Liver X Receptor α Interferes with SREBP1c-mediated Abcd2 Expression

NOVEL CROSS-TALK IN GENE REGULATION

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The peroxisomal ATP binding cassette (ABC) transporter adrenoleukodystrophy-related protein, encoded by ABCD2, displays functional redundancy with the X-linked adrenoleukodystrophy-associated protein, making ABCD2 up-regulation of therapeutic value. Cholesterol lowering activates human ABCD2 in cultured cells. To investigate in vivo regulation by sterols, we first characterized a sterol regulatory element (SRE) in the murine Abcd2 promoter that is directly bound by SRE-binding proteins (SREBPs). Intriguingly, this element overlaps with a direct repeat 4, which serves as binding site for liver X receptor (LXR)/retinoid X receptor heterodimers, suggesting novel cross-talk between SREBP and LXR/retinoid X receptor in gene regulation. Using fasting-refeeding and cholesterol loading, SREBP accessibility to the SRE/direct repeat 4 was tested. Results suggest that adipose Abcd2 is induced by SREBP1c, whereas hepatic Abcd2 expression is down-regulated by concurrent activation of LXRα and SREBP1c. In cell culture, SREBP1c-mediated Abcd2 induction is counteracted by ligand-activated LXRα. Finally, hepatic Abcd2 expression in LXRα,β-deficient mice is inducible to levels vastly exceeding wild type. Together, we identify LXRα as negative modulator of Abcd2, acting through a novel regulatory mechanism involving overlapping SREBP and LXRα binding sites.

X-linked adrenoleukodystrophy (X-ALD; OMIM 300100) is a neurodegenerative disorder caused by mutations in the ABCD1 (ALD) gene, encoding the peroxisomal ATP binding cassette (ABC) half-transporter adrenoleukodystrophy protein (ALDP) (1). Currently, no satisfying therapy is available for X-ALD patients.

Next to ALDP, three additional mammalian peroxisomal ABC half-transporters have been identified: adrenoleukodystrophy-related protein (ALDPRP), 70-kDa peroxisomal membrane protein (PMP70), and PMP70-related protein (P70R), sharing 66, 39, and 25% amino acid identity with ALDP, respectively. Thus, the ABCD2-encoded ALDPRP is the protein most closely related to ALDP and upon overexpression can functionally compensate for ALDP deficiency in X-ALD fibroblasts and Abcd1-deficient mice (2, 3). Therefore, pharmacological stimulation of ABCD2 expression has been targeted as an alternative therapeutic strategy for X-ALD (4), requiring detailed knowledge about how the ABCD2 gene is transcriptionally regulated.

We recently showed that human ABCD2 is induced upon cholesterol depletion in cultured cells via a mechanism requiring the activation and binding of sterol regulatory element (SRE)-binding proteins (SREBPs) (5). SREBPs, which are synthesized as membrane-bound precursors and cleaved to generate the active nuclear form, are a class of transcription factors known to play a major role in regulating genes involved in fatty acid and cholesterol synthesis (reviewed in Ref. 6). To date, three SREBPs have been identified: SREBP1a and SREBP1c, which are produced from a single gene and preferentially regulate fatty acid synthesis, and SREBP2, encoded by a separate gene and controlling expression of cholesterogenic genes (7). Actively growing cultured cells predominantly produce SREBP1a, whereas in vivo, SREBP1c is much more abundant (8).

Abcd1-deficient mice mimic the biochemical defect found in X-ALD patients, i.e. accumulation of very long-chain fatty acids in tissues (9–11). Thus, a model is available to evaluate the efficacy of cholesterol-lowering drugs on SREBP maturation and Abcd2 induction as a future therapeutic agent for X-ALD. This requires investigation of in vivo accessibility and inducibility of Abcd2 by SREBPs. Intriguingly, the Abcd2 SRE overlaps with a direct repeat separated by 4 nucleotides (DR-4) that fits the consensus sequence of the 3,5,3′-triiodothyronine thyroid hormone receptor (TR) or the liver X receptor (LXR) response element. Thus, in this particular case, TR or LXR could possibly interfere with binding and activation of the Abcd2 promoter by SREBP.

