Translational studies in X-linked adrenoleukodystrophy

Engelen, M.

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Chapter 2

Cholesterol-deprivation increases mono-unsaturated very long-chain fatty acids in skin fibroblasts from patients with X-linked adrenoleukodystrophy

Marc Engelen\textsuperscript{1,2}, Rob Ofman\textsuperscript{2}, Petra A.W. Mooijer\textsuperscript{1}, Bwee Tien Poll – The\textsuperscript{2}, Ronald J.A. Wanders\textsuperscript{1} and Stephan Kemp\textsuperscript{1,2}

\textsuperscript{1}Department of Clinical Chemistry, Laboratory Genetic Metabolic Diseases; \textsuperscript{2}Department of Pediatric Neurology/Emma Children’s Hospital, Academic Medical Center, University of Amsterdam, The Netherlands

Abstract

X-linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder and is characterized by a striking and unpredictable variation in phenotypic expression. It ranges from a rapidly progressive and fatal cerebral demyelinating disease in childhood (CCALD), to the milder slowly progressive form in adulthood (AMN). X-ALD is caused by mutations in the ABCD1 gene that encodes a peroxisomal membrane located ABC half-transporter named ALDP. Mutations in ALDP result in reduced beta-oxidation of very long-chain fatty acids (VLCFA, >22 carbon atoms) in peroxisomes and elevated levels of VLCFA in plasma and tissues. Previously, it has been shown that culturing skin fibroblasts from X-ALD patients in lipoprotein-deficient medium results in reduced VLCFA levels and increased expression of the functionally redundant ALD-related protein (ALDRP). The aim of this study was to further resolve the interaction between cholesterol and VLCFA metabolism in X-ALD. Our data show that the reduction in 26:0 in X-ALD fibroblasts grown in lipoprotein-deficient culture medium (free of cholesterol) is offset by a significant increase in both the level and synthesis of 26:1. We also demonstrate that cholesterol-deprivation results in increased expression of stearoyl-CoA-desaturase (SCD) and increased desaturation of 18:0 to 18:1. Finally, there was no increase in [1-14C]-26:0 beta-oxidation. Taken together, we conclude that cholesterol-deprivation reduces saturated VLCFA, but increases mono-unsaturated VLCFA. These data may have implications for treatment of X-ALD patients with lovastatin.
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Introduction

X-linked adrenoleukodystrophy (X-ALD; MIM300100) is a severe metabolic disorder characterized by impaired peroxisomal beta-oxidation of very long-chain fatty acids (VLCFA, >22 carbon atoms) and elevated VLCFA levels in plasma and tissues of patients (Moser et al 1981). The clinical presentation of X-ALD is highly variable, even among affected members of the same family (Moser et al 2001). In the most severe phenotype, affected patients develop cerebral demyelination with inflammation which is rapidly progressive and usually fatal within 2 years after onset. This phenotype presents most frequently in early childhood (childhood cerebral ALD; CCALD), but can also occur in adolescence or adulthood. The adrenomyeloneuropathy (AMN) phenotype usually develops between the 3rd and 4th decade of life and is gradually progressive. The main symptoms are spastic paraparesis and incontinence, caused by progressive myelopathy and peripheral neuropathy. Adrenocortical and testicular insufficiency can occur in isolation (Addison-only), but also in combination with any of the other phenotypes.

X-ALD is caused by mutations in the ABCD1 gene (MIM 300371) (Mosser et al 1993), that encodes a peroxisomal transmembrane protein named the adrenoleukodystrophy protein (ALDP), which is classified as a member of the ATP-binding cassette subfamily D of transporters (Mosser et al 1993; Mosser et al 1994). Although the precise function of ALDP is not clear, absence or dysfunction of ALDP causes impaired peroxisomal beta-oxidation of VLCFA in human skin fibroblasts (Singh et al 1981).

