Simvastatin does not normalize very long chain fatty acids in adrenoleukodystrophy mice

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Abstract X-linked adrenoleukodystrophy (ALD) is a genetic demyelinating disorder characterized by accumulation of very long chain fatty acid (VLCFA) in tissues. Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, normalizes VLCFA in fibroblasts and plasma from ALD patients. We dietary treated ALD mice with simvastatin, an analog of lovastatin with similar pharmacokinetics and effects on plasma VLCFA in ALD patients at 20 or 60 mg/kg/day for 6–12 weeks. No decrease of VLCFA content was observed in mouse tissues, including the brain. A significant increase of VLCFA was rather observed in the brain of ALD mice at 60 mg/kg/day.

Key words: Adrenoleukodystrophy; Very long chain fatty acid; Statin

1. Introduction

X-linked adrenoleukodystrophy (ALD) is characterized by progressive demyelination within the central nervous system, adrenal insufficiency and accumulation of very long chain fatty acids (VLCFA) in tissues and plasma due to an impairment of their β-oxidation in peroxisomes [1]. This results from reduced function of activation of VLCFA to their coenzyme A (CoA) derivatives [2] that involves a peroxisomal VLCFA-CoA synthetase and the protein (ALDP) encoded by the ALD gene. ALDP belongs to a family of ATP-binding cassette (ABC) hemi-transporters [3] that need to dimerize to exert their function. Three homologs of ALDP have been identified: the ALD-related (ALDR) protein (66% identity) [4,5], the more distantly related PMP70 protein (38% identity to ALDP) [6,7] and the PMP70-related (P70R) protein (27% identity to ALDP) [8,9]. Using the two-hybrid system and co-immunoprecipitation, we have recently demonstrated that ALDP forms homodimers and heterodimers with ALDRP and PMP70 and identified missense mutations that abolish the homo- and heterodimerizations of ALDP [10]. It is however unclear whether ALDP homodimers or heterodimers transport VLCFA (or VLCFA-CoA) into peroxisomes or a substrate necessary to VLACS activation.

The link between VLCFA accumulation and cerebral demyelination remains controversial [11]. Studies of phospholipid bilayers enriched in VLCFA suggest however that incorporation of VLCFA in components of the multilamellar myelin membrane might destabilize ALD myelin [12]. At present, no satisfactory therapy for ALD is available. Bone marrow transplantation can reverse or stabilize cerebral demyelination, provided that it is performed at an early stage of the disease [13]. This procedure is however associated with a high risk of mortality and has no effect in advanced form of the disease. Thus, other therapeutic strategies are needed, particularly those aiming at reducing the accumulation of VLCFA within the brain from ALD patients.

It was recently shown that lovastatin, a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor, suppresses the induction of inducible nitric oxide synthetase and proinflammatory cytokines (in particular tumor necrosis factor α) involved in the pathogenesis of cerebral demyelination in ALD [14]. Lovastatin was also shown to normalize the levels of VLCFA in fibroblasts [15] an plasma [16] from ALD patients, through activation of VLCFA oxidation activity. This prompted us to examine the in vivo effects of simvastatin, an analog of lovastatin which differs only by the addition of a methyl group, on the accumulation of VLCFA in tissues from ALD mice.

2. Materials and methods

2.1. Mice and dietary simvastatin treatment

Three to four-month-old ALD male mice and wild-type littermates on a C57BL/6×129 hybrid background [17] were fed by gavage with simvastatin at a dose of 20 mg or 60 mg/kg/day for 4 and 12 weeks. Simvastatin-treated and control groups of wild-type and ALD mice each consisted of four animals per timepoint. Total plasma cholesterol was measured by spectrophotometry using the oxidase method [17]. Statistical analyses were done using the Student *t*-test.