Recently, direct binding of TRβ homodimer or TRβ/RXRα heterodimer complexes to the rat Abcd2 SRE/DR-4 and inducibility by triiodothyronine have been demonstrated (12). In contrast, no study has addressed a possible role of LXRα in Abcd2 regulation. The LXR subfamily of nuclear receptors, consisting of LXRα and LXRβ, emerged as key mediator of cholesterol homeostasis (13). LXRs function as cholesterol sensors that respond to elevated sterol concentrations by initiating expression of genes involved in cholesterol efflux, bile acid production, and lipid transport (14). LXRα has a dual role in gene expression: the ligand-receptor complex up-regulates many target genes, whereas the unliganded receptor suppresses gene expression by recruiting corepressor proteins (15). In addition, activated LXRα down-regulates transcription of a subset of genes, as in the case of matrix metalloproteinase-9 or 11β-hydroxysteroid dehydrogenase type 1 (16, 17). However, so far no investigation has demonstrated that the underlying mechanism

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1 The abbreviations used are: X-ALD, X-linked adrenoleukodystrophy; ABCD2/Abcd2, ATP binding cassette transporter subfamily D member 2 gene; ALDPRP, adrenoleukodystrophy-related protein; Abcd2, ATP binding cassette transporter subfamily D member 2 gene; ALDRP, adrenoleukodystrophy-associated protein; DR-4, direct repeat spaced by 4 nucleotides; LXRα, liver X receptor α; LXR, LXR response element; RXRα, retinoid X receptor α; SRE, sterol regulatory element; SREBP, SRE-binding protein; TRβ, thyroid hormone receptor β; 22-β-hydroxycholesterol; 22-β-hydroxycholesterol; EMA, enzyme-linked immunosorbent assay; QRT, quantitative reverse transcriptase; PPAR, peroxisome proliferator-activated receptor; nt, nucleotide.

2 The abbreviations used are: X-ALD, X-linked adrenoleukodystrophy; ABCD2/Abcd2, ATP binding cassette transporter subfamily D member 2 gene; ALDRP, adrenoleukodystrophy-associated protein; Abcd2, ATP binding cassette transporter subfamily D member 2 gene; ALDP, adrenoleukodystrophy-related protein; DR-4, direct repeat spaced by 4 nucleotides; LXRα, liver X receptor α; LXR, LXR response element; RXRα, retinoid X receptor α; SRE, sterol regulatory element; SREBP, SRE-binding protein; TRβ, thyroid hormone receptor β; 22-β-hydroxycholesterol; 22-β-hydroxycholesterol; EMA, enzyme-linked immunosorbent assay; QRT, quantitative reverse transcriptase; PPAR, peroxisome proliferator-activated receptor; nt, nucleotide.

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SREBP and LXRα Cross-talk in Abcd2 Regulation

involves a direct interaction of LXR with LXREs in the repressed target genes.

The aim of the present study was to (a) evaluate whether murine Abcd2 is inducible by SREBP, (b) investigate the relevance of the SRE motif for in vivo transcriptional regulation of Abcd2 by either fasting-refeeding or dietary “cholesterol loading” or “cholesterol depletion” of mice, (c) characterize the physiological relevance of the overlapping SRE and DR-4 motifs for Abcd2 expression, and (d) evaluate a putative modulatory effect of LXR on Abcd2 mRNA levels in cell culture and in mice.

EXPERIMENTAL PROCEDURES

Animals and Treatment—Male 4–6-month-old wild type C57BL/6 mice were kept at a temperature of 22 °C with equal periods of darkness and light. For high cholesterol or lovastatin/colestipol treatment, mice were fed ad libitum either a standard diet, a standard diet enriched with 2% cholesterol, or a standard diet supplemented with 0.1% lovastatin and 2% colestipol (sniff). Groups consisting of three animals were treated for either 1 or 6 weeks and sacrificed in the middle of the light phase.

For fasting-refeeding, 3-month-old wild type mice were divided into three groups, each consisting of three mice: nonfasted, fasted, and fasted/refed. The nonfasted group was fed ad libitum a standard chow (sniff). The fasted group was starved for 24 h (sacrificed at the end of the light phase), and the fasted/refed group was fasted for 24 h and then refed a high carbohydrate/low fat diet (sniff) for 12 h prior to study (sacrificed at the beginning of the light phase).

For studies involving treatment of wild type and LXRαβ-deficient mice with the synthetic RXRα agonists T0901317 (50 mg/kg body weight) or LG268 (30 mg/kg body weight) or both. Control animals (number 7001; Teklad) supplemented with either T0901317 (50 mg/kg body weight) or LG268 (30 mg/kg body weight) or both. Control animals were dosed with vehicle. Animals were treated for 12 h and sacrificed at the beginning of the light phase.

