).

ALD Coriell (GM17819)							ALD-3					
	GSK(17)		22S-HC		22R-HC		GSK(17)		22S-HC		22R-HC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C20:0	1.10	0.25	1.02	0.37	1.08	0.32	1.07	0.14	1.09	0.15	1.27	0.25
C22:0	0.79	0.23	0.87	0.34	1.26	0.32	1.08	0.18	1.10	0.51	0.86	0.21
C24:0	0.94	0.26	1.09	0.39	1.10	0.22	1.04	0.19	0.97	0.19	0.75	0.20
C26:0	1.19	0.40	1.04	0.08	0.98	0.15	1.13	0.19	1.36	0.17	1.05	0.30
C20:1 n − 9	1.04	0.02	0.63	0.09	1.58	0.44	0.87	0.10	0.81	0.23	1.30	0.23
C22:1 n − 9	1.12	0.49	0.46	0.001	1.72	0.96	0.79	0.12	0.60	0.21	1.32	0.23
C24:1 n − 9	0.92	0.07	0.63	0.16	1.38	0.15	0.85	0.11	0.76	0.26	1.06	0.20
C26:1 n − 9	1.15	0.35	1.11	0.12	1.56	0.64	0.92	0.09	1.03	0.31	1.36	0.30
C20:5 n − 3	1.04	0.11	0.93	0.10	1.66	0.34	0.93	0.10	1.41	0.42	1.74	0.43
C22:6 n − 3	1.16	0.26	0.78	0.05	1.38	0.41	1.00	0.12	0.95	0.14	1.18	0.19
C24:6 n − 3	0.76	0.43	0.53*	0.10	1.21	0.39	0.93	0.10	0.54	0.09	0.90	0.17
C20:4 n − 6	1.05	0.27	0.89	0.03	1.44	0.70	0.91	0.14	1.21	0.44	1.23	0.26
C22:5 n − 6	1.39	0.60	0.76	0.02	1.37	0.61	0.99	0.13	0.91	0.21	1.02	0.23

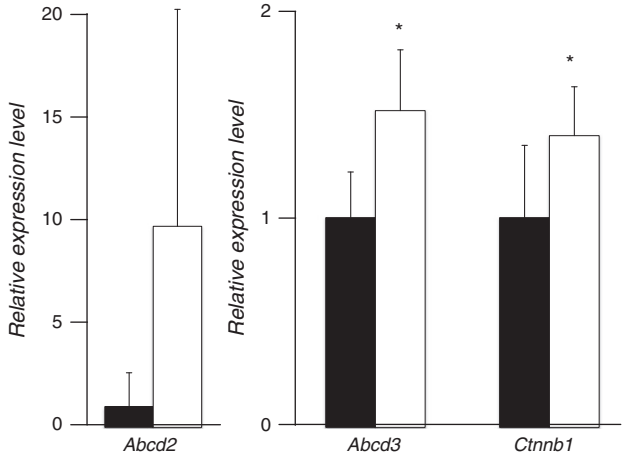

Fig. 4. 22S-HC induces *Abcd2*, *Abcd3* and *Ctnbb1* (β-catenin) gene expression in rat liver. Rats were randomly divided into 2 groups with 6 animals each, receiving high-fat diet (HFD) ± 22S-HC for 21 days. Tissue samples were prepared as described in Methods. Total RNA was extracted, reversely transcribed and analyzed by RT-qPCR. Results are normalized to levels of *36B4* and presented as mean ± SD (n = 6) of two PCR analyses. Expression level in untreated rats (black) is arbitrary taken equal to 1.*$p \leq 0.05$ *vs* control.

expression level (liver, placenta, fetal spleen and placenta) showed that *ABCD2* expression is very low (Fig. 5A). In whole brain and in substructures of central nervous system, expression level of *NR1H3* is relatively low and inversely correlates with the highest expression level of *ABCD2* (Fig. 5B). The whole results showing the compared expression level of *ABCD2* and *NR1H3* in tissues belonging to cardiovascular system, immune system, digestive system, fetal and other adult tissues show a quite equivalent inverse correlation with some exceptions for instance in heart and lymph nodes (Fig. S1).

