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Peroxisomal cholesterol biosynthesis and Smith-Lemli-Opitz syndrome

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Abstract

Smith-Lemli-Opitz syndrome (SLOS), caused by 7-dehydrocholesterol-reductase (DHCR7) deficiency, shows variable severity independent of *DHCR7* genotype. To test whether peroxisomes are involved in alternative cholesterol synthesis, we used [1-^{14}C]C24:0 for peroxisomal β-oxidation to generate [1-^{14}C]acetyl-CoA as cholesterol precursor inside peroxisomes. The HMG-CoA reductase inhibitor lovastatin suppressed cholesterol synthesis from [2-^{14}C]acetate and [1-^{14}C]C8:0 but not from [1-^{14}C]C24:0, implicating a peroxisomal, lovastatin-resistant HMG-CoA reductase. In SLOS fibroblasts lacking DHCR7 activity, no cholesterol was formed from [1-^{14}C]C24:0-derived [1-^{14}C]acetyl-CoA, indicating that the alternative peroxisomal pathway also requires this enzyme. Our results implicate peroxisomes in cholesterol biosynthesis but provide no link to phenotypic variation in SLOS.

Keywords: Peroxisome; Cholesterol synthesis; Smith-Lemli-Opitz syndrome

For many years, the peroxisome has been speculated to be a source of alternative cholesterol biosynthesis [1]. This is based on (a) reports demonstrating additional peroxisomal localization of a number of cholesterogenic enzymes [2–4] and (b) the finding that some steps of cholesterol biosynthesis, e.g., the conversion of lanosterol to cholesterol, could also be performed by peroxisomes [5,6]. In addition, disturbed cholesterol homeostasis has been found in peroxisome-deficient *Pex2* knockout mice [7]. However, contradictory results have been published on this subject including reports demonstrating that peroxisome deficiency is not accompanied by a reduction of cholesterol levels in Pex5-deficient mice, another mouse model lacking peroxisomes [7–10]. In view of these discrepancies, the role of peroxisomes in cholesterol biosynthesis is not clear.

Cholesterol is an essential structural component of cellular membranes and serves as the starting point for the production of steroid hormones, bile acids, and oxysterols. These different important functions necessitate that cholesterol homeostasis is tightly regulated and the availability of cholesterol for usage in variable pathways needs to be controlled independently. The obvious advantage of cholesterol biosynthesis in peroxisomes would be the compartmentalization provided by an additional independently regulated pathway for special requirements. It could be speculated that this would result in two complete separate pools of cholesterol to be used for different purposes, e.g., cholesterol derived from the endoplasmatic reticulum (ER) for usage as membrane constituent and cholesterol synthesized in peroxisomes for production of steroid hormones. Intriguingly, this hypothesis of a specialized peroxisomal pathway would explain why overt adrenal insufficiency is uncommon in patients with the cholesterol biosynthesis defect Smith-Lemli-Opitz syndrome (SLOS, OMIM 270400).

SLOS is an autosomal recessive disorder of cholesterol biosynthesis that is caused by mutations in the gene *DHCR7* encoding 7-dehydrocholesterol reductase, which

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reduces 7-dehydrocholesterol to cholesterol in the final step of cholesterol biosynthesis [11–13]. SLOS manifests as a broad spectrum of phenotypic abnormalities including severe craniofacial and limb malformations, as well as incomplete development of the male genitalia [14]. Additionally, SLOS presents with variable plasma sterol levels even between patients carrying the same *DHCR7* mutation [15,16], suggesting that additional genetic or environmental modifiers are involved. Next to alterations in cholesterogenic gene expression and maternal factors like apolipoprotein E (*ApoE*) genotype [17], the existence of an alternative pathway of cholesterol biosynthesis, not requiring DHCR7, has been suggested [18]. Accordingly, *Dhcr7*-deficient mice present cholesterol in the brain [19,20], a finding that may not be explainable by maternal transfer of cholesterol during pregnancy as de novo synthesis was demonstrated to be the only source of fetal brain cholesterol [21].

