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Cholesterol regulates *ABCD2* expression: implications for the therapy of X-linked adrenoleukodystrophy

Isabelle Weinhofer, Sonja Forss-Petter, Mihaela Žigman and Johannes Berger*

Brain Research Institute, University of Vienna, Austria

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X-linked adrenoleukodystrophy (X-ALD) is a severe neurodegenerative disorder with impaired very long-chain fatty acid (VLCFA) metabolism. The disease-associated *ABCD1* (*ALD*) gene encodes a peroxisomal membrane protein, which belongs to the superfamily of ATP-binding cassette transporters. Several treatment regimes have been tried without satisfactory clinical benefit. Recently, the cholesterol-lowering drug lovastatin was reported to normalize VLCFA levels in two out of three clinical studies. This investigation aimed to disclose the molecular mechanism of successful reduction of VLCFA accumulation in order to fill in the gap in the understanding how dietary cholesterol lowering affects the levels of VLCFA in patients with X-ALD and to allow more efficacious treatment. Overexpression of *ABCD2* (ALDR), the closest relative of *ABCD1*, restores VLCFA accumulation in cultured *ABCD1*-deficient cells. Here we show by real-time PCR that the *ABCD2* gene is induced in cultured human fibroblasts and monocytes upon sterol depletion via a mechanism requiring the activation of sterol regulatory element-binding proteins (SREBPs), a family of transcription factors that control the metabolism of cholesterol and fatty acids. This is unexpected and the first report that extends the mechanism of transcriptional regulation by SREBPs to a peroxisomal protein, thus providing a closer link between peroxisomes, cholesterol and fatty acid biosynthesis. Using reporter gene studies, site-directed mutagenesis and gel shift assays, we identified a functional sterol regulatory element in the proximal promoter region of *ABCD2*. Finally, we demonstrated that *ABCD2* induction by sterol depletion significantly reduced the accumulation of VLCFA in X-ALD fibroblasts. Thus, lowering cholesterol leads to SREBP maturation, increased *ABCD2* expression and reduced VLCFA accumulation.

INTRODUCTION

Adrenoleukodystrophy-related protein [ALDRP; or ATP-binding cassette transporter subfamily D member 2 (ABCD2)] is a peroxisomal transmembrane protein with high homology to the disease-associated adrenoleukodystrophy protein (ALDP or ABCD1). Mutations in the *ABCD1* gene lead to the severe neurodegenerative disorder X-linked adrenoleukodystrophy (X-ALD; McKusick 300100), characterized biochemically by the accumulation of very long-chain fatty acids (VLCFA) (1). The most severe form of the disease, childhood cerebral ALD, with progressive inflammatory demyelination in the central nervous system, leads to death or a persistent vegetative state within a few years. Currently, no satisfactory therapy is available for X-ALD. The only treatment with some documented benefit for patients is bone marrow transplantation when performed at an early stage of the disease (2). Recently, the cholesterol-lowering drug lovastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase inhibitor, was proposed as a new therapeutic agent for X-ALD (3) and a cause-and-effect relationship between lovastatin therapy and plasma VLCFA levels was observed when plasma VLCFA and serum cholesterol levels were compared (4). However, statin treatment successfully normalized VLCFA levels in the plasma of X-ALD patients in only two out of three studies; the underlying mechanism was unclear (4–6).

Our previous work demonstrated that ABCD2 is able to functionally replace ABCD1 in cultured fibroblasts of X-ALD patients lacking ABCD1 protein (7). However, at the intrinsic level of expression, *ABCD2* does not compensate for *ABCD1* deficiency in X-ALD patients or in mice. The reason for this appears to be the complementary expression patterns of *ABCD1* and *ABCD2* (8), and a low level of *ABCD2* expression does not compensate for loss of ABCD1 protein in disease-relevant cell types. Thus, induction of *ABCD2* expression could represent a novel therapeutic strategy for X-ALD. Furthermore, *ABCD2* mRNA is downregulated when primary human monocyte-derived macrophages are loaded with acetylated low-density lipoprotein (9).

*To whom correspondence should be addressed at: Brain Research Institute, University of Vienna, Spitalgasse 4, A-1090 Vienna. Tel: +43 1427762812; Fax: +43 142779628; Email: johannes.berger@univie.ac.at

