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

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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, ≥ 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 β-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.[8,9] However, it did not show a significant reduction in C26:0 in a randomized, double-blind clinical trial.[10] 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 metabolite, 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*.[11] 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 neuroinflammation 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

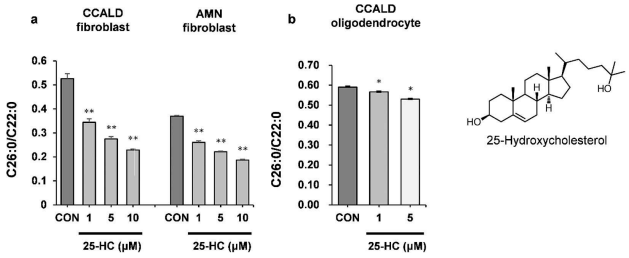

Figure 1. Changes in C26:0/C22:0 by 25-HC treatment. a) C26:0/C22:0 ratio was reduced by adding 25-HC at indicated concentrations in CCALD and AMN fibroblasts. b) Addition of 25-HC reduced the level of C26:0/C22:0 ratio in oligodendrocytes differentiated from patient-derived iPS cells. The fibroblasts and oligodendrocytes were treated with 25-HC for 3 days. Data are shown as mean from three independent experiments ± S.D. ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA with Tukey's post-hoc test.

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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 VLCFA levels. This reduction in VLCFA 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 VLCFA 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

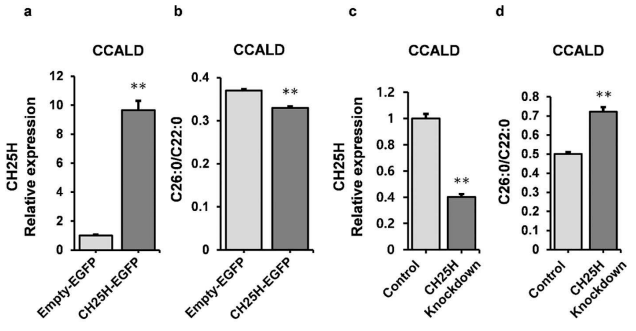

Figure 2. Changes of C26:0/C22:0 ratio according to *CH25H* expression level in CCALD fibroblasts. a) mRNA expression level of *CH25H* by transfection of CH25H-EGFP, which was analyzed by quantitative real time PCR. b) C26:0/C22:0 ratio under ectopic expression of *CH25H*. c) mRNA expression level of *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 ($n = 2$), $^{**}p < 0.01$, Student's *t*-test.

VLCFA. The overexpression of *CH25H* did not result in great changes in the VLCFA 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 VLCFA production more than *CH25H*. In contrast, knock-down of *CH25H* using siRNA resulted in significant increases of VLCFA. These data suggest that endogenous 25-HC may contribute to suppression of VLCFA accumulation. However, increased levels of VLCFA 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 VLCFA levels. Alternatively, part of the endogenous 25-HC may exist in an inactivated form unable to bind targets related to the reduction of VLCFAs, such as 5-cholesten-3β, 25-diol 3-sulfate (25HC3 S), a sulfated metabolite of 25-HC that acts in contrast to 25-HC in the expression of sterol regulatory element binding protein-1 (SREBP-1) and fatty acid synthase (FAS) in hepatocytes.[18]

Regarding studies on mode of action of 25-HC, we examined whether exogenous addition of 25-CH affects expression of VLCFA-specific elongase 1 (*ELOVL1*), which is the elongase responsible for C26:0 and 26:1 fatty acid synthesis.[19,20] It has been reported that knockdown[19] or inhibition

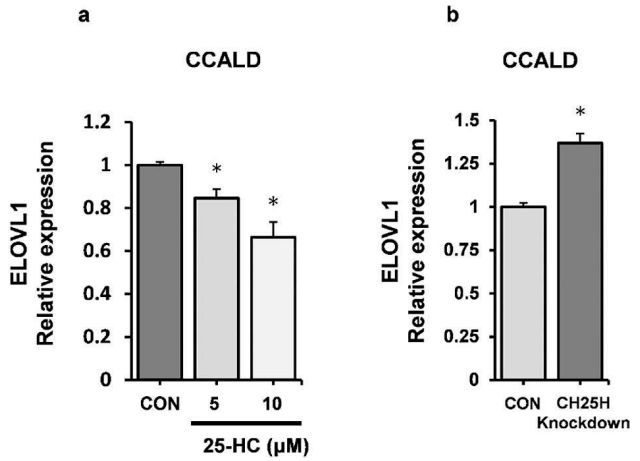

Figure 3. Relative mRNA expression levels of *ELOVL1* under 5 and 10 μM of 25-HC and *CH25H* knockdown in CCALD fibroblasts. a) Addition of 5 μM and 10 μM of 25-HC for 3 days reduced expression level of *ELOVL1*. b) The knockdown of *CH25H* increased expression level of *ELOVL1*. The control was transfected 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.