In contrast to the conversion of lanosterol to cholesterol in the last steps of cholesterol biosynthesis, the preceding steps of producing lanosterol from farnesyl-diphosphate are believed to occur exclusively at the ER, as the involved enzymes have been solely localized to this cellular compartment. Accordingly, it could be speculated that cholesterol biosynthesis proceeds in peroxisomes until the formation of farnesyl-diphosphate, which then leaves the peroxisome to be used for cholesterol synthesis at the ER. However, as peroxisomes have been demonstrated to be able to produce cholesterol from lanosterol, a mechanism for efficiently transporting this intermediate from the ER back to peroxisomes has been suspected [5,6].

A number of reports have addressed the contribution of peroxisomes to cholesterol biosynthesis in cultured cells [22–24]. In these studies, radioactively labeled acetate was used as a precursor for cholesterol synthesis. However, when considering the extensive use of acetate in different metabolic reactions, e.g., citric acid cycle and fatty acid synthesis, the net-flux of labeled acetate into cholesterol biosynthesis is expected to be low and should even further decrease for putative peroxisomal cholesterol biosynthesis. In addition, to our knowledge no mechanism has been described explaining the transport of acetyl-CoA units into peroxisomes. To circumvent this low amount of labeled peroxisomal acetyl-CoA, we directly generated acetyl-CoA units in peroxisomes using radioactively labeled lignoceric acid (C24:0) as substrate. C24:0 is a very long-chain fatty acid that is degraded exclusively by the peroxisomal fatty acid β-oxidation system, thus providing peroxisomal acetyl-CoA that could be efficiently used for cholesterol synthesis.

Using this approach of targeting labeled acetyl-CoA as substrate for cholesterol biosynthesis directly to the peroxisome, our work aimed (a) to determine whether peroxisomes are involved in cholesterol biosynthesis and (b) to elucidate whether a peroxisomal pathway of cholesterol biosynthesis is dependent on the presence of DHCR7, the enzyme deficient in SLOS patients.

Materials and methods

Cell culture. The monkey kidney Cos-7 cell line (CRL-1651, ATCC) and human primary skin fibroblasts derived from a healthy control (630/95, kindly provided by Dr. B. Molzer, Medical University of Vienna, Austria) or a SLOS patient with the IVS8-1G>C/W151X *DHCR7* genotype and no detectable residual DHCR7 [25] were cultivated in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 100 μg/ml streptomycin (Biowhittaker). For cholesterol depletion, standard FCS was substituted with 10% lipoprotein-deficient (LPD) FCS (Sigma). Lovastatin (VWR) was dissolved in DMSO and used at a concentration of 50 μM for 24 h.

Measurement of cholesterol biosynthesis. To assess cholesterol biosynthesis, 3.7×10^5 cells (Cos-7 or primary fibroblasts) were incubated in DMEM containing 10% LPD-FCS for 24 h. Subsequently, the medium was changed to DMEM containing 10% LPD-FCS with added [1-^{14}C]C24:0 (0.1 μM, 53.1 mCi/mmol, ARC); [1-^{14}C]C8:0 (0.1 μM, 55 mCi/mmol, ARC) or [2-^{14}C]acetate (0.1 μM, 56.7 mCi/mmol, ARC) and cells were incubated for another 24 h. After washing cells twice with ice-cold buffer A (5 mM Tris–HCl, 150 mM NaCl, and 0.2% BSA, pH 7.4) and twice with buffer B (buffer A without BSA), cells were lysed and aliquots taken for Bradford-based protein estimation (Bio-Rad Protein Assay). Lipids were saponified with ethanol/75% KOH, at 120 °C for 45 min including a [^{3}H]cholesterol thin-layer chromatography (TLC) recovery standard. For measurement of cholesterol biosynthesis in Cos-7 cells, the extracted lipids were separated by TLC with chloroform as a mobile phase. Lipid spots were visualized using iodine vapor and identified by co-chromatography with known standards. After excision of the cholesterol containing band, radioactivity was quantitated by liquid scintillation counting. For studies involving SLOS fibroblasts, extracted lipids were separated on AgNO3-coated TLC plates (Macherey-Nagel) with chloroform/acetone (9:1) as a mobile phase, in order to differentiate between cholesterol and 7-dehydrocholesterol. Lipid spots were identified by co-chromatography with known standards (cholesterol, lathosterol, and 7-dehydrocholesterol) and visualized by exposure to a phosphoimager screen (Bio-Rad).