4. Discussion

LXR activation has been shown to down-regulate the *ABCD2* expression *in vitro* [28]. The availability of GSK(17), a very efficient synthetic LXR antagonist [32], prompted us to study the effect of this molecule in different cell lines. Treatments with the antagonist 22S-HC and the endogenous agonist 22R-HC were carried out in parallel. Our results clearly show that treatment with LXRα antagonists induces the expression of *ABCD2* in HepG2 hepatoma cells and X-ALD skin fibroblasts and confirm that 22R-HC treatment results in the opposite effect. However, in HepG2 cells, the observed variations remain quite low (1.5–2-fold increase) as compared with the levels of up-regulation measured after a treatment with thyroid hormone and thyromimetics [20]. Interestingly, both *ABCD3* and *CTNNB1* genes were found to be induced by LXR antagonists whereas expression of *ACOX1* remained unchanged whatever the treatment and the cell type. This latter result was unexpected since the well-characterized LXR synthetic ligand, T0901317, was demonstrated to induce a small increase of hepatic *Acox1* expression in mice [38]. Ligand differences might explain this discrepancy and it is also possible that the concentrations used in our assays were not sufficient to observe any changes.

To see whether the use of LXR antagonists could improve the redox status and reduce the VLCFA accumulation, we treated X-ALD fibroblasts with the LXR antagonists GSK(17) and 22S-HC. Although VLCFA content was not significantly modified, MUFA (C20:1, C22:1 and C24:1) as well as C24:6 n − 3 levels appeared to be reduced by the treatments with LXR antagonists, mainly 22S-HC. In mirror, MUFA levels were found to be increased by the 22R-HC treatment. With the exception of saturated fatty acids, these observations, which have to be statistically consolidated, are in quite good agreement with the suspected role of ABCD2 towards these fatty acids [13–15]. However, these results may not be strictly dependent on ABCD2 expression since the level of *ABCD2* expression in human primary fibroblasts is very low [4] and that the induction level due to LXR antagonists is not elevated. Besides, the stearoyl-CoA desaturase-1 (SCD1) which converts C18:0 to C18:1 has been shown to be induced by LXR agonists [39]. It is therefore consistent to find an increase level of MUFA in 22R-HC treated cells and the opposite in cells treated with LXR antagonists. Concerning ROS production, a decrease was observed in X-ALD fibroblasts treated with the LXR antagonists while 22R-HC treatment resulted in increased ROS production. Oxidative stress changes may result from the moderated alterations observed in MUFA and be a consequence of ABCD2 induction or repression. However, it is also tempting to speculate that an effect of LXR antagonists on other targets mediated the ROS related effects. For instance, the Wnt/β-catenin pathway should be explored in detail since an important role of β-catenin signaling for liver protection against oxidative stress has previously been demonstrated [40].

Reinvestigation of the samples collected from the study of the effect of 22S-HC in rats by Kase et al. [34], showed increased expression levels of *Abcd2*, *Abcd3*, and *Ctnnb1* genes upon 22S-HC treatment. Further studies would be necessary to see whether the GSK(17) compound is also efficient *in vivo* and to see whether induction of the target genes can be found in other tissues related to X-ALD such as brain, spinal

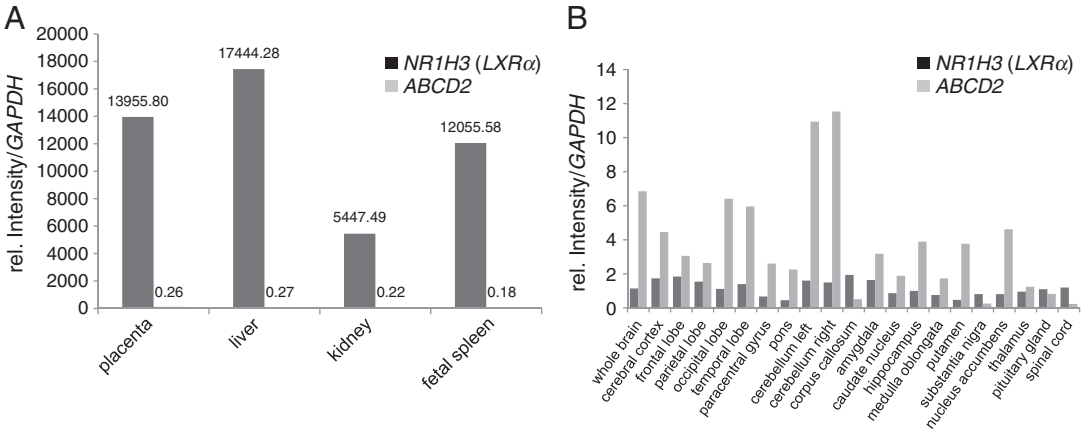

Fig. 5. Expression profile of *ABCD2* and *NR1H3* (LXRα) in human tissues. A human multiple tissue expression (MTE™) array RNA dot blot was hybridized with ^{32}P-labeled cDNA probes for *ABCD2*, *NR1H3* and *GAPDH* as described in Methods. (A) In placenta, liver, kidney and fetal spleen, *NR1H3* is highly expressed whereas only minor amounts of *ABCD2* mRNA are present. (B) In the central nervous system, *ABCD2* is relatively highly expressed and correlates with low expression of *NR1H3*.