Cholesterol regulates transcription of several genes. Each of these sterol-sensitive genes contains at least one sterol regulatory element (SRE) or palindromic sequences called E boxes within their promoter regions, through which a class of transcription factors known as sterol regulatory element-binding proteins (SREBPs) activate transcription (10,11). SREBPs are transcription factors that belong to the basic helix–loop–helix leucine zipper family. In contrast to other members of this family, SREBPs are synthesized as precursor proteins that remain bound to the endoplasmatic reticulum and the nuclear envelope in the presence of sufficient sterol concentrations. Upon sterol deprivation, the SREBP precursor protein associated with the SREBP cleavage-activating protein (SCAP) moves from the endoplasmic reticulum to the Golgi apparatus, where the SREBP precursor undergoes a sequential two-step cleavage process to release the N-terminal portion (12). This mature SREBP then enters the nucleus and activates the transcription of genes involved in cholesterol and fatty acid synthesis (13,14). Currently, three forms of SREBP have been characterized: SREBP1a and SREBP1c are derived from a single gene through the use of alternate promoters, while SREBP2 is derived from a different gene. SREBP1a is the more common isoform, and is a stronger activator of transcription with a wider range of target genes than SREBP1c because of a longer transactivation domain (15). Transgenic mouse studies have shown that SREBP1c plays a more active role in regulating the transcription of genes involved in fatty acid synthesis than those involved in cholesterol synthesis, whereas SREBP1a activates both (15,16). SREBP2 is known to be actively involved in the transcription of cholesterogenic enzymes (17). For a review, see (18).

In this study, we demonstrate that SREBPs stimulate the human *ABCD2* promoter through their binding to an upstream promoter region. Furthermore, we show that the *ABCD2* gene is induced in cultured cells after sterol depletion and that this induction significantly reduced the accumulation of VLCFA in X-ALD fibroblasts. Thus, we identified *ABCD2* as a candidate for mediating the effect of the cholesterol-lowering drug lovastatin on VLCFA in X-ALD patients.

RESULTS

Cholesterol affects *ABCD2* expression in cell lines and primary fibroblasts

Lovastatin inhibits HMGCoA-reductase, the rate-limiting enzyme in cholesterol biosynthesis, thereby reducing cellular cholesterol. To determine how the cholesterol content affects *ABCD2* expression, we loaded and depleted human or mouse monocytes (THP-1 and WEHI-3), mouse microglial cells (BV-2) and human X-ALD (*ABCD1*-deficient, lack ALD protein as demonstrated by immunofluorescence; data not shown) or healthy control fibroblasts by incubation with sterols, lipid-depleted medium or medium containing standard fetal calf serum (FCS) for 2 days and quantified the *ABCD2* mRNA level by real-time RT–PCR (Fig. 1). Cholesterol loading resulted in decreased *ABCD2* mRNA levels in all cell types examined whereas lipid reduction increased the *ABCD2* mRNA, indicating that intracellular cholesterol regulates *ABCD2* expression. This regulation appears to be more prominent in the macrophage/monocyte lineage compared with the primary fibroblast lines used. The data obtained also show that *ABCD2* mRNA levels in the X-ALD fibroblast cell line are lower than in healthy control fibroblasts. However, this could possibly be due to variability among individuals. In conclusion, the findings are in good agreement with data demonstrating that *ABCD2* mRNA is downregulated in human primary macrophages during cholesterol influx mediated by acetylated low-density lipoprotein (9).

Sterol depletion reduces VLCFA accumulation in X-ALD fibroblasts

Overexpression of *ABCD2* leads to reduced VLCFA accumulation in cultured fibroblasts from X-ALD patients (7). Therefore, we investigated whether the induction of *ABCD2* expression by cholesterol depletion correlates with a normalization of the diagnostic VLCFA (C26:0) accumulation in cultured human X-ALD fibroblasts. Gas-chromatographic analysis of VLCFA levels in primary fibroblasts from X-ALD patients or healthy controls showed a significant reduction of VLCFA after 10 days of culturing in sterol-depleted medium (Fig. 2). Thus, reduced intracellular cholesterol activates *ABCD2* gene expression and consequently prevents VLCFA accumulation in X-ALD fibroblasts. Next, we attempted to identify the molecular mechanism underlying the induction of *ABCD2* expression by cholesterol depletion.

ABCD2 expression is induced by SREBP—characterization of an SRE in the *ABCD2* promoter

To determine whether SREBPs are involved in the activation of *ABCD2* expression and to identify the responsive regions, we constructed plasmids containing 1200 bp (ABCD2–1200luc) or 460 bp (ABCD2–460luc) of the human *ABCD2* promoter linked to the firefly luciferase reporter gene. These reporter constructs were used to transiently transfect immortalized human X-ALD fibroblasts in the presence or absence of a plasmid encoding the mature form of human SREBP1a, SREBP1c or SREBP2, respectively (Fig. 3A). The luciferase activity clearly increased in a SREBP-dependent manner with both *ABCD2* promoter constructs and with all three SREBP isoforms. Although the shorter promoter construct was somewhat less responsive, especially to SREBP2, expression was still SREBP-inducible, strongly suggesting a proximal localization of the SRE. Accordingly, within the 460 bp *ABCD2* promoter sequence, we identified two putative SREs: element E1 at position −232/−223 and element E2 at −401/−391 (Fig. 3B), with striking homology to the SREs of the acetyl-CoA carboxylase (19) at position −280/−272 and the stearoyl-CoA desaturase 1 gene (20) at position −423/−413, respectively. These elements were cloned upstream of a minimal β-globin promoter into a luciferase reporter construct to generate pGl–E1luc and pGl–E2luc. Co-transfection of X-ALD fibroblasts with these constructs and SREBP expression vectors clearly identified element E2 as a functional SRE, whereas E1 did not enhance luciferase activity in response to SREBP1a (Fig. 3C). The isolated E2 element conferred strong induction and the E1 element remained unresponsive also when