2.2. Analysis of VLCFA concentrations in tissues

Mice were killed by bleeding. Brain, adrenal glands, liver and heart were frozen and analyzed separately from each mouse. Frozen tissues were homogenized by sonication. Protein concentration was determined in aliquot from each sample using the method of Bradford [18]. VLCFA were then extracted and measured by gas/liquid chromatography/mass spectrometry (GLC/MS) as described [19]. [3,3,5,5-$^2H_4$]-Docosanoic (C22:0-d_4) and [3,3,5,5-$^2H_4$]-hexacosanoic (C26:0-d_4) acids (gift from Dr. H.J. ten Brink, Amsterdam, The Netherlands) were used as internal standard. Ions at *m/z* 397, *m/z* 401, *m/z* 453 and *m/z* 457 corresponding to the [M-57] ions of 22:0, 22:0-d_4, 26:0 and 26:0-d_4 TBDMS (butyldimethylsilyl) derivatives were selectively monitored using electron impact. The corresponding peaks were integrated and the content of C22:0 and C26:0 fatty acids was calculated according to a calibration curve using C22-d_4 and C26-d_4 as internal standards. Results were expressed as C26:0/C22:0 ratios and μg/mg protein for C26:0 concentrations. The GLC/MS method used in this study was previously shown to detect with sensitivity and reproducibility a difference of 10% in the absolute measure of C26:0 or C22:0 concentrations [19]. The coefficient of analytical variation was less than 1.55%. Statistical analyses were done using the Student *t*-test.

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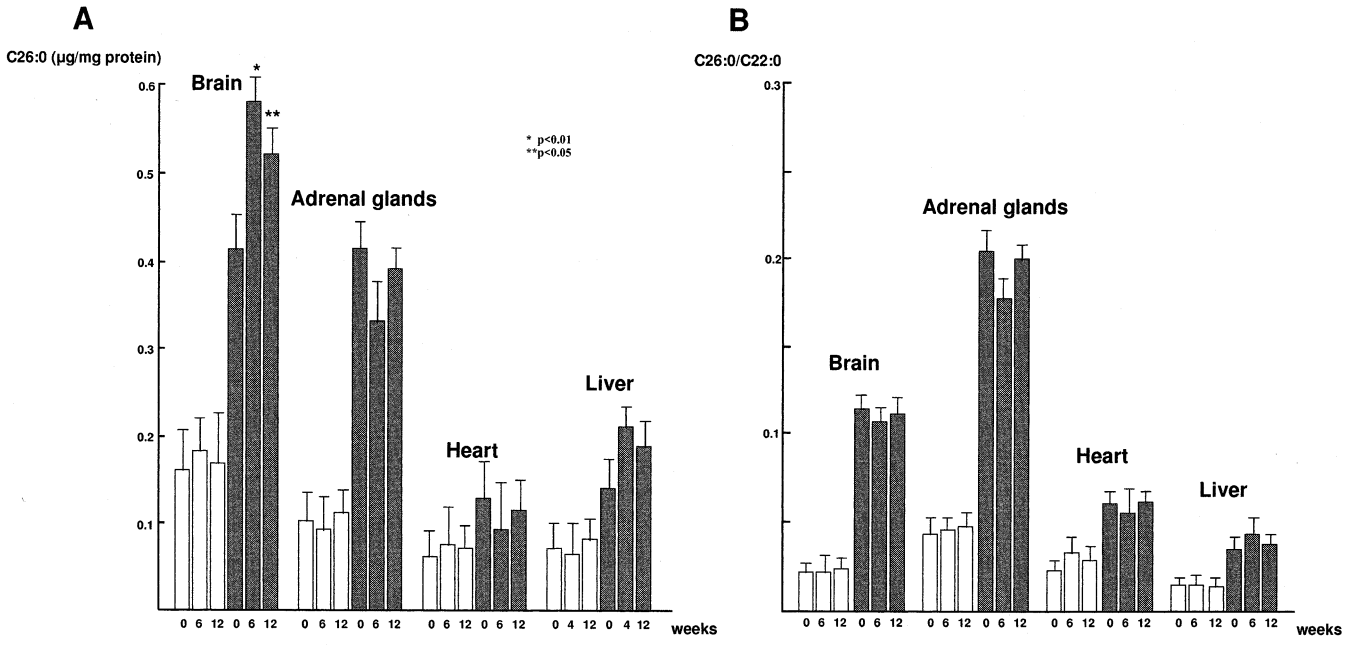

Fig. 1. C26:0 concentrations (A) and C26:0/C22:0 ratio in brain, adrenal glands, heart and liver from control wild-type (open bars) and ALD (hatched bars) mice before and after 6 and 12 weeks of simvastatin treatment at 20 mg/kg/day. Each bar represents the mean ± S.D. value of C26:0 levels or C26:0/C22:0 ratio of four animals. *P* values are given for tissues showing statistically significant changes.

