Very Long Chain Fatty Acid β-Oxidation in Astrocytes: Contribution of the ABCD1-Dependent and -Independent Pathways

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Very long chain fatty acid (VLCFA) metabolism in astrocytes is important for the maintenance of myelin structure in central nervous system. To analyze the contribution of the ABCD1-dependent and -independent pathways to VLCFA metabolism in astrocytes, we prepared human glioblastoma U87 cells with a silencing of ABCD1 and primary astrocytes from abcd1-deficient mice, and measured fatty acid β-oxidation in the presence or absence of a potent inhibitor of carnitine palmityltransferase I, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA). In U87 cells, C24:0 β-oxidation was decreased to ca. 70% of the control in the presence of POCA, and the activity was further decreased to ca. 20% by the silencing of ABCD1. In mouse primary astrocytes, C24:0 β-oxidation was also decreased to ca. 70% of the control in the presence of POCA. The C24:0 β-oxidation in Abcd1-deficient primary astrocytes was ca. 60% of the wild-type cells and the activity was further decreased to ca. 25% in the presence of POCA. Compared to human skin fibroblasts, in which VLCFA β-oxidation is not significantly inhibited by POCA, approximately one-third of the overall VLCFA β-oxidation was inhibited in both types of astrocytic cells. These results suggest that VLCFA is indeed β-oxidized in ABCD1-dependent pathway, but the ABCD1-independent peroxisomal and mitochondrial β-oxidation pathways significantly contribute to VLCFA β-oxidation in astrocytic cells.

Key words very long chain fatty acid β-oxidation; astrocyte; ABCD1; peroxisome; mitochondria

Peroxisomes are single membrane organelles that are present in almost all eukaryotic cells. The peroxisomes are involved in a variety of metabolic processes, including the β-oxidation of fatty acids, especially very long chain fatty acids (VLCFA, >C22), and the synthesis of ether phospholipids and bile acids in mammals.1) Transport of substrates for fatty acid β-oxidation across the peroxisomal membrane is an essential step in this metabolism. Peroxosomal ATP-binding cassette (ABC) proteins have been implicated in this transport.

To date, three ABC proteins, classified into “subfamily D,” have been identified in mammalian peroxisomes. These are adrenoleukodystrophy protein (ALDP/ABCD1),2) ALDP-related protein (ALDRP/ABCD2)3) and a 70-kDa peroxisomal membrane protein (PMP70/ABCD3).4) Dysfunction of ABCD1 causes the human genetic disorder X-linked adrenoleukodystrophy (X-ALD)5) characterized by an accumulation of very long chain fatty acid (VLCFA) metabolism in astrocytes is important for the maintenance of myelin structure in central nervous system. Fatty acid β-oxidation may provide acetyl-CoA for the demyelination. Astrocytes were also reported to be enriched in the lipid synthetic pathway, including cholesterol and docosahexaenoic acid (DHA) synthesis, and to supply these lipids to other neural cells for myelination and synaptogenesis. Fatty acid β-oxidation may provide acetyl-CoA for de novo fatty acid and cholesterol synthesis in the brain. It is thus likely that VLCFA β-oxidation in astrocytes has an important role in remyelination and the maintenance of the myelin structure in the CNS.

In the present study, to characterize the impact of ABCD1 on overall VLCFA β-oxidation, we examined the VLCFA β-oxidation pathway using human glioblastoma U87 cells with a silencing of ABCD1 and primary astrocytes from abcd1-deficient mice. To distinguish between peroxisomal and mitochondrial β-oxidation, we measured the activities under the condition in which mitochondrial β-oxidation was inhibited by 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA), a potent inhibitor of carnitine palmityltransferase I. Our results corroborate ABCD1 contributes to peroxisomal fatty acid oxidation pathways.