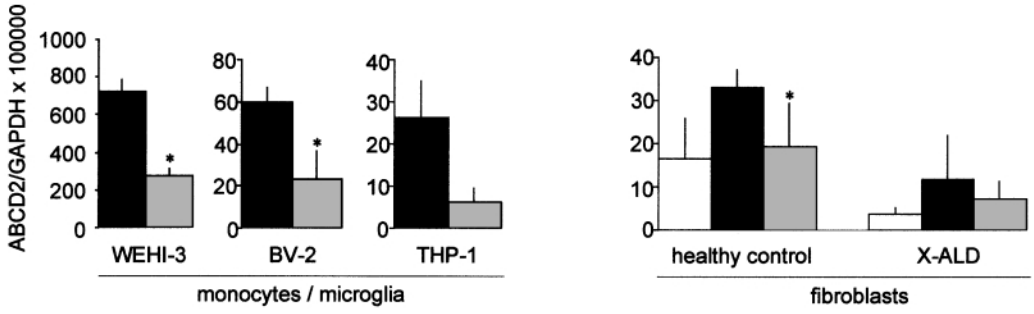

Figure 1. Cholesterol regulates *ABCD2* expression in cell lines and primary fibroblasts. Mouse (WEHI-3 and BV-2) or human (THP-1) rnicroglia/monocyte cell lines and primary human fibroblasts (from healthy controls and X-ALD patients) were cultured in medium containing standard FCS (white bars), lipid-deficient FCS (black bars) or lipid-deficient FCS with added sterols (gray bars). The number of *ABCD2* mRNA copies was evaluated by quantitative real-time RT–PCR in duplicate wells and was normalized to the number of *GAPDH* mRNA copies. Data represent the mean ± SEM of three independent experiments. Statistically significant differences by Student's *t*-test are indicated by an asterisks ($P < 0.05$).

co-expressed with SREBP1c and SREBP2 (data not shown). For more detailed functional analyses, we focused on SREBP1a.

To confirm the activity of the identified regulatory element in SREBP-mediated induction of the *ABCD2* promoter, we incorporated two different mutated versions of the putative SRE both into the *ABCD2* promoter constructs (ABCD2–1200M1luc, ABCD2–1200M2luc; ABCD2–460M1luc, ABCD2–460M2luc) and into the minimal β-globin promoter context (pGl–E2M1luc, pGl–E2M2luc) (Fig. 3B). Both mutations significantly impaired the activation of the reporter constructs in response to SREBP co-transfection (Fig. 3D).

Electrophoretic mobility shift assay

In order to establish whether the *ABCD2* induction is mediated directly by binding of SREBP, we performed electrophoretic mobility shift assays (Fig. 4). The incubation of a ^{32}P-labeled *ABCD2*–SRE oligonucleotide probe with *in vitro* translated SREBP1a resulted in the formation of a protein–DNA complex (Fig. 4: lane 2). A 50-fold molar excess of unlabeled oligonucleotide reduced the binding of SREBP1a protein to the labeled DNA probe (Fig. 4: lane 4), whereas the unrelated competitor mGH3 (mouse growth hormone receptor 3) DNA was unable to inhibit the formation of the complex (Fig. 4: lane 3). Cross-competition with the SRE from the mouse stearoyl-CoA desaturase1 (Scd1) promoter to assess the specificity of the protein–DNA interaction resulted in reduced amounts of the complex (Fig. 4: lane 5). When a ^{32}P-labeled *ABCD2*–SRE oligonucleotide probe with the incorporated point mutations M2 was incubated with *in vitro* transcribed and translated SREBP1a, the formation of only a weak complex was observed, indicating that the introduced mutations interfere with binding of the protein in accordance with the data obtained in the cell-based reporter assay (data not shown).

DISCUSSION

Our data suggest that the molecular mechanism by which lovastatin treatment lowers VLCFA storage in X-ALD patients is based on a reduction of the intracellular cholesterol content, resulting in the proteolytic maturation of SREBP. Activated SREBP then binds the *cis*-active SRE of the *ABCD2* promoter and stimulates *ABCD2* expression, which leads to complementation of the impaired *ABCD1* gene function and restored VLCFA metabolism.