Two other peroxisomal transmembrane proteins belonging to the class of ATP-binding cassette transporters have been identified in mammals. These include ALD-related protein (ALDRP) (Lombard-Platet et al 1996) and PMP70 (Kamijo et al 1990). It has been well established that these proteins show functional redundancy, at least partially, and have overlapping substrate specificities. Over-expression of either PMP70 or ALDRP in X-ALD fibroblasts corrects VLCFA beta-oxidation (Kemp et al 1998; Netik et al 1999). Furthermore, in the Abcd1 knockout mouse in which ALDRP was overexpressed the biochemical abnormalities normalized and the neurological AMN-like phenotype was reversed (Pujol et al 2004).

This prompted research with the aim to identify ways to induce the expression of ALDRP, encoded by the ABCD2 gene, which is normally expressed at low levels in most tissues (Berger et al 1999). It has been shown that 4-phenylbutyrate (4-PBA) can induce ALDRP expression in human and mouse primary fibroblasts (Kemp et al 1998). Unfortunately, the dosage needed to obtain a biological effect in humans makes it unpractical for clinical application (Kemp et al 1998; Moser et al 2001).

In 1998 Singh et al (Singh et al 1998a) reported that beta-oxidation of VLCFA was increased markedly by incubating fibroblasts from X-ALD patients with lovastatin, a cholesterol lowering drug that belongs to the class of HMG-CoA-reductase inhibitors. Later it was shown that culturing fibroblasts from X-ALD patients in medium with lipoprotein-deficient fetal calf serum resulted in a reduction in 26:0 levels and increased ABCD2 expression (Weinhofer et al 2002). Since cholesterol depletion leads to activation of the SREBP-pathway (Brown and Goldstein, 1997), a likely mechanism for the observed effects on 26:0 metabolism would be the increased expression of ALDRP. Subsequent studies showed that the promoter of the
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ABCD2 gene indeed contains a functional sterol regulatory element (SRE) (Weinhofer et al. 2002). Based on these observations patients with X-ALD have been treated with lovastatin and a low-fat diet in a small open-label study. An approximately 40% reduction in plasma 26:0 levels was observed in 7 out of 12 patients (Singh et al. 1998b; Pai et al. 2000). In another study in which patients were treated with simvastatin but without dietary fat restriction, no reduction in plasma VLCFA was observed (Verrips et al. 2000). Still, based on the aforementioned observations many patients with X-ALD are being treated with lovastatin. Unfortunately, further studies showed that in Abcd1 knockout mice lovastatin (Yamada et al. 2000) and simvastatin (Cartier et al. 2000) were unable to reduce VLCFA. On the contrary, even higher levels of VLCFA in tissues, notably the brain, were found. With respect to lovastatin treated patients no data is available on the effect on VLCFA levels in tissues other than plasma and erythrocytes. There are, however, indications that interspecies differences are important. Mouse fibroblasts cultured in lipoprotein-deficient culture medium showed a 40% reduction in 26:0 levels compared to a 80% reduction in human fibroblasts (Weinhofer et al. 2005). Therefore, the data from mice experiments cannot be extrapolated to humans directly.

The aim of the experiments described in this study was to further resolve the interaction between cholesterol and VLCFA metabolism in X-ALD using skin fibroblasts from patients with X-ALD and controls.

**Materials and Methods**

**Fatty acid substrates**
Deuterium-labeled free fatty acids 16,16,16-D$_3$-16:0 and 17,17,17,18,18-D$_5$-18:0 were purchased from CDN isotopes (Pointe-Claire, Canada) and 12.5 mM stock solutions in DMSO were prepared. Prior to usage stock solutions were vortex mixed and diluted in Ham F-10 tissue culture medium to their final concentration. All chemicals used were of analytical grade.