References

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\(\beta\)-oxidation, but the ABCD1-independent peroxisomal and mitochondrial \(\beta\)-oxidation pathways also significantly contribute to VLCFA \(\beta\)-oxidation in astrocytes. The ABCD1-independent pathway in astrocytes is a potential therapeutic target in X-ALD.

MATERIALS AND METHODS

**Materials** [\(1^{14}\)C]Lignoceric acid (53 mCi/mmol) and [\(1^{14}\)C]palmitic acid (53 mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA, U.S.A.). The pcDNA4/HisMax TOPO TA Expression Kit was from Invitrogen (Carlsbad, CA, U.S.A.). Antipain, chymostatin, leupeptin, and pepstatin A were from the Peptide Institute (Osaka, Japan). The MessageMuter shRNAi Production kit was from EPICENTRE. FibrOut (System 4, Mouse) was purchased from Tika (Konstanz, Germany). ECL Plus, a Western blotting ECLPlus was purchased from Byk Gulden Pharmazeu- 

**Primary Culture of Mouse Astrocytes** Wild-type mice (C57BL6) and abcd1-deficient mice (kindly provided by Dr. N. Hashimoto, National Institute for Longevity Sciences, Kokufu, Japan) were maintained at the animal facility of University of Hashimoto, National Institute for Longevity Sciences, Kokufu, Japan. Mouse wild-type ABCD1 and abcd1-de 

**Human Fibroblasts and a Glioblastoma Cell Line** Human glioblastoma U87 cells from the American Type Culture Collection were cultured in Eagle's minimal essential medium (EMEM) (Nissui, Tokyo, Japan) containing 10% foetal calf serum (FCS). Human skin fibroblasts (kindly provided by Dr. T. Yamada (Kyushu University, Japan).

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glioblastoma U87 cells were seeded on dishes containing coverslips in EMEM with 10% FBS. The cells on the coverslips were washed three times in PBS and fixed with 3% formaldehyde in PBS for 20 min. The fixed cells were made permeable with a solution of 1% Triton X-100 in PBS for 5 min and immediately rinsed with PBS. The cells were incubated with primary antibodies followed by secondary antibodies. The samples were examined under fluorescence microscopy (Olympus, AX80TRF-65). The protein concentration was determined by the Lowry method using bovine serum albumin as the standard. The p values were calculated using unpaired Student’s t-test. A p-value of <0.05 was used as the criterion for statistical significance.

RESULTS

The VLCFA β-oxidation Capacity of Mitochondria and Peroxisomes in Astrocytic Cells In human skin fibroblasts, degradation of VLCFA and LCFA are known to be largely dependent on the peroxisomal and mitochondrial fatty acid β-oxidation pathway, respectively.11 To test the contribution of peroxisomes and mitochondria to the degradation of C24:0 in astrocytic cells, we first examined the effect of the mitochondrial respiratory chain inhibitors antimycin A/rotenone on the β-oxidation activities in U87 cells along with human skin fibroblasts as control. As shown in Fig. 1, in the presence of antimycin A/rotenone (0.2 μg/mL), the C24:0 β-oxidation activity in human skin fibroblasts was decreased by only 10% under conditions where the C16:0 β-oxidation activity decreased by 80%, confirming previous observations that VLCFA and LCFA were selectively oxidized in peroxisomes and mitochondria, respectively.11 On the other hand, in the presence of antimycin A/rotenone, the C24:0 β-oxidation activity in U87 cells decreased by 40% under the conditions where C16:0 β-oxidation activity decreased by 90%. These data suggest that mitochondria are involved in VLCFA β-oxidation in U87 cells.