In three reports, X-ALD patients were treated with lovastatin or simvastatin to normalize the pathological level of VLCFA in the plasma (4–6). In one study, plasma VLCFA levels failed to normalize (6), although plasma cholesterol was successfully lowered in all three studies. Interestingly, in the two successful studies, lovastatin treatment was accompanied by a low-fat diet (4), whereas in the third study (6), the diet was normal. The main actions of lovastatin are to block cellular cholesterol synthesis by inhibiting the key enzyme HMGCoA-reductase and to lower liver cholesterol by upregulating the LDL receptor, consequently resulting in reduced plasma LDL cholesterol. In normal human monocyte-derived macrophages, lovastatin treatment produces a significant decrease in total (free plus esterified) cholesterol. However, lovastatin does not change the cellular cholesterol content in cells loaded with LDL-cholesterol (21), which may reflect the situation in some X-ALD patients on a regular diet. Under this condition, SREBP would not be activated and *ABCD2* expression not induced, possibly explaining the different outcomes of statin treatment of X-ALD patients.

Even though the pathogenesis of X-ALD is not fully understood, it is generally accepted that the accumulation of VLCFA in body fluids and tissues as well as inflammation of the cerebral white matter play key roles in this process. However, it still remains to be elucidated if correction of the plasma and tissue levels of VLCFA alone by diet and Lorenzo's oil therapy could change the natural history of X-ALD (1). The therapeutic potential of lovastatin for X-ALD arises from its ability to correct plasma VLCFA levels and to block the induction of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines in astrocytes and microglia, therefore also addressing the inflammatory component of the disease (22). The goal of the small-scale study carried out by Pai and colleagues (4) was to demonstrate that statin treatment can lower or normalize plasma and tissue VLCFA levels in X-ALD patients. Further studies are needed to evaluate the clinical efficacy of lovastatin for X-ALD patients.

Expression of both the murine *Abcd2* and human *ABCD2* genes has also been shown to be upregulated by other

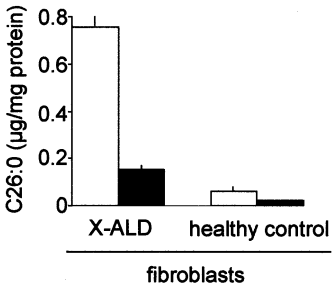

Figure 2. Cholesterol affects VLCFA levels in primary X-ALD fibroblasts. Human X-ALD and healthy control fibroblasts were cultured in medium containing standard FCS (cholesterol loading, white bars) or lipid-deficient FCS (cholesterol depletion, black bars) for 10 days. The C26:0 levels of fibroblasts ($n = 2$) were determined by gas chromatography.

pharmacological agents, such as 4-phenylbutyrate (4PBA), fenofibrate and retinoic acid (23–25). 4PBA was reported to increase expression of the *ABCD2* gene and oxidation of VLCFAs in cultured fibroblasts from X-ALD patients and to normalize VLCFA levels in brain and adrenal gland of *in vivo* treated *Abcd1* knockout mice (23). Although the details of the molecular mode of 4PBA action are unknown, it seems unlikely that this drug, which is normally used in urea cycle disorders, induces *ABCD2* expression by an SREBP-dependent mechanism. Expression of the murine and rat *Abcd2* genes has also been shown to be upregulated in the liver by oral administration of the peroxisome proliferator fenofibrate, an activator of the transcription factor peroxisome proliferator receptor (PPAR) α (24). However, several investigations failed to demonstrate a direct activation of the human or rodent *ABCD2*/*Abcd2* promoter (25,26; J. Berger *et al.*, unpublished data), indicating that the effect of fenofibrate on *ABCD2* mRNA levels might be indirect. Since fenofibrate is used as a cholesterol-lowering drug in human medicine, the molecular mechanism of *Abcd2* induction in the liver of mice could be mediated through an SREBP-dependent mechanism. We are currently investigating this issue. It was also shown that *ABCD2* transcription can be upregulated in the presence of 9-*cis*-retinoic acid (25). It is suggested that the molecular mechanism is via binding of a thyroid hormone receptor/RXR nuclear receptor heterodimer to the putative DR4 motif located in the human *ABCD2* and murine *Abcd2* promoters (25).