Cell Culture—COS-7 and THP-1 cells (ATCC) were cultivated in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, and 100 μg/ml streptomycin (Bio-whittaker). For cholesterol depletion, standard fetal calf serum was substituted with 10% lipoprotein-deficient fetal calf serum (Sigma). 22-(R)-hydroxycholesterol (22-(R)-HC; Sigma) was used at 10 μM for 24 h.

Plasmid Construction and in Vitro Mutagenesis—Abcd2 promoter fragments were generated by PCR using the antisense primer containing a XhoI site (number 7001; Teklad) supplemented with either T0901317 (50 mg/kg body weight) or LG268 (30 mg/kg body weight) or both. Control animals were dosed with vehicle. Animals were treated for 12 h and sacrificed at the beginning of the light phase.

For studies involving treatment of wild type and LXRαβ-deficient mice with the synthetic RXRα agonists T0901317 (50 mg/kg body weight) or LG268 (30 mg/kg body weight) or both. Control animals were dosed with vehicle. Animals were treated for 12 h and sacrificed at the beginning of the light phase.

Transient Transfection Experiments—COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen) with 0.5 μg of pGLUC or pGL3basic construct, 0.1 μg of SREBP1α/1c/2-expression vector (kindly provided by Dr. Timothy Osborne, University of California, Irvine, CA), 0.1 μg of LXRE expression vector (19), and 0.05 μg of PCMV-β-Gal (Clontech). Transfected cells were incubated with or without 22-(R)-HC for 24 h and assayed for luciferase and β-galactosidase activity (5).

Electrophoretic Mobility Shift Assay (EMSA)—SREBP1α, LXRαβ, and RXRα were synthesized in vitro using the TNT T7 Quick for PCR in vitro transcription/translation system (Promega) and the primers SREBP1α 5'-GGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGCAGGCGGCTGAGG-3' (forward), 5'-T5ACTATGTCAGGGCTCAGGTGCTGAGG-3' (reverse); LXRα 5'-GGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGG-3' (forward), 5'-T5ACTATGTCAGGGCTCAGGTGCTGAGG-3' (reverse); RXRα 5'-GGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGG-3' (forward), 5'-T5ACTATGTCAGGGCTCAGGTGCTGAGG-3' (reverse) and the SREBP1α, LXRαβ, and RXRα expression plasmids as templates, respectively. Complementary oligonucleotides were 5'-GGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGG-3' (reverse) and the SREBP1α, LXRαβ, and RXRα expression plasmids as templates, respectively. Complementary oligonucleotides were 5'-GGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGG-3' (reverse) and the SREBP1α, LXRαβ, and RXRα expression plasmids as templates, respectively.

Oligonucleotides for competition experiments were murine growth hormone receptor 3 (Ghr3) 5'-CTGGTTAGTCTATTGCTTACACTACACCCCTTTGGGAAGGGAACGCAAGCGAGATTGGGTG-3' and 5'-TGGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGG-3' (reverse); LXRα 5'-GGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGG-3' (forward), 5'-T5ACTATGTCAGGGCTCAGGTGCTGAGG-3' (reverse); RXRα 5'-GGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGG-3' (forward), 5'-T5ACTATGTCAGGGCTCAGGTGCTGAGG-3' (reverse) and the SREBP1α, LXRαβ, and RXRα expression plasmids as templates, respectively. Complementary oligonucleotides were 5'-GGATCTTAACTAGCTACTATAGGAAAGCCTACCCTGAGCAGGCGGCTGAGG-3' (reverse) and the SREBP1α, LXRαβ, and RXRα expression plasmids as templates, respectively.

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SREBP and LXRα Cross-talk in Abcd2 Regulation

A Functional SRE Is Present in the Murine Abcd2 Promoter That Is Preferentially Activated by SREBP1—A computer-based search of 3.5 kb of murine Abcd2 promoter sequence (GenBank™ accession code AF302500) led to the identification of an SRE-like motif located at a similar position as in the human Abcd2 gene (5) and differing by only two bp (Fig. 1). To test the functionality of the putative SRE, various extents between 0.5 and 3.5 kb (see "Experimental Procedures") of the murine Abcd2 promoter were fused to a luciferase gene. These reporter constructs were used to transiently transfect COS-7 cells, together with a plasmid encoding either the mature forms of SREBP1a, SREBP1c, or SREBP2 or the empty vector pCMV5 (Fig. 2a). The luciferase activity increased in an SREBP-dependent manner with all three Abcd2 promoter constructs. The highest levels of induction were observed after cotransfection with cDNAs encoding the SREBP1 isoforms. In contrast, cotransfection with SREBP2 resulted only in a low and, with the exception of the intermediate promoter construct (Abcd2–1486luc), barely statistically significant increase in reporter activity (Fig. 2a).