**Cell lines and cell culture**
Human primary skin fibroblasts cell lines were obtained from patients with X-ALD through the neurology outpatient clinic of the Academic Medical Centre. From each patient written informed consent was obtained. Five cell lines from X-ALD patients were used. The clinical phenotype of the patient, the ABCD1 mutation and the effect of the mutation on ALDP expression by means of immunofluorescence were: ALD005 (AMN, p.Ser290X, absent); ALD009 (AMN, p.Glu471fs, absent); ALD011 (Addison-only, p.Arg464X, absent); ALD013 (AMN, p.Met1Val, absent) and ALD014 (AMN, p.Leu220Pro, reduced). Fibroblasts from anonymous controls were obtained from the laboratory cell bank. Cells were grown in HAM F-10 tissue culture medium supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 U/mL) and amphotericin B (250 ng/mL) or in Dulbecco’s Modified Eagle Medium (DMEM), additionally supplemented with HEPES (25 mmol/L). All cell lines were used with passage numbers below 20. For cholesterol deprivation 10% lipoprotein-deficient fetal calf serum, containing less than 3 µmol/L of cholesterol, was used (Bodinco B.V., Alkmaar, The Netherlands). For comparison, regular fetal calf serum contains approximately
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1.5 mmol/L of cholesterol. For cholesterol loading, the lipoprotein-deficient fetal calf serum was supplemented with 10 µg cholesterol (dissolved in EtOH) and 1 µg 25-OH-cholesterol (dissolved in EtOH) per mL serum. Culture media were refreshed every 5 days.

**Fatty acid synthesis and fatty acid desaturation**

Cells were seeded in T75 tissue culture flasks at approximately 50% confluency and the medium was refreshed the next day. VLCFA synthesis was measured by incubating the cells for 10 days in the presence of increasing concentrations of D$_3$-16:0. Culture media and D$_3$-16:0 were refreshed after 5 days.

For the measurement of long chain fatty acid desaturation activity, cells were incubated for 24 hours in the presence of 100 µM D$_5$-18:0.

After incubation, cells were harvested with trypsin, washed twice with phosphate-buffered saline (PBS), once with 0.9% NaCl and taken up in 200 µL deionized water. After sonication protein concentration was determined using BCA (Smith et al 1985). Total cellular fatty acids were analyzed using the electrospray ionization mass spectrometry (ESI-MS) method described previously (Valianpour et al 2003; Kemp et al 2004).

**Measurement of VLCFA beta-oxidation**

Cells were cultured in the different media for 7 days and the effect on the peroxisomal beta-oxidation activity was measured using [1-$^{14}$C]-26:0 as described previously (Wanders et al 1995). Measurements were performed in triplicate for each cell line. As an internal control, mitochondrial beta-oxidation was determined using [1-$^{14}$C]-16:0 (Wanders et al 1995).

**Quantitative RT-PCR analysis of SCD mRNA levels**

The expression levels of SCD mRNA were related to the expression levels of GAPDH (Glyceraldehyde-phosphate-dehydrogenase) mRNA using the LightCycler system (Roche, Mannheim, Germany). Total RNA was isolated from primary skin fibroblasts growing in log phase with TRIzol® reagent (Invitrogen, Carlsbad, USA) and cDNA was synthesized using the first-strand cDNA synthesis kit (Roche, Mannheim, Germany). Quantitative real-time PCR analysis of SCD in skin fibroblasts was performed using the LightCycler FastStart DNA Master SYBR green I kit (Roche, Mannheim, Germany). The following primers for SCD were used: forward 5’-CACCCAGCTGTCAAAGAGAA-3’ and reverse 5’-TCACCCACAGCTCCAAGT-3’. For GAPDH the following primers were used: forward 5’-ACCACCATGGAGAAGGCTGC-3’ and reverse 5’-CTCAGTGCCCAGGATGC-3’. The specificity of amplification was confirmed by agarose gel electrophoresis. Data were analyzed using LightCycler software version 3.5 (Roche) and the LinReg PCR program version 7.5 for analysis of RT-PCR data (Ramakers et al 2003).