Klucken and colleagues (9) investigated the effect of cholesterol loading on mRNA levels of other ABCD transporter family members in human primary monocyte-derived macrophages. They demonstrated that *ABCD4* (*P70R*) mRNA levels were not regulated by cholesterol, indicating the absence of an SRE sequence in the promoter. Concerning *ABCD1* (*ALD*) mRNA levels, the data show induction after cholesterol loading. It seems unlikely that genes upregulated by high cholesterol levels would utilize an SREBP-dependent mechanism. In addition, the human *ABCD1* promoter and co-transfected SREBP showed no activity in reporter assays (data not shown), demonstrating the absence of a functional SRE. For *ABCD3* (*PMP70*), no data are available, since it is not expressed in the primary human-derived macrophages used in the study by Klucken and colleagues (9). However, a computer-based search of 3 kb of the 5′ flanking region of the *ABCD3* gene did not reveal an element with similarity to the SRE of the human *ABCD2* promoter.

Cholesterol loading of murine monocyte or microglial cell lines resulted in substantially decreased *Abcd2* mRNA levels, suggesting that the murine *Abcd2* gene is also SREBP-responsive. Inspection of the mouse *Abcd2* promoter indeed revealed an SRE-like region that is located at a similar position and differs from the SRE found in the human *ABCD2* promoter by only 2 bp (mouse *Abcd2* promoter: nucleotide −389 5′-AGCAGCTGACC-3′; human *ABCD2* promoter: −401 5′-AGCAGATGGCC-3′). Investigations are in progress to study the SRE-dependent inducibility of the *Abcd2* gene in the mouse.

In two reports, the effect of dietary statins on the accumulation of VLCFA were assessed in tissues of *Abcd1*-deficient mice (27,28). In both studies, statin treatment did not result in a decrease of VLCFA in the plasma or tissues of these mice. However, several reports demonstrate that statin treatment has no significant effect on plasma or liver cholesterol levels in rodents (29,30). This indicates that, although a direct causes-and-effect relationship between plasma VLCFA and serum cholesterol levels was demonstrated in X-ALD patients (4), it might not be possible to lower VLCFA in *Abcd1*-deficient mice by statin treatment.

Cholesterol-enriched feeding reduces the amount of mature SREBP1 and SREBP2 in the liver of hamsters (31). However, depletion of cholesterol by combined treatment with a bile acid-binding resin (colestipol) and an HMGCoA-reductase inhibitor (lovastatin) caused a paradoxical decline in the amount of mature SREBP1, whereas the amount of SREBP2 increased (10,31). The latter effect was consistent only when the two drugs were given together, indicating that lovastatin alone is not sufficient to induce SREBP2 expression (32). In patients with primary hypercholesterolemia, combined therapies consisting of simvastatin and low-dose colestipol as bile acid sequestrant were found to be more effective in reducing serum cholesterol levels (33). Since SREBP maturation depends critically on a reduced cholesterol load of cells, this suggests that a combinatorial treatment of X-ALD patients could have beneficial effects on *ABCD2* expression and, thus, on the prevention of VLCFA accumulation. Although dietary cholesterol cannot pass directly through the blood–brain barrier, a low-cholesterol diet will influence brain cholesterol, since changes in the cholesterol balance across the whole body cause alterations in sterol recycling and apolipoprotein E expression within the central nervous system (34). SREBP is expressed in normal rodent and primate brain (35) and lovastatin can efficiently cross the blood–brain barrier, partially inhibiting the rate of cholesterol synthesis in the brain (36). Thus, the combination of a low-cholesterol diet and lovastatin treatment carefully adapted to individual patients (possibly accompanied by colestipol) could have beneficial effects on the lowering of VLCFA in the brain, the most severely affected tissue in X-ALD.

A recent report (37) demonstrates data showing that long-term users of statins are at a 4–14-fold increased risk of developing idiopathic polyneuropathy compared with the background population. The study cannot rule out the possibility that the neuropathy observed in the patients is caused not by the treatment but by the underlying disorder,