Respiratory chain inhibitors such as antimycin A and rotenone might have a nonspecific effect on peroxisomal fatty acid β-oxidation by the reduction of the cellular ATP or NADH level.29 Therefore, we next used POCA, a potent inhibitor of carnitine palmitoyltransferase I.27 When we examined the effect of POCA on the C16:0 β-oxidation activity in U87 cells and mouse primary astrocytes, the activity decreased progressively with the increases in the concentrations of POCA up to 25 μM, and the C16:0 β-oxidation activity was decreased by approximately 80% and 70% in U87 cells and mouse primary astrocytes, respectively (Figs. 2A, C). However, even at the higher concentrations of POCA, the C16:0 β-oxidation activity was observed in these cells, indicating that 25 μM POCA is sufficient for the inhibition. We then measured C24:0 β-oxidation activity in the presence of 25 μM of POCA. As shown in Figs. 2B and D, the C24:0 β-oxidation activity was decreased by ca. 30% in both U87 cells and mouse primary astrocytes, which were more weakly inhibited compared with the result shown in Fig. 1. Under the same conditions, the C24:0 β-oxidation in human fibroblasts was only slightly inhibited (ca. 15%) (data not shown). Taken together, in these astrocytic cells, mitochondria contribute approximately 1/3 of the overall C24:0 β-oxidation and the mitochondrial contribution is higher than that in human skin fibroblasts.

Fig. 1. Effect of Antimycin A/Rotenone on Fatty Acid β-Oxidation in Human Glioblastoma U87 Cells and Human Skin Fibroblasts

The fatty acid β-oxidation was measured in the presence or absence of antimycin A/rotenone at the final concentration of 0.2 μg/mL. Antimycin A and rotenone, in ethanol solution, were co-evaporated under N2 gas with the fatty acid substrate and mixed with the reaction mixture. C16:0 and C24:0 β-oxidation activities in U87 cells (4.4±0.44, 0.70±0.036 nmol/mg h, respectively) and skin fibroblasts (3.1±0.19, 1.2±0.057 nmol/mg h, respectively) were represented as 100%. A similar result was observed in the presence of the inhibitors at concentrations up to 20 μg/mL.

Contribution of ABCD1 to VLCFA β-Oxidation in U87 Cells Our laboratory and others have reported that in X-ALD fibroblasts the C24:0 β-oxidation activity is decreased by ca. 70%, while the C16:0 β-oxidation activity is unaffected.27 Therefore, ABCD1 is clearly involved in the VLCFA β-oxidation in these cells. To clarify the role of ABCD1 in peroxisomal VLCFA β-oxidation in astrocytic cells, we prepared U87 cells with a silencing of ABCD1 and analyzed the activity in the presence of POCA. When ABCD1-specific shRNA was transfected into U87 cells, the silencing effect continued for 5 d post-transfection, as shown by immunofluorescence analysis (Fig. 3A), and the expression of ABCD1 was reduced to less than 10% when compared with control (scramble) cells (Fig. 3B). Therefore, in the following experiments the β-oxidation activities were analyzed at 4 d post-transfection. As a positive control, we also silenced ACOX1, since this enzyme is responsible for the first step of the peroxisomal β-oxidation of VLCFA. When ACOX1-specific shRNA was transfected into U87 cells, the ACOX1 protein was also reduced to less than 20% at 4 d post-transfection (Fig. 3B).

As expected, C24:0 β-oxidation activity was reduced to ca. 30% by the silencing of ACOX1 as compared with control (scramble) in the presence of POCA (Fig. 3C). Similarly, by the silencing of ABCD1, C24:0 β-oxidation activity was decreased to ca. 30% of control. These results suggest that ABCD1 is mainly involved in peroxisomal C24:0 β-oxidation. Next, we overexpressed ABCD1 in fusion with histidine-tag (HisABCD1) in U87 cells and analyzed 24:0 β-oxidation activities in the presence of POCA. Immunoblotting studies showed that the expression level was approximately 3-fold that of endogenous ABCD1 (Fig. 4A). In HisABCD1-overexpressing cells, the C24:0 β-oxidation activity was increased by 35% (Figs. 4A, B). It thus seems likely that the endogenous expression level of ABCD1 is sufficient for peroxisomal VLCFA β-oxidation in U87 cells.