**Results**

**Effect of cholesterol deprivation on endogenous VLCFA**

Culturing fibroblasts from X-ALD patients and control subjects in lipoprotein-deficient medium resulted in a 0.29 nmol/mg protein (35%) reduction of 26:0 levels (Fig. 1A and B). Interestingly, these conditions caused a 1.03 nmol/mg protein (70%) increase in the 26:1 levels in fibroblasts from X-ALD patients, but not in control fibroblasts (Fig. 1C and D). This
experiment was repeated with cells cultured in DMEM instead of HAM F-10 to compare our results with the data from Weinhofer et al who did not investigate the effect on 26:1 levels (Weinhofer et al 2002). The data in Fig. 1B and Fig. 2A show a comparable effect on 26:0 levels: deprivation of cholesterol resulted in a 40% reduction in 26:0 levels in X-ALD cells independent of the type of medium used. Under both culture conditions there was a significant increase in 26:1 (Fig. 1D and 2B). Remarkably, both the 26:0 as well as the 26:1 levels were higher when cells were cultured in DMEM. Compared to Ham F10, 26:0 levels were approximately 1.5-fold higher (compare Fig. 1B with Fig. 2A), and 26:1 levels were about 4-fold higher (compare Fig. 1D with 2B). This could be related to differences in composition between DMEM and Ham F10 culture medium. For example there are clear differences in the concentration of D-glucose (25 mmol/L versus 6 mmol/L) and L-glutamine (1 mmol/L versus 4 mmol/L).

**Effect of cholesterol deprivation on VLCFA synthesis from D3-16:0 and 26:0 beta-oxidation**

To gain more insight into the mechanism by which cholesterol deprivation influences the levels of saturated and mono-unsaturated VLCFA, we measured the effect of cholesterol deprivation on the synthesis of both D$_3$-26:0 and D$_3$-26:1 from D$_3$-16:0. Furthermore, VLCFA beta-oxidation was measured. To this end, X-ALD fibroblasts were cultured in the presence of D$_3$-16:0 for 10 days and the amount of D$_3$-VLCFA was determined. Cholesterol deprivation did not affect net synthesis of saturated VLCFA from D$_3$-16:0 (Fig. 3A). However, the synthesis

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**Figure 1:** Endogenous levels of 26:0 (A and B) and 26:1 (C and D) in fibroblasts from controls (A and C) and X-ALD patients (B and D). Fibroblasts were cultured for 10 days in HAM F10 medium supplemented either with 10% fetal calf serum (open bars) or with 10% lipoprotein-deficient fetal calf serum (black bars). Fatty acid levels are expressed as nmol/mg protein. Statistical significance was determined with Student’s unpaired t-test. Error bars represent the standard deviation: (*) p < 0.05; (**) p < 0.01 and (***) p < 0.001.
of D₃-26:1 from D₃-16:0 was found to be increased 7-fold under low-cholesterol conditions (Fig. 3B).

When X-ALD fibroblasts were grown in lipoprotein-deficient medium, the rate of [1-¹⁴C]-26:0 beta-oxidation was not affected (Fig. 4). Furthermore, the beta-oxidation of [1-¹⁴C]-16:0 was not different between X-ALD fibroblasts and controls and between the different culture conditions (data not shown).

**Effect of cholesterol deprivation on SCD expression and desaturation activity**

In mammalian cells, the synthesis of mono-unsaturated fatty acids from saturated fatty acids is catalyzed by stearoyl-CoA-desaturase (SCD), an enzyme that introduces a cis-double bond in the ω-9 position (Ntambi and Miyazaki, 2003). The main substrates for this enzyme are palmitoyl-CoA (16:0-CoA) and stearoyl-CoA (18:0-CoA). We investigated whether the increase in mono-unsaturated VLCFA could be explained by an increased expression and activity of SCD. To this end, we performed qPCR analysis using control and X-ALD cell lines cultured on standard HAM F10 medium, lipoprotein-deficient medium, or lipoprotein-deficient medium supplemented with free cholesterol and 25-OH-cholesterol. When

![Figure 2: Endogenous 26:0 (A) and 26:1 levels (B) in fibroblasts from X-ALD patients cultured for 10 days in DMEM supplemented either with 10% fetal calf serum (open bars) or with 10% lipoprotein-deficient fetal calf serum (black bars). Fatty acid levels are expressed as nmol/mg protein. Statistical significance was determined with Student’s unpaired t-test. Error bars represent the standard deviation: (*) p < 0.05; (**) p < 0.01.](image)

![Figure 3: Net synthesis of D₃-26:0 (A) and D₃-26:1 (B) from D₃-16:0 in fibroblasts from X-ALD patients. Fibroblasts were incubated with increasing concentrations D₃-16:0 for 10 days in HAM F10 medium supplemented either with 10% fetal calf serum (open squares) or with 10% lipoprotein-deficient fetal calf serum (grey circles). Fatty acid levels are expressed as nmol/mg protein. Error bars represent the standard deviation.](image)
fibroblasts from both controls and X-ALD patients were cultured in lipoprotein-deficient medium, the SCD mRNA levels increased 4 to 5-fold (Fig. 5). This increase was abolished by the addition of cholesterol and 25-OH-cholesterol to the lipoprotein-deficient culture medium (Fig. 5).