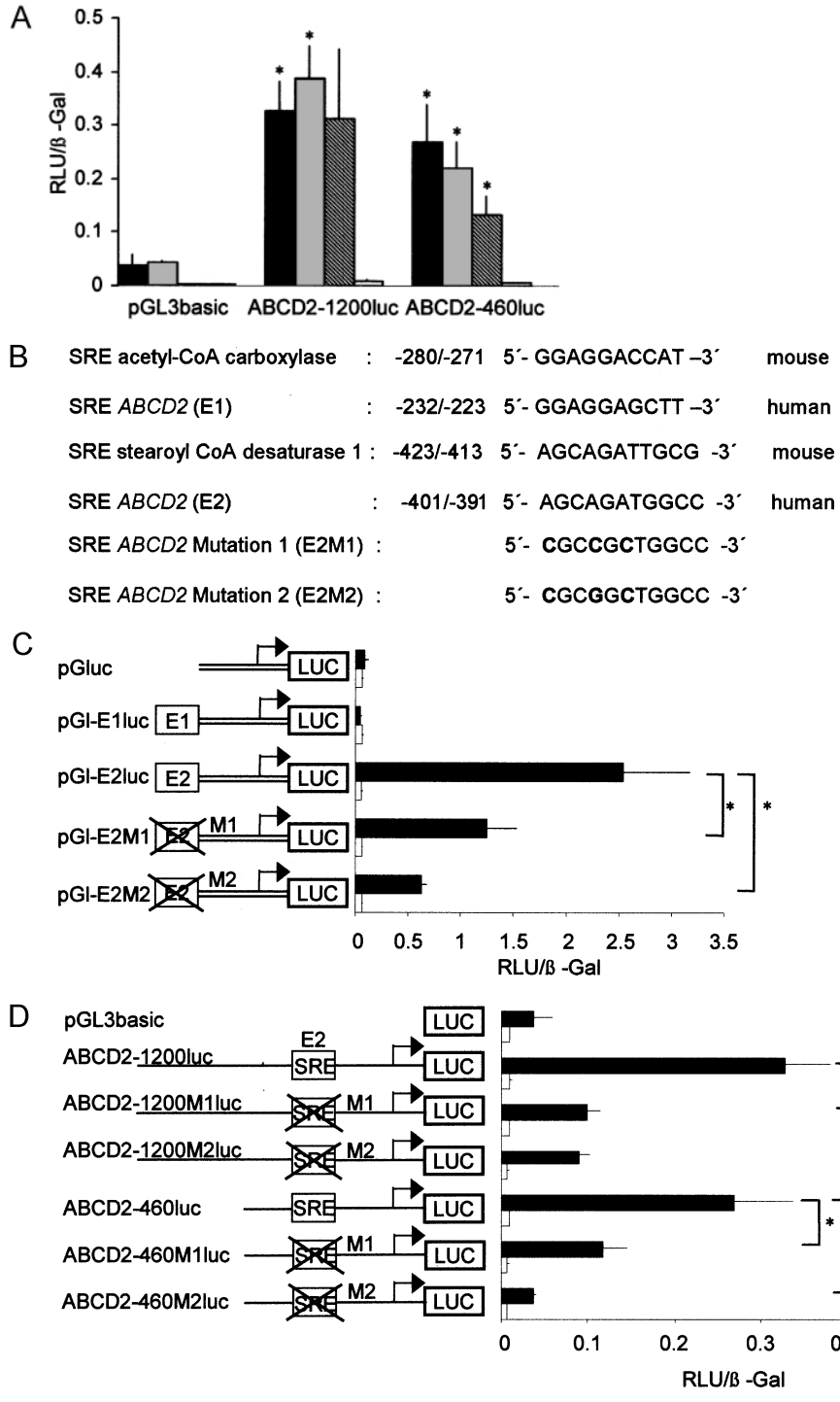

Figure 3. Characterization of a sterol regulatory element (SRE) in the human *ABCD2* promoter. Immortalized human X-ALD fibroblasts were transiently co-transfected with (**A**) wild-type *ABCD2* promoter luciferase reporter constructs, (**C**) constructs with wild-type or mutated putative *ABCD2* SRE sequences cloned into the β-globin minimal promoter context or (**D**) *ABCD2* promoter constructs with mutated SRE motifs, together with expression plasmids encoding either human mature SREBP1a (black bars), SREBP1c (gray bars), SREBP2 (hatched bars) or the empty vector pCMV as a negative control (white bars), as well as pCMV–βGal to normalize the transfection efficiency. The sequences of identified putative SRE sequences (E1 and E2) as well as the point mutations M1 and M2 introduced into the SRE sequence to generate pG1–E2M1 or -M2 and ABCD2–460M1 or -M2 are shown in (**B**). Data represent the mean ± SEM of three independent experiments performed in duplicate wells. Statistically significant differences by Student's *t*-test are indicated by an asterisks ($P < 0.05$).

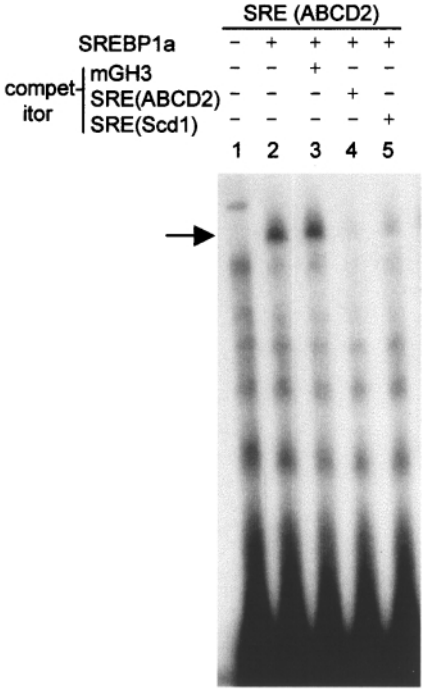

Figure 4. SREBP1a specifically binds the SRE identified in the human *ABCD2* promoter. Electrophoretic mobility shift assays using *in vitro* translated SREBP1a and a labeled SRE (ABCD2) oligonucleotide probe that corresponds to the identified E2 element in the human *ABCD2* promoter show a specific band shift (lane 2), which could not be competed by a 50-fold molar excess of unlabeled unspecific oligonucleotide mGH3 (lane 3), but with SRE-containing oligonucleotides SRE (ABCD2) (lane 4) or SRE (Scd1) (lane 5). Incubation of the labeled probe with reticulocyte lysate instead of *in vitro* translated SREBP1a did not result in the formation of a complex (lane 1). Specific bands are marked by an arrow.