cord, testis and adrenals. Meanwhile, these results together with the results obtained *in vitro* show for the first time that LXR antagonists can induce *ABCD2* and other genes of interest both *in vitro* and *in vivo*.

In order to gain additional knowledge on the expression level of *ABCD2* and *NR1H3* (LXRα) genes in human tissues, we performed multiple tissue expression arrays. The general conclusion of this exploration underlines a possible inverse correlation between the expression level of these two genes since *ABCD2* appears very weakly expressed in the tissues containing the highest level of *NR1H3* and *vice versa*. Obviously, it is not a strict correlation and the expression level of *ABCD2* cannot be restricted to a simple and unique dependency to LXRα. LXRβ is probably another actor involved in the regulation of *ABCD2* as well as many other nuclear receptors and transcription factors. Interestingly, *ABCD2* was recently demonstrated to be regulated by β-catenin [29] and we show in this study that *CTNBB1* expression increases through LXR antagonist treatments. These results suggest the presence of a possible loop of amplification involving both the metabolic adaptation due to LXRs and oxysterol levels and the cell signaling pathways in relation with β-catenin. Besides, as discussed in the introduction, *ABCD2* is a target gene for various pathways in relation with LXR activation (PPARα, TRα/β, SREBPs …). Many cross-talks exist between these molecular pathways. For instance, deiodinase 2, which converts T4 to T3, is repressed by 22R-HC in HepG2 cells [41] and unliganded TRβ represses LXRα transactivation [42,43]. Indirect regulation of PPARα ligands such as fibrates was supposed to involve LXRα [18]. The effects of PUFAs on *ABCD2* expression probably involve several mechanisms, one of them being possibly LXR antagonization [23]. The regulatory pathways and cross-talks targeting *ABCD2* promoter are summarized in Fig. 6 and confirm that *ABCD2* is a main target of several converging regulatory pathways in relation with each other. Such a complex regulation is likely to be linked to a crucial functional role of the ABCD2 protein into the peroxisomal membrane for the peroxisomal metabolism and important physiological functions that remains to be discovered. This is coherent with the role attributed to ABCD2 in the fatty acid homeostasis [14]. In the context of X-ALD, the crosstalk between LXRα and the Wnt/β-catenin pathway might be particularly interesting since positive consequences might be expected towards inflammation control [44] and myelination process [31,45]. The roles of LXR receptors (LXRα and LXRβ) in lipid homeostasis [46], inflammation [47], and steroid synthesis [48] are very well documented in peripheral tissues. In the nervous system, both LXR isoforms are expressed and are thought to control cholesterol homeostasis [49]. The observed disturbances in LXR double-knockout mice demonstrate the importance of LXR in brain [50]. LXR activation in brain is apparently associated with deleterious effects. The levels of hydroxycholesterol species (22R-HC, 24S-HC, and 25-HC which are ligands of both LXR isoforms) are usually altered in neurodegenerative diseases. 24S-HC, also known as cerebrosterol, seems to be a key element of inflammation and demyelination [51]. In neural cells, 24S-HC induces inflammatory genes such as cyclooxygenase-2 and phospholipase A2. It is therefore tempting to speculate that the use of LXR antagonists, if they can cross the blood–brain barrier and remain sufficiently bioavailable, may be interesting in the context of neurodegenerative diseases.

