Exogenous Addition of 25-Hydroxycholesterol Reduces Level of Very Long-Chain Fatty Acids in X-Linked Adrenoleukodystrophy


X-Linked adrenoleukodystrophy (X-ALD) is a severe metabolic disorder characterized by the accumulation of very long-chain fatty acids (VLCFAs). Recently, we demonstrated that levels of 25-hydroxycholesterol (25-HC) and cholesterol 25-hydroxylase (CH25H) were found to be elevated in X-ALD. Herein, we report that the exogenous addition of 25-HC significantly reduces C26:0 levels in X-ALD patient-derived fibroblasts and oligodendrocytes differentiated from induced pluripotent stem cells (iPSCs) derived from X-ALD patients. Moreover, 25-HC treatment was found to down-regulate the expression of ELOVL1, a key enzyme for the synthesis of C26. In addition, activation of liver X receptor (LXR), a molecular target of endogenous 25-HC, also reduced C26:0 level. The reduction of C26:0 levels by 25-HC treatment might result, at least partially, from the decrease of ELOVL1 expression as well as the activation of LXR. Our findings could provide a better understanding of the role of 25-HC in X-ALD and useful information to find therapeutic agents to treat X-ALD.

Aberrant accumulation of very long chain fatty acids (VLCFA, \( \geq \) C24:0), resulting from mutation of ATP-binding cassette transporter subfamily D member 1 (ABCD1), is a key feature of X-linked adrenoleukodystrophy (X-ALD).[1,2] It has been reported that due to the impaired peroxisomal \( \beta \)-oxidation of VLCFAs, C26:0 and C24:0 levels were 4–6 fold and 10–30% higher in fibroblasts from patients with X-ALD, respectively, compared to controls, whereas C22:0 levels were unaffected.[3–5] VLCFA accumulation is critically involved in the process of tissue damage.[1,2] Therefore, prevention of VLCFA accumulation can be an effective treatment for X-ALD. Lorenzo’s oil has been used as a dietary supplement in patients with X-ALD. However, it does neither affect C26:0 levels in the nervous system, nor prevent disease progression.[6,7] Lovastatin, a potent HMG-CoA reductase inhibitor, was also a high potential candidate to overcome X-ALD.[8] However, it did not show a significant reduction in C26:0 in a randomized, double-blind clinical trial.[9] To date, there are no clinically useful approaches to sufficiently reduce VLCFA levels to inhibit X-ALD progression.

Recently, we demonstrated that upregulation of a cholesterol metabolic, 25-hydroxycholesterol (25-HC) and its synthesizing enzyme, cholesterol 25-hydroxylase (CH25H), was observed in Childhood Cerebral Adrenoleukodystrophy (CCALD) patients-derived cells and ex vivo.[10] Further, dysfunction of ABCD1 is implicated in the induction of CH25H expression.[11] Moreover, it was reported that 25-HC is a factor contributing to neuro-inflammation in X-ALD.[11]

25-HC has been identified as a key regulator of lipid metabolism.[12] It also has been demonstrated that 25-HC acts as an intrinsic lipid effector of the interferon antiviral response and suppresses viral infection for a broad range of viruses including zika virus.[13–16] In addition, 25-HC significantly reduced LPS-induced inflammatory response through interaction with myeloid differentiation protein 2.[17]

In this study, we have undertaken further investigation on the pathophysiological role of 25-HC in X-ALD and revealed significant reduction of VLCFA (C26:0) by exogenous addition of 25-HC.

Exogenous addition of 25-HC significantly reduced the level of VLCFA in CCALD patient-derived fibroblasts (CCALD-fibroblast), as shown in Figure 1. When CCALD-fibroblasts were treated with 25-HC, a significant reduction in C26:0 was observed.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Changes in C26:0/C22:0 by 25-HC treatment. a) C26:0/C22:0 ratio was reduced by adding 25-HC at indicated concentrations in CCALD and AMN fibroblasts. b) Addition of 25-HC reduced the level of C26:0/C22:0 ratio in oligodendrocytes differentiated from patient-derived iPSCs. The fibroblasts and oligodendrocytes were treated with 25-HC for 3 days. Data are shown as mean from three independent experiments \( \pm \) S.D. \((n = 3)\). *p < 0.05, **p < 0.01, one-way ANOVA with Tukey’s post-hoc test.
treated with 1 μM of 25-HC, significant reduction of C26:0/C22:0 ratio was observed. Further, the VLCFA levels decreased in a concentration-dependent manner, such that the higher the concentration of 25-HC, the greater the decrease in VLCA levels. This reduction in VLCA by 25-HC addition was consistently observed in adrenomyeloneuropathy (AMN) patient-derived fibroblasts and oligodendrocytes (CCALD-oligodendrocytes) differentiated from induced pluripotent stem cells (iPSC) derived from CCALD patients.