i.e. hyperlipidemia. However, this report might be of concern regarding the use of statins in the treatment of X-ALD patients.

Recently, a new class of hypolipidemic drugs that directly trigger the proteolytic maturation of SREBPs and their migration to the nucleus have been described (38,39). Thus, their mechanism of action is different from that of statins, which act through depletion of cellular cholesterol and subsequent derepression of the SREBP cleavage-activating protein (SCAP). As this new class of hypolipidemic drugs act directly on SREBP maturation, independent of the cholesterol load of the cells, and *ABCD2* gene expression is mediated by an SREBP-dependent mechanism, it remains to be elucidated if these drugs would allow a more specific treatment of X-ALD patients.

MATERIALS AND METHODS

Cell culture

The cell lines used were fibroblasts from X-ALD patients and non-diseased persons (kindly provided by Drs Ernst Conzelmann, University of Würzburg, Germany and Brunhilde Molzer, University of Vienna, Austria, respectively), SV40-transformed X-ALD fibroblasts (a gift from Dr Kirby Smith, Kennedy Krieger Institute, Baltimore, MD), and mouse (WEHI-3) and human (THP-1) monocyte cell lines (ATCC, Manassas, VA) and mouse microglia cell line BV-2 (kindly obtained from Dr Helmut Kettenmann, Max-Delbruck Center for Molecular Medicine, Germany). All cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin and 100 µg/ml streptomycin (Biowhittaker, Walkersville, MD). For cholesterol depletion, standard FCS (typically containing 33 mg cholesterol per 100 ml) was substituted with 10% lipoprotein-deficient FCS (typically containing 9 mg cholesterol per 100 ml; Sigma, St Louis, MO); for cholesterol loading of cells, 1 µg/ml 25-hydroxycholesterol and 10 µg/ml cholesterol (Sigma) were added to the lipid-deficient FCS.

Biochemical analyses of VLCFA

For gas-chromatographic (GC) analyses of VLCFA, fibroblasts were harvested from a confluent 75 cm^2 culture flask by trypsinization. After adding C23:0 as an internal standard, total lipids were extracted, converted to methyl esters, purified by thin-layer chromatography and subjected to capillary GC analyses as described previously (40).

Plasmid construction and *in vitro* mutagenesis of the *ABCD2* SRE

Human *ABCD2* promoter fragments were generated by PCR using sense primers containing a *Kpn*I site (nucleotide −1275 5′-GAAGTAAGGTaCcAGAGAAGCTATTTC-3′, 1200 bp fragment; nucleotide −518 5′-CTGTTTGCGG-TAcCTACTTGAAATCTT-3′, 460 bp fragment), a common antisense primer containing an *Sma*I site (nucleotide −56 5′-CACAGAAATCCCggGCAAATGTTTTAG-3′), and human genomic liver DNA as the template. The PCR fragments were cloned directionally into the *Kpn*I/*Sma*I-digested vector pGL3 basic (Promega, Mannheim, Germany) upstream of the promoterless luciferase gene. The expression vector pGLUC (26), a modified pGL2 vector (Promega) containing the minimal β-globin promoter upstream from a luciferase gene, was used for studies of selected response elements. The pairs of annealed oligonucleotides with *Hin*dIII and *Bam*HI overhangs (E1: 5′-agcttatcgatGCCTGGGAGGGAGGAGCTTGGTGCAG-CTg-3′, 5′-gatccAGCTGCACCAAGCTCCTCCCTCCCAGG-Cacgata-3; E2: 5′-aGCttGCAGATGGCCTGATTCGACCTCT-CCG-3′, 5′- gatcCGGAGAGGTCGAATCAGGCCATCTGC-3′) were ligated into *Bam*HI/*Hin*dIII-digested pGLUC upstream from the β-globin promoter to generate the plasmids pG1–E1luc and pG1–E2luc. The point mutations M1 and M2 were introduced into the SRE of the *ABCD2* promoter by *in vitro* mutagenesis usind a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. PCR was conducted using complementary degenerate primers (M1: nucleotide −419 5′-GCCGGTTTTGTTCG-CCcGCcGcTGGCCTGATTCGACCTC-3′ and M2: nucleotide −419 5′-GCCGGTTTTGTTCGCCcGCgGcTGGCCTGATTC-GACCTC-3′, ABCD2–1200luc and ABCD2–460luc promoter constructs; M1: 5′-CCCGGGAAGCTcGCcGcTGGCCTGA-TTCGACCTC-3′ and M2: 5′-CCCGGGAAGCTcGCgGcTGG-CCTGATTCGACCTC-3′, pG1–E2luc construct), introducing three single-point mutations. All constructs were verified by

DNA sequence analysis using the GL primer 2 and RV primer 3 (Progema).