The fact that the shortest Abcd2 promoter construct (Abcd2–499luc) was still activated by SREBP strongly suggested a proximal location of the SRE. To test whether the candidate SRE sequence (5′-AGCACGTGCCACC-3′, nucleotide −389 to −379) is sufficient to confer SREBP-dependent activation, it was cloned into a luciferase reporter construct upstream from a minimal β-globin promoter to generate pGL-mAbcd2-SREluc. Cotransfection with this construct and SREBP expression vectors clearly identified this element as a functional SRE (Fig. 2b). Again, induction was best conferred by SREBP1, reflecting the situation seen with the reporter constructs harboring the SRE in its natural context of the Abcd2 promoter (Fig. 2a).

To confirm the requirement of the identified regulatory element in SREBP-mediated induction of the Abcd2 promoter, we replaced the putative SRE in the minimal β-globin promoter context with two different mutated versions (pGL-mAbcd2-SRE-M1luc and pGL-mAbcd2-SRE-M2luc in Fig. 2c). For both constructions, the activation in response to SREBP1 cotransfection was significantly impaired, whereas only mutation M2 abrogated the induction by SREBP1a (Fig. 2c).

SREBP Directly Interacts with the SRE from the Abcd2 Gene—To establish whether the Abcd2-SRE can be directly bound by SREBP, EMSAs were carried out (Fig. 2d). The incubation of a 32P-labeled Abcd2 SRE oligonucleotide probe with in vitro produced mature SREBP1a resulted in the formation of a protein-DNA complex (Fig. 2d, lane 4). SREBP1c was not incorporated in this study because its DNA-binding domain is identical to that of SREBP1a. To assess the specificity of this protein-DNA interaction, cross-competition using a 50-fold molar excess of unlabeled oligonucleotides containing the SRE from the murine stearyl-CoA desaturase 1 (Scd1) promoter was performed, resulting in reduced amounts of complex formation (Fig. 2d, lane 5). In contrast, the unrelated competitor mG3 (murine growth hormone receptor 3) DNA was unable to inhibit SREBP1a binding (Fig. 2d, lane 6). As a positive control, complex formation between labeled oligonucleotide containing the Scd1 SRE and SREBP1a is shown (Fig. 2d, lane 3). Thus, the results indicate that a direct interaction is possible between the SRE from the Abcd2 gene and SREBP1.

In Fasted-Refed Mice, Abcd2 Expression Correlates with SREBP1c-activated Genes in Adipose and PPARα-regulated Genes in the Liver—To determine whether SREBP1c also regulates Abcd2 expression in vivo, a fasting-refeeding protocol (see "Experimental Procedures") was employed. In this paradigm, typical SREBP1c-regulated genes like fatty acid synthase (Fas) are induced after fasting when mice are re-fed a high carbohydrate/low fat diet (23). When mice were fasted, Abcd2 mRNA levels markedly declined in white adipose tissue and were re-induced when mice were re-fed a high carbohydrate/low fat diet (Fig. 3a). These results show that during a fasting-refeeding protocol, adipose Abcd2 expression correlates with the level of mature SREBP1c and thus with genes induced under conditions of fatty acid and triglyceride synthesis.

In the liver, fasting led to increased Abcd2 mRNA levels that returned to pre-fasted levels upon refeeding (Fig. 3, b and c). As expected, hepatic Fas expression was found to be strongly induced in the refed state.
whereas fasting induced acyl-CoA oxidase, encoding the key enzyme of the fatty acid degradation pathway, which is controlled by the nuclear peroxisome proliferator-activated receptor (PPAR) α (Fig. 3c). Thus, in accordance with our previous studies involving synthetic PPARα agonists (20, 24), hepatic Abcd2 regulation correlated with a PPARα and not with a SREBP1c target gene, although several investigations failed to...
localize a PPARα binding site in the human or rodent ABCD2 promoter (18, 24, 25).