3. Results

Treatment with 20 mg/kg/day of simvastatin for 6 or 12 weeks did not lead to significant difference in body weight or plasma cholesterol levels of ALD and control wild-type mice (Table 1). In contrast, treatment with 60 mg/kg/day of simvastatin led in ALD and control wild-type mice to a 16% ($P < 0.05$, Student's *t*-test) and 22.5% ($P < 0.01$) reduced body weight after respectively 6 and 12 weeks of treatment (Table 1). Plasma cholesterol levels decreased by 18% ($P < 0.05$) and 31% ($P < 0.01$) in ALD and control wild-type mice after 6 and 12 weeks of simvastatin treatment (Table 1). No statistically significant differences in body weight and plasma cholesterol levels were observed between ALD and control wild-type mice on treatment with 20 or 60 mg/kg/day of simvastatin.

Treatment with 20 mg/kg/day of simvastatin for 6 or 12 weeks did not normalize the C26:0 levels and C26:0/C22:0 ratio in brain, adrenal glands, heart and liver from ALD mice (Fig. 1). In fact, the C26:0 levels increased by 41% ($P < 0.01$) and 26% ($P < 0.05$) in the brain from ALD mice (Fig. 1A) without changes in the C26:0/C22:0 ratio (Fig. 1B) after treatment for 6 and 12 weeks. No significant changes of C26:0 levels and C26:0/C22:0 ratio was observed in other tissues.

Treatment with 60 mg/kg/day of simvastatin for 6 weeks produced an increase of C26:0 levels by 481% in the brain ($P < 0.001$), 58% ($P < 0.01$) in adrenal glands, 74% ($P < 0.01$) in the heart and 207% ($P < 0.001$) in the liver from ALD mice (Fig. 2A). The C26:0/C22:0 ratio increased by 17% ($P < 0.02$), 29% ($P < 0.01$) and 71% ($P < 0.001$) in brain, adrenal glands and liver without significant changes in heart (Fig. 2B), reflecting that simvastatin treatment increased also the C22:0 levels in these tissues. After 12 weeks of treatment, the C26:0 concentrations decreased but remained higher than at baseline. The C26:0 levels were still increased by 200% ($P < 0.001$), 21.5% ($P < 0.02$), 87% ($P < 0.001$) and 46% ($P < 0.01$) in brain, adrenal glands, heart and liver from ALD mice (Fig. 2A). The C26:0/C22:0 ratio was unchanged in heart and adrenal gland but was increased by 46% ($P < 0.001$) and 33% ($P < 0.01$) in the brain and liver. In control wild-type mice, treatment with 60 mg/kg/day of simvastatin led to no significant change in the C26:0/C22:0 ratio after 6 or 12 weeks of treatment, excepting the adrenal glands

Table 1
Effect of simvastatin on total plasma cholesterol and body weight in ALD and control mice

Treatment/duration	ALD mice		Control mice	
	cholesterol (mmol/l)	weight (g)	cholesterol (mmol/l)	weight (g)
20 mg/kg/day				
untreated ($n = 4$)	2.83 ± 0.26	22.8 ± 1.0	2.92 ± 0.30	22.5 ± 1.4
6 weeks ($n = 4$)	2.64 ± 0.22	22.6 ± 1.2	3.06 ± 0.21	21.5 ± 1.7
12 weeks ($n = 4$)	2.68 ± 0.28	21.8 ± 1.5	2.81 ± 0.31	20.5 ± 1.8
60 mg/kg/day				
untreated ($n = 4$)	2.83 ± 0.26	22.8 ± 1.0	2.92 ± 0.30	22.5 ± 1.4
6 weeks ($n = 4$)	2.32 ± 0.21[a]	19.0 ± 2.33[a]	2.39 ± 0.22[a]	18.9 ± 2.1[a]
12 weeks ($n = 4$)	1.94 ± 0.25[b]	17.6 ± 1.6[b]	2.01 ± 0.26[b]	17.5 ± 1.9[b]

[a] $P < 0.05$.
[b] $P < 0.01$.