Transient transfection experiments

SV40-transformed X-ALD fibroblasts were transfected using Lipofectamine (Invitrogen, Groningen, The Netherlands) with 0.5 µg SREBP expression vector (41) (kindly provided by Dr Timothy Osborne, University of California, Irvine, CA) and 0.05 µg pCMV–β-Gal (Clontech, Palo Alto, CA). The transfected cells were incubated for 48 h and assayed for luciferase and β-galactosidase activity using a Luciferase–β-Gal 1 step kit (Aureon Biosystems, Vienna, Austria) and a Mediators PhL luminometer (Aureon Biosystems) according to the manufacturer's instructions.

Real-time quantitative PCR analysis

Total RNA was extracted from cells using an RNeasy Mini kit (Quiagen, Bothell, WA); cDNA was generated by reverse transcription (GeneAmp RNA PCR Kit, Applied Biosystems, Branchburg, NJ) and analyzed by quantitative PCR using an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). The forward primers nucleotide 1959 5′-CACAGCGTGCACCTCTAC-3′ (mouse) or nucleotide 1883 5′-TCCTACACAATGTCCATCTCT-3′ (human) and reverse primer nucleotide 2032 (mouse) or nucleotide 1961 (human) 5′-AGGACATCTTTCCAGTCCA-3′ as well as the TaqMan fluorescent probe nucleotide 1986 (mouse) or nucleotide 1915 (human) 5′-CAAAGAGAAGGAGGATGGGATGC-3′ were used for amplification and detection of *ABCD2* mRNA. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were used as a control. Primers were nucleotide 525 (mouse) or nucleotide 560 5′-AGGTCATCCATGACAACTTT-3′ (forward) and nucleotide 601 (mouse) or nucleotide 636 (human) 5′-AGTCTTCTGGGTGGCAGT-3′ (reverse), for the probe nucleotide 562 (mouse) or 597 (human) 5′-CATGACCACAGTCCATGCCA-3′. Standard curves for quantification were generated using plasmids containing the human or mouse *ABCD2* or *GAPDH* cDNA (24). For each assay, 1.6 ng (*GAPDH*) and 6–360 ng (*ABCD2*) of reverse-transcribed RNA was used for the PCR analysis of *GAPDH* and *ABCD2* mRNA, respectively. The thermocycler was programmed to give 95°C for 10 min followed by 50 cycles at 95°C for 20 s and 58°C for 50 s.

Electrophoretic mobility shift assay (EMSA)

SREBP1a was synthesized *in vitro* using a TNT T7 Quick for PCR DNA *in vitro* transcription/translation system (Promega) and the primers 5′-GGATCCTAATACGACTCACTATAGGGAACAGCCACCATGGACGAGCCACCCTTCAG-3′ (forward) and 5′-$T_{(29)}$ACTATGTCAGGCTCCGAGTCACTGCCA-3′ (reverse) and the SREBP1a expression plasmid as template according to the manufacturer's instructions. As control for correct synthesis, a reaction containing biotinylated lysine tRNA (Promega) was run in parallel. The oligonucleotides corresponding to the SRE motif from the human *ABCD2* promoter were 5′-GTTCGCCAGCAGATGGCCTGATTCGA-3′ and 5′-AGGTCGAATCAGGCCATCTGCTGG-3′. The oligonucleotides corresponding to the SRE motif with the incorporated point mutations M2 were 5′-GTTCGCCCGCGGCTGGCCTGATTCGA-3′ and 5′-AGGTCGAATCAGGCCAGCCGCGGG-3′. The probe was labeled by filling-in with [α-^{32}P]dCTP, and the binding experiments were performed by preincubating 3 µl of SREBP1a synthesis mixture (or reticulocyte lysate for the negative control) with oligonucleotides as described previously (26). Oligonucleotides used for competition experiments were: Scd1 SRE 5′-AGGCAGAGGGAACAGCAGATTGTG-3′ and 5′ TCTGCACAATCTGCTGTTCCCTC-3′ mGHR 5′-CTGGTTAGTTCATATTGTTGTTCCACCAATAGGGTTG-3′ and 5′-GTCTGCAACCCTATTGGTGGAACAACAATATGAACTAA-3′.

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