[20] of *ELOVL1* reduces C26:0 level in X-ALD fibroblasts. As shown in Figure 3 (Figure S1 for AMN fibroblasts), treatment of CCALD fibroblasts with 25-HC resulted in decrease of *ELOVL1* expression levels. Therefore, it seems that the effect of 25-HC on VLCFA levels comes, at least, partially from downregulation of *ELOVL1*. Moreover, the knockdown of *CH25H*, corresponding to reduced endogenous 25-HC, increased expression of *ELOVL1* (Figure 3b). These data suggest that downregulation of *CH25H* may lead to reduction of endogenous 25-HC, which can increase C26:0 levels.

To explore changes of VLCFA levels according to variation in the key functional group, hydroxy group, some analogues were evaluated. A dehydrated 25-HC, desmosterol showed activity, though much less than 25-HC (Figure 4). Analogs of 25-HC, MSX-101 and MSX-102 were evaluated in CCALD fibroblasts. Acetylated 25-HC (MSX101) showed similar activity to 25-HC, but ether analogue (MSX-102) did not reduce C26:0 levels, which has a tetrahydrofuran moiety for solubility (Figure 4). It appears that two alcohol moieties or their bioisosteres may take part in binding targets related to reduction of VLCFAs. Further investigations are required to clarify the structure-activity relationship of 25-HC.

Cholesterol and its many metabolites, including desmosterol and 25-HC have been known as liver X receptor (LXR) ligands.[21] Recently, Liu et al. reported that 25-HC induces expression of *CH25H* via activation of LXR.[22] Hence, a widely used potent LXR agonist, TO901317 was used to explore whether it could lower VLCFA levels. As shown in Figure 5, TO901317 significantly reduced VLCFA levels in CCALD and AMN fibroblasts. In addition, the LXR agonist showed reduction of VLCFA levels, even in normal human dermal fibroblasts containing relatively low levels of C26:0.

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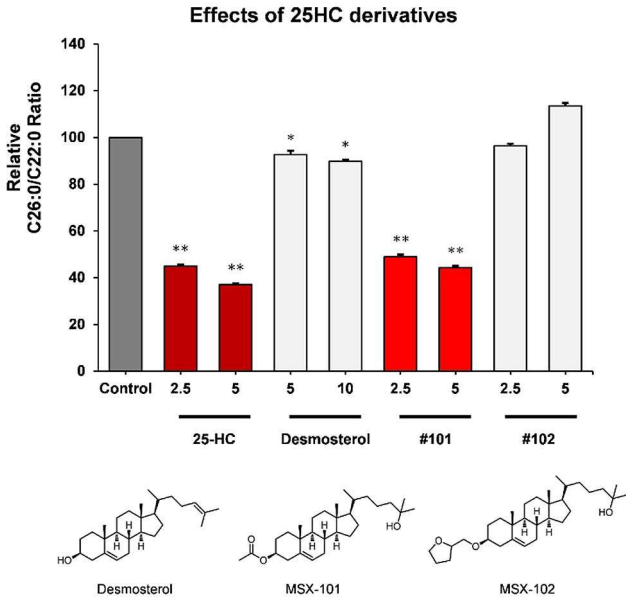

Figure 4. Effect of 25-HC analogs on C26:0/C22:0 ratio in in CCALD fibroblasts. Desmosterol showed significantly approximately 10% reduction of C26:0/C22:0 ratio. Activity of MSX101 was similar to 25-HC, whereas MSX102 was no active. Compounds were applied for 3 days. Data are normalized to control treated 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.

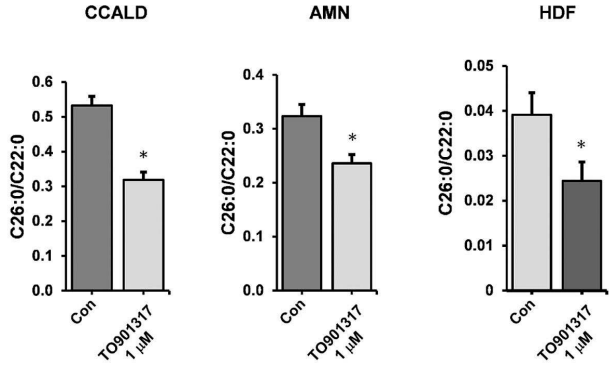

Figure 5. Effect of T0901317 on level of VLCFAs. Addition of T0901317 for 3 days reduced C26:0/C22:0 ratio in a) CCALD fibroblasts, b) AMN fibroblasts, and c) normal human dermal fibroblasts. Data are shown as mean ± S.D. from three independent experiments ($n = 3$), *$p < 0.05$, Student's *t*-test.

To our knowledge, it has never reported that LXRs are involved in biosynthesis of C26:0, although LXRs have been identified as key regulators of lipid metabolism.[23] Expression of *ELOVL1* is not be effected by LXR agonist T0901317.[24] 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.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Keywords: 25-hydroxycholesterol · X-linked adrenoleukodystrophy · very long chain fatty acids · liver X receptor · derivatives

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