Hepatic Abcd2 Expression Is Down-regulated by Dietary Cholesterol—To investigate whether SREBP1c plays a role in regulating hepatic Abcd2 expression under conditions when PPARα is not activated, we studied the effect of a dietary modulation of cholesterol levels in mice fed a diet containing 2% cholesterol (for comparison, the standard mouse chow contains only 0.01% cholesterol). In this feeding paradigm, excess cholesterol results in LXR activation, which induces SREBP1c transcription but also cholesterol storage and efflux through conversion to bile acids (26). Hepatic Abcd2 expression was evaluated after 1 and 6 weeks by QRT-PCR and Northern blot analysis (Fig. 4, a and b). Surprisingly, a high cholesterol diet led to significantly reduced Abcd2 mRNA levels in the liver after 1 week and a further down-regulation after 6 weeks of treatment (Fig. 4, a and b). In contrast, SREBP1c was consistently induced after 1 and 6 weeks of cholesterol feeding. In addition, cholesterol 7α-hydroxylase (Cyp7A1) mRNA, encoding the rate-limiting enzyme of bile acid synthesis, was up-regulated after 1 week (Fig. 4b). As both genes are directly controlled by LXRα, their induction serves as a positive control for the treatment paradigm, indicating that LXRα was activated by cholesterol feeding. At the protein level, high cholesterol feeding resulted in increased levels of cleaved SREBP1c, whereas SREBP2 was barely detectable by Western blot analysis before and after feeding a cholesterol-enriched diet (Fig. 4c), thus confirming previous studies. From these results, it appears that although a functional SRE is present in the Abcd2 promoter and SREBP1c is amply available, SREBP recruitment is not possible under conditions of concomitant LXα activation.

Abcd2 Expression Correlates Inversely with LXα-mediated SREBP1c Induction in the Liver of Cholesterol-depleted Mice—To clarify whether depletion of sterols also affects hepatic Abcd2 expression, mice were treated with a diet supplemented with 0.1% lovastatin and 2% colesterol for 1 and 6 weeks. This feeding paradigm is known to decrease the hepatic cholesterol content by (a) lovastatin inhibiting HMG-CoA reductase, the rate-controlling enzyme of cholesterol biosynthesis, and (b) colesterol binding bile acids in the intestine, thus decreasing their reabsorption and stimulating the liver to convert more cholesterol to bile acids. Abcd2 mRNA levels were quantified using QRT-PCR and Northern blot analysis (Fig. 4, a and d). One week after treatment, Abcd2 mRNA levels were reduced in the liver, comparable with the down-regulation seen after feeding a cholesterol-enriched diet. After 6 weeks of treatment, however, Abcd2 mRNA returned to pretreatment levels (Fig. 4, a and d). Northern blot analysis verified the results obtained by QRT-PCR and revealed that both SREBP1c and Cyp7A1 mRNA levels were moderately induced after 1 week of cholesterol depletion but returned to pretreatment or lower levels after 6 weeks (Fig. 4e). The moderate and transient increase in SREBP1c expression observed after 1 week was also reflected by an increase in mature SREBP1c (Fig. 4f). As expected, cleaved SREBP2 was barely detectable by Western blot analysis in mice fed normal chow but strongly increased when the diet was supplemented with lovastatin/colesterol for 1 week, thus reflecting a response aimed at inducing hepatic de novo cholesterol synthesis (Fig. 4f). After 6 weeks, the amounts of mature SREBP2 returned to pretreatment levels, indicating a transient effect of lovastatin/colesterol on hepatic cholesterol metabolism in mice (Fig. 4f). In summary, these results suggest that a combinatorial treatment of lovastatin/colesterol for 1 week stimulates cholesterol and bile acid synthesis through activation of SREBP2 and LXα, respectively, as shown by maturation of SREBP2 and induction of SREBP1c and Cyp7A1 expression, which correlates with a down-regulation of Abcd2 mRNA levels.

LXα Interferes with SREBP1c-mediated Stimulation of Abcd2 Promoter Reporter Constructs—Because Abcd2 expression inversely correlates with induction of SREBP1c in vivo, we considered LXα as a potential candidate that could interfere with the access of SREBP1c to the Abcd2 SRE by binding to the overlapping DR-4 element (Fig. 1). To test this hypothesis, we next examined whether LXα directly affects SREBP1c-dependent stimulation of the Abcd2 promoter. COS-7 cells were cotransfected with the reporter construct containing 3.5 kb of the

**FIGURE 4.** Hepatic Abcd2 expression in cholesterol- or lovastatin/colestipol-treated mice inversely correlates with LXα induction. Wild type C57BL/6 mice were fed a diet supplemented with either 2% cholesterol (a–c) or 0.1% lovastatin and 2% colesterol (d–f) for 1 or 6 weeks. Total RNA was isolated from the liver to evaluate Abcd2 mRNA levels using quantitative real-time RT-PCR (a, d) or Northern blot analysis (b, e), with detection of SREBP1c and Cyp7A1 mRNAs as controls for the feeding paradigm. 36B4 was used as a control for equal loading and transfer. c, Western blot analysis of equal amounts of homogenized liver samples (100,000 × g pellet) using antibodies recognizing mature SREBP1c (mSREBP) or mature SREBP2 (mSREBP2). As a loading control for the Western blot analysis, detection of β-actin is shown. a and d, results were normalized to the amount of hydroxy-phosphoribosyl transferase mRNA in each sample and are shown as mean ± S.E. with the number of analyzed mice in parentheses. Each sample was analyzed in triplicate.
SREBP and LXRα Cross-talk in Abcd2 Regulation