Subsequently, in order to obtain information on the activity of SCD, we incubated X-ALD fibroblasts and controls with labeled D₅-18:0 and the amount of D₅-18:1 present after 24 hours was measured. Figure 6 shows that the synthesis of D₅-18:1 was increased under conditions of cholesterol-deprivation which was reversed by the addition of cholesterol and 25-OH-cholesterol to the lipoprotein-deficient culture medium.
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Figure 6: The effect of cholesterol-deprivation on the activity of SCD was analyzed by measuring the amount of D$_5$-18:1 formed from D$_5$-18:0. Control (A) and X-ALD (B) cells were cultured for 24 hours in HAM F10 with 100 μM D$_5$-18:0 supplemented either with 10% fetal calf serum (open bars), with 10% lipoprotein-deficient fetal calf serum (black bars), or with 10% lipoprotein-deficient fetal calf serum with 10 μL cholesterol and 1 μL 25-OH-cholesterol (grey bars). Fatty acid levels are expressed as nmol/mg protein. Statistical significance was determined with Student’s unpaired t-test. Error bars represent the standard deviation: (*) p < 0.05; (**) p < 0.01.

Discussion

The data presented in this paper confirm the earlier observation that X-ALD fibroblasts grown in lipoprotein-deficient culture medium have a reduced level of 26:0 (Fig. 1B) (Weinhofer et al 2002). However, we also found a large increase in the 26:1 level (Fig. 1D). This has not been reported before.

VLCFA are mostly derived from endogenous synthesis and not from dietary sources (Moser et al 2001), and it has been shown previously that X-ALD fibroblasts have increased 26:1 synthesis from oleate (18:1) (Kemp et al 2005). Saturated VLCFA are synthesized from palmitate (16:0) by chain elongation (Jakobsson et al 2006), whereas elongation of palmitoleate (16:1) yields mono-unsaturated VLCFA. Mono-unsaturated fatty acids in humans are synthesized by SCD, an enzyme that introduces a cis double bond at the ω-9 position. The preferred substrates for SCD are palmitoyl-CoA (16:0-CoA) or stearoyl-CoA (18:0-CoA), and not fatty acyl-CoAs with longer chain length (Ntambi and Miyazaki, 2003). Our results show that cholesterol depletion of the culture medium results in increased 26:1 levels (Fig. 1D) and increased synthesis of D$_5$-26:1 from D$_5$-16:0 in X-ALD fibroblasts (Fig. 3B).

Interestingly, this increase in 26:1 was accompanied by an increase in the expression of SCD and increased conversion of D$_5$-18:0 to D$_5$-18:1. SCD is regulated at the transcriptional level and it is a known target of the SREBP class of transcription factors (Ntambi and Miyazaki, 2003). 25-OH-cholesterol is a potent inhibitor of SREBP activation (Brown and Goldstein, 1997), and addition of cholesterol and 25-OH-cholesterol will abolish SREBP activation and can serve as a control condition. The fact that the SREBP signaling pathway is activated by cholesterol depletion (Brown and Goldstein, 1997), may well explain the increase in SCD mRNA levels when X-ALD fibroblasts were cultured in lipoprotein-deficient medium (Fig. 5A). This finding is supported by the increased conversion of D$_5$-18:0 to D$_5$-18:1 by SCD (Fig. 6A). The observation that both SCD mRNA levels and increased conversion of D$_5$-18:0 to D$_5$-18:1 can be reversed by adding free cholesterol and 25-OH-cholesterol to the lipoprotein-deficient medium strongly suggests that the observed effects are indeed attributable to
cholesterol deprivation and not to other differences between the media.