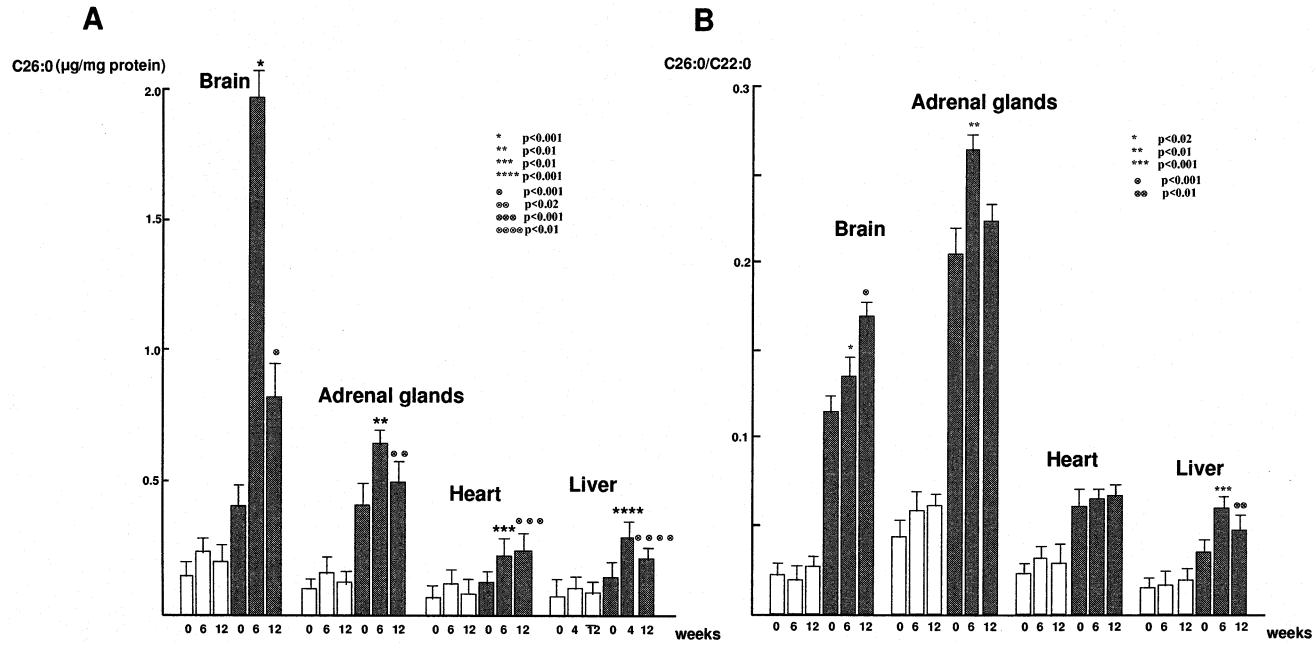

Fig. 2. C26:0 concentrations (A) and C26:0/C22:0 ratio in brain, adrenal glands, heart and liver from control wild-type (open bars) and ALD (hatched bars) mice before and after 6 and 12 weeks of simvastatin treatment at 60 mg/kg/day. Each bar represents the mean ± S.D. value of C26:0 levels or C26:0/C22:0 ratio of four animals. *P* values are given for ALD tissues showing statistically significant changes.

($P < 0.05$). The C26:0 levels remained unchanged after 12 weeks of treatment but were increased by 51% ($P < 0.01$), 49% ($P < 0.01$), 80% ($P < 0.001$) and 41% ($P < 0.01$) in brain, adrenal glands, heart and liver after 6 weeks of treatment (Fig. 2A).

Altogether, these data demonstrate that not only simvastatin did not lead to a substantial decrease in VLCFA concentration in tissues from ALD mice at any timepoint but that simvastatin led to a significant increase of VLCFA content in most tissues, particularly within the brain.

4. Discussion

Mice with a targeted disruption in the ALD gene lack a clinical phenotype but accumulate VLCFA in the brain and adrenal glands together with a decrease in VLCFA β-oxidation activity in vitro [20–22]. The 3–5-fold accumulation of VLCFA in the brain from ALD mice is similar to that observed in the brain from ALD patients at an early stage of the disease. This animal model is therefore suitable to evaluate the effect of drugs aiming at reducing VLCFA levels within the brain.