**FIGURE 5.** LXRα interferes with SREBP1c-mediated stimulation of Abcd2. COS-7 cells were transiently cotransfected with a luciferase reporter construct driven by 3.5 kb of the murine Abcd2 promoter (Abcd2–3515luc) (a) or a 3.5-kb Abcd2 promoter reporter construct with a mutated DR-4 motif and expression plasmids encoding human LXRα, the mature form of human SREBP1c, or the empty vector pCMV5 as a negative control (b), as indicated. To allow correction of transfection efficiency, pCMV-βgal was cotransfected in all experiments. The mutations that were introduced in the DR-4 of the Abcd2 gene are indicated in bold at the bottom of the reporter construct (LXRE-luc) and used as a positive control for LXRα stimulation. Data represent the mean ± S.E. of six samples. Statistically significant differences by Student’s t test are indicated by asterisks (p < 0.05).

Abcd2 promoter (Abcd2–3515luc; compare Fig. 2a) and expression plasmids encoding either LXRα or SREBP1c or both. Because COS-7 cells endogenously express RXXα , the heterodimer partner of LXRα, cotransfection with a RXRα expression plasmid was not necessary. Neither unactivated LXRα nor 22-(R)-HC-activated LXRα influenced expression from the Abcd2 promoter reporter construct (Fig. 5a). When the cells were cotransfected with LXRα and mature SREBP1c, the reporter gene was activated to a level comparable with the induction mediated by SREBP1c alone. However, cotransfection with SREBP1c and LXRα in the presence of the LXRα ligand 22-(R)-HC resulted in repression of SREBP1c-mediated stimulation of the Abcd2 promoter (Fig. 5a). To confirm that activated LXRα directly interferes with SREBP1c stimulation via the DR-4 element, two point mutations were introduced into the DR-4 half-site that does not overlap with the SRE of the Abcd2 promoter reporter construct. When this mutated construct, designated DR-4 mutated Abcd2–3515luc, was cotransfected with SREBP1c and LXRα expression plasmids, 22-(R)-HC-activated LXRα was no longer capable of repressing SREBP1c stimulation of the Abcd2 promoter (Fig. 5b). These results strongly suggest that ligand-activated LXRα has a negative effect on Abcd2 gene activation by SREBP and that the intact DR-4 element is necessary to mediate this repression.

**LXRα/RXRα Interacts Directly with the DR-4 from the Abcd2 Gene—**The ability of LXRα/RXRα to specifically bind to the SRE/DR-4 sequence from the Abcd2 gene was tested by EMSA (Fig. 6). The wild type oligonucleotides were radiolabeled with [α-32P]dCTP and incubated with in vitro synthesized LXRα and RXRα. In the presence of both receptors, the formation of a protein-DNA complex was observed (Fig. 6a, lane 3) that was not dependent, as assessed by the incorporation of the LXRα agonist 22-(R)-HC (Fig. 6a, lane 4). The specificity of binding was shown by the ability of a 50-fold molar excess of unlabeled RXRα oligonucleotides from the chicken acetyl-CoA carboxylase gene to directly interfere with binding of LXRα/RXRα heterodimer (Fig. 6a, lane 6), whereas the unrelated competitor DNA mGhr3 was unable to inhibit the formation of the complex (Fig. 6a, lane 5). As a positive control, complex formation between LXRα/RXRα and labeled oligonucleotides containing the acetyl-CoA carboxylase-LXRE is shown (Fig. 6a, lane 2). To test whether LXRα interferes with binding of SREBP1a to the SRE/DR-4 from the Abcd2 promoter, in vitro synthesized SREBP1a was incorporated in the assay system (Fig. 6b). As expected, incubation of the mAbcd2-SRE/DR-4-containing oligonucleotides with SREBP1a alone resulted in formation of a DNA-protein complex (Fig. 6b, lane 1). In the presence of both SREBP1a and LXRα/RXRα, two specific bands corresponding to SREBP1a and LXRα/RXRα protein-DNA complexes were formed (Fig. 6b, lane 2) that were not affected by the addition of 22-(R)-HC (Fig. 6b, lane 3). However, no supershifted bands appeared that would indicate a direct interaction between SREBP and LXRα. In summary, these results show that the LXRα/RXRα heterodimer directly binds the Abcd2 SRE/DR-4 element and furthermore suggest that a concurrent binding of SREBP1 and LXRα/RXRα to this sequence is not possible.