Results

Cholesterol synthesis from peroxisomally generated acetyl-CoA is not significantly inhibited by statin treatment

The entire cholesterol biosynthesis is initiated from the two-carbon acetate group of acetyl-CoA that among others can be generated from β-oxidation of short, medium, and long or very long-chain fatty acids in mitochondria or peroxisomes, respectively. Thus, we first tested whether acetyl-CoA generated in peroxisomes or mitochondria could be used for cholesterol biosynthesis. Cos-7 cells were incubated with either very long-chain fatty acid [1-^{14}C]C24:0 or medium-chain fatty acid [1-^{14}C]C8:0 or with [2-^{14}C]acetate and cholesterol synthesis was measured using TLC and liquid scintillation counting. The [1-^{14}C]acetyl-CoA produced by degradation of [1-^{14}C]C24:0 or [1-^{14}C]C8:0 was utilized for cholesterol biosynthesis (Fig. 1a). In these experiments, [1-^{14}C]acetyl-CoA originating from fatty acid β-oxidation was used with equal or even higher efficiency than [2-^{14}C]acetate-derived [^{14}C]acetyl-CoA (Fig. 1a). However, next to differences in cellular uptake of the precursors, [1-^{14}C]acetyl-CoA is diluted with unknown amounts of unlabeled acetyl-CoA in the peroxisomal and cellular compartment. Thus, it is not possible to exactly

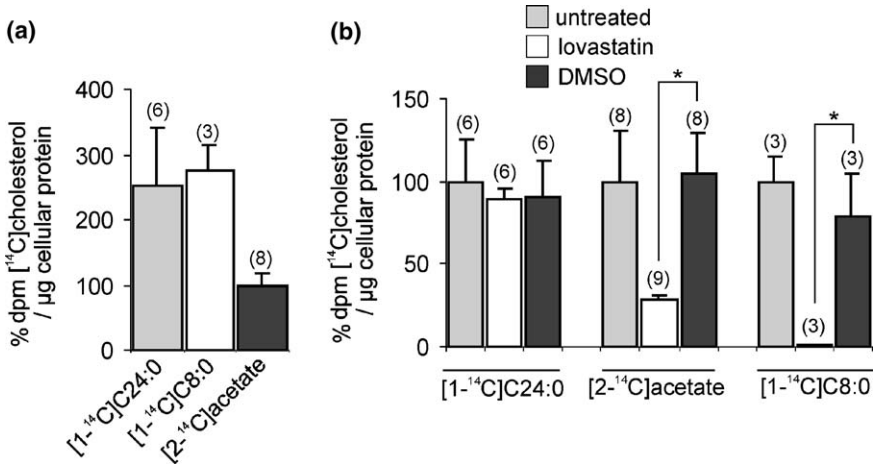

Fig. 1. Cholesterol biosynthesis from [1-^{14}C]C24:0-derived acetyl-CoA is not impaired by statin treatment. (a) Cos-7 cells were incubated with either [1-^{14}C]C24:0, [2-^{14}C]acetate or [1-^{14}C]C8:0 and (b) treated with 50 μM lovastatin or the solvent DMSO for 24 h. To assess cholesterol biosynthesis, lipids were extracted from cell lysates, separated by TLC and the radioactivity of the cholesterol fraction was quantified by liquid scintillation counting. Results were normalized to cellular protein content and to an internal [^{3}H]cholesterol recovery standard. Data are shown as means ± SD with the number of analyzed samples in parentheses. Statistically significant differences (Student's *t*-test) are indicated by asterisks ($p < 0.05$).

determine the quantitative differences between cholesterol biosynthesis from [1-^{14}C]fatty acid or [2-^{14}C]acetate-derived [^{14}C]acetyl-CoA.