Several compounds were recently shown to lower VLCFA in ALD fibroblasts, including 4-phenylbutyrate [23], rolipram [24] and lovastatin [15]. Lovastatin and other related statins are most promising because lovastatin was shown to reduce the plasma levels of VLCFA in ALD patients [16], and statins are approved for therapeutic usage in hyperlipidemia [25] and the prevention of coronary heart disease [26]. Lorenzo's oil, a dietary therapy enriched in oleic and erucic acids, normalizes also plasma VLCFA of ALD patients within 4–6 weeks [27]. It has however no effect on the clinical course of the disease, perhaps because erucic acid, the main active ingredient of Lorenzo's oil, does not get into the brain. It is thus essential to determine whether lovastatin or related statins show efficacy in experimental models before engaging in human trials. We decided to evaluate the effect of simvastatin rather than that of lovastatin because the former drug is more readily available in different countries around the world. Simvastatin and lovastatin have similar pharmacokinetics and effects on cholesterol metabolism in human and mouse [28].

Our study demonstrates that simvastatin administered orally at 20 mg/kg/day or 60 mg/kg/day for 6–12 weeks does not correct the accumulation of VLCFA in the body tissues from ALD mice, including the brain. The effect of simvastatin on plasma VLCFA was not studied in ALD mice because this animal model, in contrast to ALD patients, does not accumulate VLCFA in blood. The accumulation of VLCFA in plasma reflects mainly the impaired capacity of hepatocytes to degrade them. A possible explanation accounting for the discrepancy between ALD patients and ALD mice is the absence of ALDP expression in mouse hepatocytes, in contrast to that observed in human hepatocytes [29]. An other related half-peroxisomal ABC transporter plays likely the function of ALDP in mouse hepatocytes.

The lack of efficacy of simvastatin to reduce tissue VLCFA levels is unlikely due to the requirement of higher doses than that required for lowering cholesterol levels. A significant decrease in the body weight of ALD and control wild-type mice, as well as in their plasma cholesterol levels was observed at a dose of 60 mg/kg/day, which is close to toxic levels.

Insufficient brain distribution of simvastatin could be an explanation for its absence of efficacy in this tissue. Simvastatin, as lovastatin, is a lipophilic prodrug which undergoes in vivo transformation into active hydroxy-acid forms [30]. Simvastatin, like lovastatin, is efficiently taken up by hepatocytes after oral administration [31]. In our experiments, simvastatin was unable to lower the levels of VLCFA in liver from ALD mice at 20 or 60 mg/kg/day, despite its high hepato selectivity. Although the brain selectivity of simvastatin (and lovastatin) is much less documented, it is therefore unlikely that the lack

of effect of simvastatin in brain was due to its low uptake by this tissue.

Our study demonstrates that simvastatin at 60 mg/kg/day increased significantly the levels of C26:0 and at a lesser extent the levels of C22:0 in most tissues from ALD mice. VLCFA accumulate predominantly in cholesterol ester fractions in ALD tissues. In normal human monocyte-derived macrophages loaded with cholesterol, lovastatin was shown to hinder the delivery of intracellular cholesterol to the plasma membrane, resulting in increased free cholesterol and lower levels of cholesteryl esters [32]. In non-loaded cells where virtually all cholesterol is of endogenous origin and normally translocated to the cell membrane, lovastatin prevented this process leading to an increase in the concentration of cholesteryl esters, even free and total cholesterol levels decreased. This study did not address the effects of lovastatin on VLCFA but demonstrated that saturated fatty acids were not reduced in loaded cells whereas unsaturated fatty acids increased at the highest dose (12 μM/l) of lovastatin in non-cholesterol-loaded cells. The in vivo intracellular effects of statins on VLCFA levels might thus critically be dependent on cholesterol loading of cells. The concentration of lovastatin used in these studies encompassed the plasma levels found in human subjects after standard oral administration and these effects probably apply to most if not all members of this drug class.

Although a preliminary study showed that lovastatin is able to normalize plasma VLCFA in ALD patients [16], it must be emphasized that changes in the plasma levels of VLCFA may not necessarily reflect levels of free/bound VLCFA within tissues, particularly the brain. In a therapeutic perspective in which the goal is precisely to lower VLCFA levels in the brain from ALD patients, our data support that one must be cautious on using statins in ALD patients. Additional experimental data will clarify whether these drugs may have eventually beneficial effects on brain VLCFA concentrations in ALD mice at different dosages than those used in this study.

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