**In LXRα,β-deficient Mice Hepatic Abcd2 Expression Can Be Induced to Levels Vastly Exceeding Those in Wild Type—**The effect of ligand-activated LXRα on hepatic Abcd2 expression was further examined by comparing the response to LXR agonist treatment in wild type and LXRα,β-deficient mice (Fig. 7a). By QRT-PCR, basal Abcd2 mRNA levels were found to be slightly but not significantly elevated in the liver of LXRα,β-deficient mice compared with wild type controls. In addition, when animals were treated with the synthetic LXR ligand T0901317 for 12 h, Abcd2 mRNA levels did not significantly differ from untreated controls in both genotypes. In contrast, treatment with the synthetic RXRα ligand LG268 resulted in an almost 6-fold increase of hepatic Abcd2 expression in both wild type and LXRα,β-deficient mice. As this induction was also observed in LXRα,β-deficient mice, the underlying mechanism seems not to involve LXRα as heterodimeric partner for RXRα. Strong hepatic stimulation of the LXRα target gene Abca1 requires activation by both LXRα and RXRα ligands, and thus a combinatorial treatment with T0901317 and LG268 was performed. As expected, Abca1 mRNA levels were significantly induced in the liver of wild type but not in LXRα,β-deficient mice. Surprisingly, in wild type mice administration of both LXRα and RXRα ligands abolished the induction of Abcd2 observed with the RXRα agonist alone, indicating a negative effect of activated LXR on the stimulation of Abcd2 expression. In contrast, LXRα,β-deficient mice treated with both LXRα and RXRα
ligands demonstrated hepatic Abcd2 mRNA levels vastly exceeding those observed in LG268-treated animals, thus reflecting the loss of negative LXR action on Abcd2 expression.

**Activation of LXRβ under Conditions of Induced SREBP Maturation Results in Reduced ABCD2 mRNA Levels in Human Monocytic Cells**—We have previously shown that SREBP activation by cholesterol depletion in human and murine monocyte or microglial cell lines results in substantially increased ABCD2 mRNA levels (5). To test whether activation of LXRs under cholesterol depletion influences endogenous ABCD2 expression, human monocyte THP-1...
cells were incubated with 10 μM 22-(R)-HC in sterol-deficient medium for 24 h, and ABCD2 mRNA levels were quantified by QRT-PCR (Fig. 7b). Treatment with 22-(R)-HC under concurrent SREBP activation results in a considerable down-regulation of ABCD2 expression, indicating that also in cultured human cells, activated LXRx is a negative modulator of ABCD2.

**DISCUSSION**

Pharmacological stimulation of the ABCD2 gene has been suggested as an alternative therapeutic strategy for X-ALD (4), necessitating a careful characterization of endogenous ABCD2 expression and function of the encoded protein. Our recent work has uncovered a link between human ABCD2 expression and cholesterol metabolism in cultured cells (5). Thus, the goal of the present study was to investigate the in vivo effect of cholesterol and SREBP maturation on murine Abcd2 expression.

Our cell culture experiments show that murine Abcd2 expression is also regulated by SREBPs and revealed the presence of a functional SRE in the murine Abcd2 promoter, which was preferentially stimulated by SREBP1. Interestingly, no significant stimulation of reporter gene expression was observed with SREBP2, indicating that in cell culture this SREBP isoform stimulates the human, but not the murine, ABCD2 gene.

To investigate whether in vivo SREBP1 also regulates Abcd2 expression, different feeding paradigms were applied. In adipose, fasting, reduced, and refeeding a high carbohydrate/low fat diet induced Abcd2 mRNA levels. Together with the presence of a functional SRE in the Abcd2 promoter region, this suggests that Aldrp in white adipose tissue is connected to fatty acid and triglyceride synthesis and lipid storage. This proposition is further supported by the finding that the SRE motifs present in both the murine and the human ABCD2 expression and cholesterol metabolism in cultured cells (5). Thus, the goal of the present study was to investigate the in vivo effect of cholesterol and SREBP maturation on murine Abcd2 expression.