The findings described in this paper may well be relevant for research aimed towards the generation of new therapeutic approaches for X-ALD currently ongoing and also to clinical practice. It has been shown that increased expression of ALDRP could reverse the deficient VLCFA beta-oxidation in X-ALD fibroblasts (Kemp et al 1998). Furthermore, the compound 4-phenylbutyric acid (4-PBA) reduced VLCFA levels in both human X-ALD fibroblasts as well as in the brain and adrenals of Abcd1 knockout mice. Even though the mode of action has not yet been resolved, this may be caused by peroxisome proliferation, increased ALDRP expression (Kemp et al 1998) or increased mitochondrial beta-oxidation (McGuinness et al 2003). In Abcd1 knockout mice in which ALDRP is overexpressed, there is normalization of the biochemical abnormalities and also reversal of the AMN-like phenotype occurring at the age of 18 months (Pujol et al 2004). This data supports the hypothesis that ALDRP overexpression might be of clinical benefit in X-ALD. Unfortunately, 4-PBA mediated pharmacological overexpression of ALDRP proved unsuitable for application in humans, due to the short half-life of 4-PBA (Moser et al 2001). Therefore, other means to upregulate ALDRP in humans as a treatment for X-ALD are being sought. It has been suggested that cholesterol lowering with lovastatin might accomplish this (Weinhofer et al 2002).

In 1998 the reports that lovastatin reduced the 26:0/22:0 ratio in cultured X-ALD fibroblasts (Singh et al 1998a) and also reduced plasma VLCFA (reported as the sum of 22:0 + 24:0 + 26:0) in X-ALD patients (Singh et al 1998b) offered new hope for a widely applicable and effective treatment for X-ALD. Unfortunately, a follow-up paper from the same group showed less striking effects on 26:0 levels (Pai et al 2000) and a study with simvastatin failed to show any effect on 26:0 (Verrips et al 2000). Later work by Weinhofer et al was aimed to identify the mechanism by which lovastatin reduced the 26:0/22:0 ratio in X-ALD skin fibroblasts (Weinhofer et al 2002). To study this, X-ALD skin fibroblasts were cultured in lipoprotein-deficient medium containing low levels of cholesterol. When grown in this medium, X-ALD fibroblasts showed an increase in ABCD2 expression, as well as a decrease in 26:0 levels (Weinhofer et al 2002). The observed upregulation of ALDRP was taken as a plausible explanation for the observed effect of lipoprotein-deficient culture medium and lovastatin on 26:0 levels in X-ALD fibroblasts.

Our findings, however, shed new light on the feasibility of lowering cholesterol as a therapeutic approach. The decrease in 26:0 induced by culturing X-ALD skin fibroblasts in lipoprotein-deficient medium is offset by a significant increase in 26:1. On the whole, there is no decrease in the total VLCFA pool: the 0.3 nmol/mg protein decrease in 26:0 (Fig. 1A) is smaller than the 1 nmol/mg protein increase in 26:1 (Fig. 2A). The observation that 26:0 beta-oxidation is not increased in lipoprotein-deficient medium is in line with this finding (Fig. 4).

The role of VLCFA accumulation in the pathogenesis of X-ALD is still largely unknown. Since VLCFA accumulation (predominately 26:0) is the biochemical hallmark of X-ALD it seems plausible that this accumulation is somehow related to the development of symptoms. This is supported by experiments by Whitcomb and colleagues showing a direct toxic effect of 26:0 on adrenocortical cells (Whitcomb et al 1988). Adrenocortical cells cultured in the presence of increasing concentrations of 26:0 showed a decreased response to ACTH stimulation. Also, 26:0 has disruptive effects on cell membrane structure and function (Ho
et al 1995). Whether 26:1 has different effects on cell functioning and membrane properties than 26:0 and whether it is less toxic remains to be resolved, and should be investigated.

The data presented here indicate that it is unclear if 26:0 reduction through cholesterol lowering is a feasible therapeutic approach. Caution should be taken in prescribing lovastatin to X-ALD patients, since data on for example the possible accumulation of mono-unsaturated VLCFA or VLCFA accumulation in tissues is lacking. Currently, we are conducting a clinical trial to evaluate the effect of lovastatin versus placebo on VLCFA in plasma, lymphocytes and erythrocytes of X-ALD patients.

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