Altogether, this study confirms that agonists of LXR repress *ABCD2* expression and demonstrates for the first time that LXR antagonists induce *ABCD2* expression *in vitro* in X-ALD fibroblasts and HepG2 cells as well as *in vivo* in rat liver. It makes the proof of concept that synthetic LXR antagonists, which are in development to treat dyslipidemia

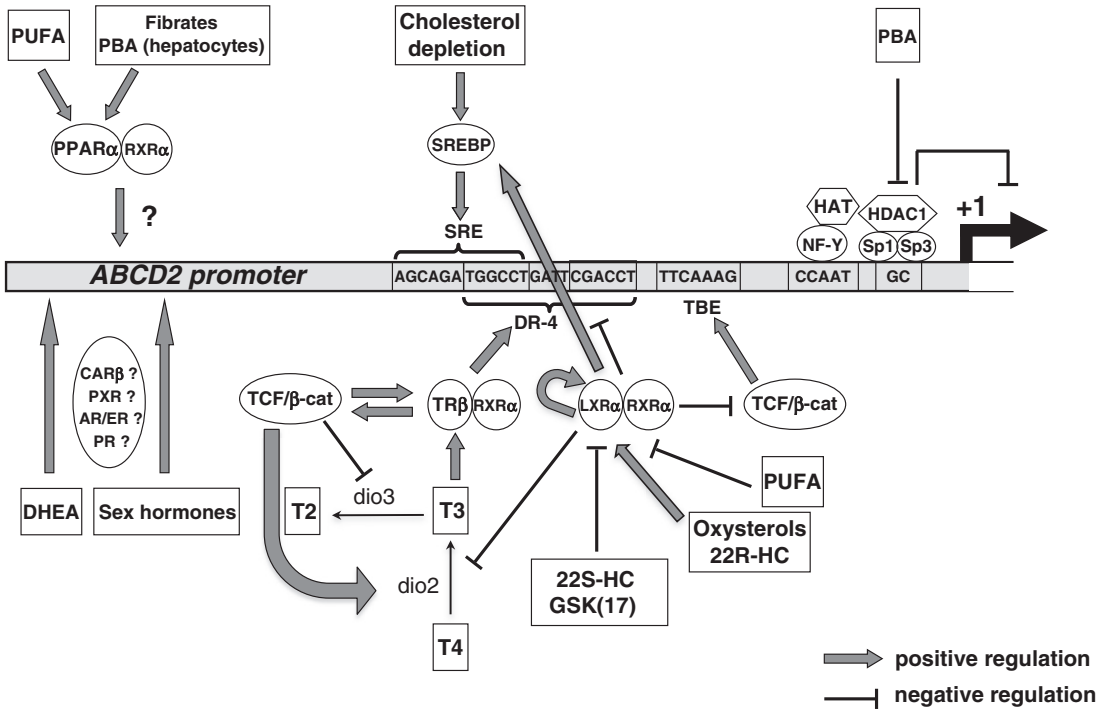

Fig. 6. Schematic and simplified representation of the transcriptional regulation pathways occurring on the *ABCD2* promoter and their crosstalks. Hormones (Dehydroepiandrosterone, sex hormones, thyroid hormone (T3)), pharmacological and synthetic regulators (22(S)-hydroxycholesterol, GSK(17), Fibrates, 4-PhenylButyrate) or endogenous regulators (22(R)-hydroxycholesterol, polyunsaturated fatty acids, cholesterol level) known to regulate *ABCD2* expression are in a square box while nuclear receptors and transcription factors are in round box. LXR agonists such as 22R-HC repress the *ABCD2* promoter through the DR4 motif while LXR antagonists (22S-HC and GSK(17)) activate the *ABCD2* promoter. LXR activation is known to induce SREBP expression. Depletion in cholesterol also results in SREBP activation, which binds to the SRE (motif overlapping the DR-4 motif) and induces *ABCD2* expression. Deiodinase 2 (dio2) converts thyroxine (T4) to the active form of thyroid hormone (T3), which is inactivated to T2 by dio3. In the absence of T3, thyroid hormone receptors repress the *ABCD2* promoter. In the presence of T3, *ABCD2* expression is induced through binding of TRs on the DR4 motif. Noteworthy, TR activation induces LXR expression. The TCF/β-catenin pathway has been shown to induce *ABCD2* expression through a TCF binding element (TBE) downstream of the DR4 motif. LXR activation suppresses the transactivation activity of β-catenin through direct interaction and transcriptional repression. TCF/β-catenin is known to potentialize the T3 formation and therefore T3-dependent transcription. β-catenin expression is induced by T3. Moreover, physical interactions between β-catenin and either TRs or LXRs have been also described improving their effects.

[32,52–54] can be used as pharmacological inducers of *ABCD2*. Further studies are needed to confirm whether such molecules, by their ability to induce *ABCD2* and to reduce oxidative stress, may have a therapeutic value in the context of X-ALD.

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