[1-^{14}C]Acetyl-CoA produced by degradation of [1-^{14}C]C24:0 is utilized for cholesterol biosynthesis. From this, it cannot be excluded that [1-^{14}C]acetyl-CoA units generated by peroxisomal β-oxidation are transported out of the peroxisome and predominantly used at the ER for cholesterol synthesis. Thus, we next examined whether the rate limiting step in cholesterol synthesis, the conversion of HMG-CoA to mevalonate catalyzed by HMG-CoA reductase (HMGCR), occurs also in peroxisomes. Besides the well-characterized ER-localized HMGCR, a second HMGCR was described in peroxisomes [26]. This enzyme was less sensitive to treatment with the HMGCR inhibitor lovastatin than the ER-localized enzyme [26]. As a consequence, cholesterol production from [1-^{14}C]C24:0 should be to a lower extent inhibited by lovastatin than cholesterol synthesis from [1-^{14}C]acetyl-CoA units derived from [2-^{14}C]acetate or generated in the mitochondria by degradation of the short-chain fatty acid [1-^{14}C]C8:0. Treatment of Cos-7 cells with lovastatin did not inhibit cholesterol biosynthesis from [1-^{14}C]C24:0 but from [2-^{14}C]acetate and [1-^{14}C]C8:0 (Fig. 1b). These results support the idea that peroxisomally generated [1-^{14}C]acetyl-CoA units do not leave the peroxisome but are rather converted to HMG-CoA and mevalonate inside peroxisomes.

SLOS fibroblasts with no residual DHCR7 activity cannot produce cholesterol from peroxisomally derived acetyl-CoA

Our experiments suggest that the first part of cholesterol biosynthesis including production of mevalonate can also be carried out within peroxisomes. To elucidate whether also proceeding steps including synthesis of cholesterol from 7-dehydrocholesterol do occur in peroxisomes and whether this pathway is independent of the ER-localized enzyme DHCR7, we next tested if cholesterol is produced in SLOS fibroblasts lacking DHCR7 activity. As expected, no ^{14}C-labeled cholesterol was formed in this cell line after incubation with [2-^{14}C]acetate (Fig. 2a). When cells were incubated with [1-^{14}C]C24 as precursor, again no ^{14}C-labeled cholesterol was produced (Fig. 2b). This indicates that cholesterol biosynthesis from acetyl-CoA units generated within peroxisomes is dependent on the presence of a functional DHCR7.

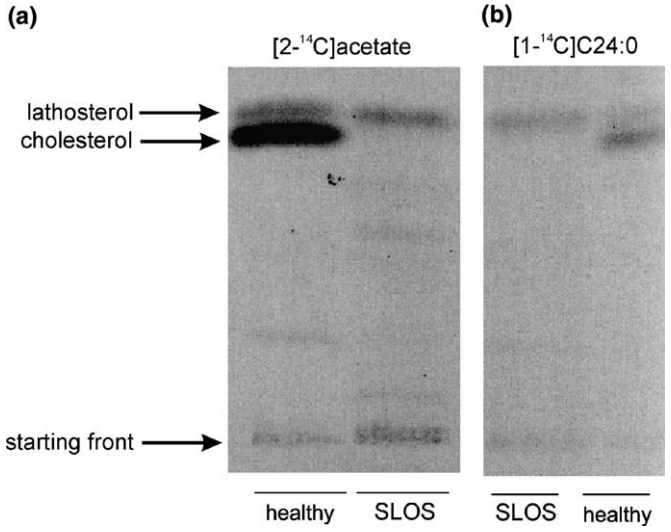

Fig. 2. Cholesterol biosynthesis from [2-^{14}C]acetate or [1-^{14}C]C24:0 in 7DHCR-deficient SLOS fibroblasts. SLOS fibroblasts lacking residual DHCR7 activity or healthy control fibroblasts were incubated with either (a) [2-^{14}C]acetate or (b) [1-^{14}C]C24:0 for 24 h. To assess cholesterol biosynthesis, lipids were extracted from cell lysates, separated by $AgNO_3$-TLC with chloroform/acetone (9:1) as the mobile phase, and directly visualized by exposure to a phosphoimager screen. Migration position of standards is indicated to the left.