To determine whether Abcd2 is also in vivo a direct target of LXRx, studies involving the synthetic LXR and RXRx ligands T0901317 and LG268, respectively, were carried out. Treatment of either wild type or LXRx,β-deficient mice with T0901317 for 12 h did not change Abcd2 mRNA levels. A possible explanation could be that the strong synthetic LXR ligand T0901317 is expected to be more potent in inducing SREBP1c expression than dietary cholesterol treatment and thus might result in a titration of the positive and negative effect of SREBP1c and LXRx, respectively, on the Abcd2 promoter. When wild type mice and LXRx,β-deficient mice were exposed to the RXRx ligand LG268, a strong induction of hepatic Abcd2 expression was observed, thus confirming previous studies demonstrating the inducibility of ABCD2 by 9-cis retinoic acid-activated RXRx in cultured cells (25). As activation of RXRx also results in stimulation of Abcd2 expression in the liver of LXRx-deficient mice, a role for LXRx in this induction can be excluded. Possibly, the RXRx heterodimeric partner TRβ, which binds and activates the Abcd2 promoter (12), is involved in this induction. An intriguing observation of this feeding experiment is the finding that co-administration of both LXRx and RXRx ligands, T0901317 and LG268, abrogated the induction of Abcd2 mRNA levels seen after RXRx activation. This suggests that with activation of both LXRx and RXRx, the repressive effect of LXRx on the Abcd2 promoter seems to overwhelm the stimulation observed with the RXRx ligand alone, possibly by competing for binding to the Abcd2 DR-4 element. This hypothesis is further supported by the finding that in LXRx-deficient mice, co-administration of both T0901317 and LG268 does not influence the induction observed after treatment with LG268 alone. Indeed, a hepatic stimulation of Abcd2 expression to levels vastly exceeding those in wild type is observed. This might be explained by involvement of other DR-4 binding nuclear receptors cross-activated by T0901317, e.g. farnesoid X receptor and pregnane X receptor (32, 33), that could possibly bind the Abcd2 SRE/DR-4 in the absence of LXRx.

The aim of our work is to pharmacologically induce ABCD2 in X-ALD patients. Thus, we also investigated whether the observation that LXRx interferes with SREBP1c-mediated Abcd2 expression is conserved between man and mouse. When human monocytic cells were cultured under conditions of induced SREBP maturation, LXRx ligand binding. In an effort to explore whether the repression of SREBP1c-induced Abcd2 expression seen under conditions of concurrent SREBP1c and LXRx activation could be explained by competitive binding of LXRx/RXRx to the DR-4 sequence, thus obstructing the SRE, reporter gene studies, in vitro mutagenesis, and gel shift assays were carried out. The results obtained indeed show that LXRx is able to directly bind the DR-4 element and that LXRx, upon ligand-binding, interferes with SREBP1c-mediated activation of the Abcd2 promoter.

In addition to LXRx/RXRx heterodimers, previous studies demonstrated that TRβ homodimers or TRβ/RXRx heterodimers also can bind the Abcd2 SRE/DR-4 sequence (12). The ability of different nuclear steroid receptors to heterodimerize with RXRx and to bind the same DR-4 response element adds an additional level to regulation of gene expression (29). In whole body metabolism SREBPs, TRβ, and LXRx are directly functionally linked through a complex transcriptional regulatory network. When steroids are in excess, LXRx mediates transcriptional activation of SREBP1c, thus providing a mechanism for inducing fatty acid synthesis necessary for storage of cholesterol (30). For the SREBP2 gene, a direct regulation by TRβ binding to a TR-response element in the 5′-flanking region could be demonstrated (31). Thus, a clear link exists between TRβ, LXRx, and SREBP1c, strongly suggesting a physiological relevance of the overlapping SRE and DR-4 elements for ABCD2 expression.
treatment resulted in reduced Abcd2 mRNA levels, indicating that also in human cells, activated LXRα is a negative modulator of Abcd2 expression.

In conclusion, our results have exposed a novel mode of cross-talk between LXRα and SREBP1c in gene regulation. It is tempting to speculate that an overlap between SRE and DR-4 elements might provide a mechanism by which a subset of SREBP1c-inducible genes are down-regulated under conditions of sustained LXRα activation. In combination with cell type or tissue-specific variation in activation or availability of LXRα, SREBP1c could stimulate Abcd2 expression in some tissues, whereas LXRα-mediated repression is obtained in others. Moreover, transcriptional regulation by LXRα is dependent on different tissue-specific cofactors, further accounting for additional effects on target genes (34). To sum up, the observation that hepatic Abcd2 is actively down-regulated under conditions of LXRα activation opens up a novel therapeutic strategy for X-ALD, targeted at bypassing repressive effects on Abcd2 expression.

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