To determine the effect of endogenous 25-HC on VLCA levels, CH25H overexpression and knockdown experiments were conducted in CCALD- and AMN-fibroblasts. As shown in Figure 2, ectopic expression of CH25H led to a slight decrease of VLCA. The overexpression of CH25H did not result in great changes in the VLCA level as compared to exogenous addition of 1 μM 25-HC, showing approximately 10% and 30% reductions, respectively. As such, it seems that 25-HC itself affects VLCA production more than CH25H. In contrast, knock-down of CH25H by siRNA resulted in significant increases of VLCA. These data suggest that endogenous 25-HC may contribute to suppression of VLCA accumulation. However, increased levels of VLCA are observed in X-ALD fibroblasts although 25-HC is upregulated.[11] This is possibly because 25-HC concentrations may not be elevated sufficiently to reduce VLCA levels. Alternatively, part of the endogenous 25-HC may exist in an inactive form unable to bind targets related to the reduction of VLCA. These data suggest that endogenous 25-HC may contribute to downregulation of CH25H expression level as compared to exogenous addition of 25-HC. Overexpression of CH25H by transfection of CH25H-EGFP, which was analyzed by quantitative real-time PCR. A dehydrated 25-HC, desmosterol, showed similar activity to 25-HC, but ether analogue (MSX-102) did not reduce C26:0 levels. Acetylated 25-HC (MSX101) showed similar activity to 25-HC, though much less than 25-HC (Figure 4). Analogs of 25-HC, MSX-101 and MSX-102 were examined whether exogenous addition of MSX-101 or inhibition of MSX-102 may lead to reduction of endogenous 25-HC, which can increase C26:0 levels.

Figure 2. Changes of C26:0/C22:0 ratio according to CH25H expression level in CCALD fibroblasts. a) mRNA expression level of CH25H by transfection of CH25H-EGFP, which was analyzed by quantitative real-time PCR. b) C26:0/C22:0 ratio under ectopic expression of CH25H. c) mRNA expression level of CH25H by transfection of CH25H or scramble siRNA. CH25H expression was reduced to approximately 60% after transfection with siRNAs against CH25H, which was analyzed by quantitative real-time PCR. d) C26:0/C22:0 ratio was significantly increased by the knockdown of CH25H. Data are shown as mean ± S.D. from two independent experiments (n = 3), **p < 0.01, Student’s t-test.

Figure 3. Relative mRNA expression levels of ELOVL1 under 5 and 10 μM of 25-HC and CH25H knockdown in CCALD fibroblasts. a) Addition of 5 μM and 10 μM of 25-HC for 3 days reduced expression level of ELOVL1. b) The knockdown of CH25H increased expression level of ELOVL1. The control was transfected with scrambled siRNA. All mRNA expression levels were analyzed by quantitative real-time PCR. Data are shown as mean ± S.D. from three independent experiments (n = 3), *p < 0.05, one-way ANOVA with Tukey’s post-hoc test.
To our knowledge, it has never reported that LXR are involved in biosynthesis of C26:0, although LXRs have been identified as key regulators of lipid metabolism. Expression of ELOVL1 is not be effected by LXR agonist T0901317. Therefore, it seems that 25-HC may act on multiple targets as well as LXR to reduce VLCFA levels. Further detailed investigations on mode of action of 25-HC are required. We believe that a novel therapeutic agent for X-ALD could be discovered from small molecules derived from 25-HC.

In summary, exogenous addition of 25-HC reduced levels of C26:0 in CCALD fibroblasts, AMN fibroblasts and oligodendrocytes differentiated from iPS cells derived from X-ALD patients. 25-HC was found to downregulate the expression of ELOVL1.

The LXR agonist T0901317 also exhibited a similar effect on C26:0 levels in X-ALD fibroblasts. Therefore, the activity of 25-HC may come at least partially from downregulation of ELOVL1 as well as LXR activation. Both of two hydroxy groups of 25-HC may be involved in target binding. We believe that our results could contribute to a better understanding of the role of 25-HC in X-ALD and to finding a therapeutic strategy for the treatment of X-ALD.

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**Conflict of interest**

The authors declare no conflict of interest.

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**Figure 4.** Effect of 25-HC analogs on C26:0/C22:0 ratio in in CCALD fibroblasts. Desmosterol showed significantly approximately 10% reduction of C26:0/C22:0 ratio. Activity of MSX101 was similar to 25-HC, whereas MSX102 was no active. Compounds were applied for 3 days. Data are normalized to control treated with DMSO and are shown as mean ± S.D. from three independent experiments (n = 3), *p < 0.05, **p < 0.01, one-way ANOVA with Tukey’s post-hoc test.

**Figure 5.** Effect of T0901317 on level of VLCFAs. Addition of T0901317 for 3 days reduced C26:0/C22:0 ratio in a) CCALD fibroblasts, b) AMN fibroblasts, and c) normal human dermal fibroblasts. Data are shown as mean ± S.D. from three independent experiments (n = 3), *p < 0.05; Student’s t-test.


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