Discussion

Peroxisomes catalyze a large variety of different biochemical pathways, most of which are associated with lipid metabolism; next to β-oxidation of fatty acids, steps in plasmalogen synthesis, biosynthesis of docosahexaenoic acid, and bile acid production have been well established to occur in peroxisomes. It has been claimed for more than 15 years but still not conclusively demonstrated that peroxisomes additionally could have a role in de novo cholesterol synthesis. Using a novel approach to generate acetyl-CoA as a substrate for cholesterol synthesis directly in the peroxisome by incubating cells with labeled C24:0 as precursor, we have sought to determine whether peroxisomes are involved in cholesterol biosynthesis. Additionally, we investigated whether such an alternative pathway of cholesterol synthesis is independent of DHCR7 enzymatic activity and thus, could at least in part account for the phenotypic variability observed in patients with the cholesterol biosynthesis disorder SLOS.

In the present work, we show that acetyl-CoA derived from peroxisomal β-oxidation of the very long-chain fatty acid, C24:0, is used for endogenous cholesterol synthesis. This result is in line with other observations demonstrating that the radioactivity of [1-^{14}C]C24:0 injected into rats was also found in the hepatic cholesterol fraction [27]. In contrast to mitochondrial β-oxidation, which has metabolic significance by providing acetyl-CoA for, e.g., fatty acid synthesis or energy production by degradation in the citric acid cycle, the physiological importance of acetyl-CoA produced by peroxisomal β-oxidation is not yet completely understood. Accordingly, it could be speculated that peroxisomal β-oxidation degrades fatty acids with the additional function to provide acetyl-CoA for the production of functional lipids, e.g., cholesterol and thus, is of anabolic significance.

Earlier reports suggested the existence of a peroxisomal HMGCR that is significantly more resistant to inhibition by statin treatment compared with the ER-localized enzyme [26]. Here we show that lovastatin suppresses cholesterol biosynthesis from [2-^{14}C]acetate and [1-^{14}C]C8:0 but not from [1-^{14}C]C24:0 as precursor. This indicates that [1-^{14}C]acetyl-CoA units generated by peroxisomal β-oxidation do not leave the peroxisome but rather are used for production of cholesterol intermediates inside peroxisomes. Furthermore, these results show that after statin treatment, the conversion of HMG-CoA to mevalonate cannot be catalyzed by the lovastatin-sensitive HMGCR localized to the ER and implicates the presence of a peroxisomal, thus lovastatin-resistant HMGCR activity. It has been suggested that these two enzymatic fractions originate from one gene through alternative splicing and targeting to the ER and peroxisome by an as yet uncharacterized mechanism [28].

Alternative peroxisomal cholesterol biosynthesis would explain phenotypic variations observed in SLOS patients with the same *DHCR7* genotype and would account for measurable amounts of cholesterol in the fetal brain of *Dhcr7*-deficient mice. Thus, we investigated whether SLOS fibroblasts lacking DHCR7 activity are able to produce cholesterol from acetyl-CoA generated in peroxisomes. Our results show that DHCR7 is required for cholesterol synthesis using either acetate or C24:0-derived acetyl-CoA as precursor, indicating that at least in cultured fibroblasts, an alternative peroxisomal pathway is dependent on this enzyme.

In summary, our results suggest that the first part of cholesterol biosynthesis including production of mevalonate from HMG-CoA is additionally carried out in peroxisomes. In addition, we provide evidence that excludes peroxisomes as a source of residual cholesterol biosynthesis that could contribute to the phenotypic variation observed in SLOS patients.

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