Contribution of Abcd1 to VLCFA β-Oxidation in Mouse Primary Astrocytes We next analyzed the contribution of
Abcd1 to peroxisomal fatty acid β-oxidation in mouse primary astrocytes. As shown in Fig. 5, the activity of C24:0 β-oxidation in Abcd1-deficient astrocytes was decreased by 40% of the activity in wild-type astrocytes in the absence of POCA. Further, the C24:0 β-oxidation in the Abcd1-deficient astrocytes was decreased by half in the presence of POCA. The results suggest that the Abcd1-dependent and -independent peroxisomal β-oxidation and mitochondrial pathways contribute equally to the overall VLCFA β-oxidation. In the case of the wild-type astrocytes, POCA decreased 24:0 β-oxidation activity by ca. 30%, roughly consistent with the decrease observed in the abcd1-deficient astrocytes.

**DISCUSSION**

VLCFA is a minor biogenic constituent, but it has an important role in the biogenesis of the myelin sheath. On the other hand, the accumulation of VLCFA in tissues induces a variety of pathological conditions. In X-ALD, it has been thought that ABCD1 dysfunction leads to a decrease in peroxisomal VLCFA β-oxidation, resulting in abnormal VLCFA accumulation in the brain and cerebral demyelination. Reducing VLCFA content in cerebral tissues is important to prevent the continued progress of X-ALD. Therefore, the stimulation of VLCFA β-oxidation by the ABCD1-independent pathway might be one of the targets for X-ALD therapy. As astrocytes seem to have an important role in the pathogenesis of X-ALD, we investigated the overall level of VLCFA β-oxidation and the contribution of ABCD1-dependent and -independent VLCFA β-oxidation pathways in astrocytic cells.

We attempted to distinguish between peroxisomal and mitochondrial VLCFA β-oxidation using mitochondrial inhibitors such as antimycin A/rotenone and POCA. The results clearly show the mitochondrial contribution to VLCFA β-oxidation in U87 cells and mouse primary astrocytes (Figs. 1, 2, 5). In addition, it is estimated that the contribution is roughly 1/3 of overall of the VLCFA β-oxidation in these cells. Under the same conditions, the C24:0 β-oxidation activity decreased only slightly in human skin fibroblasts. Other groups have also shown that VLCFA is oxidized exclusively in peroxisomes of human skin fibroblasts based on a similar VLCFA β-oxidation assay. Therefore unlike fibroblasts, mitochondria contribute to the VLCFA β-oxidation in human and mouse astrocytes. In CNS, VLCFA may be β-oxidized in mitochondria, because recently ACAD11, a new member of the acyl-CoA dehydrogenases, which have substrate specificity to C20–C24, was shown to reside in the mitochondria of CNS. In contrast to our results, Lageweg et al. showed that VLCFA is oxidized...
This discrepancy might be caused by a species difference or the difference in the substrate used. They used C26:0 as the β-oxidation substrate, which has a lower affinity to mitochondrial β-oxidation than C24:0.

Concerning the contribution of ABCD1 to the peroxisomal β-oxidation of VLCFA, our results demonstrate that the ABCD1-dependent pathway mainly contributes to peroxisomal β-oxidation, based on the following evidence. (1) In U87 cells, the contribution of peroxisomes to overall VLCFA β-oxidation is estimated to be 70% based on the inhibition of mitochondrial VLCFA β-oxidation activity by POCA (Fig. 2), and the ABCD1-dependent pathway accounts for more than 70% of the silencing of the ABCD1 protein resulted in a 70% reduction in peroxisomal 24:0 β-oxidation activity (Fig. 3). (2) In mouse astrocytes, the contribution of ABCD1-dependent pathway to overall VLCFA β-oxidation is estimated to be ca. 40% (Fig. 5), and this contribution is slightly higher than that of the mitochondrial pathway or the ABCD1-independent peroxisomal pathway. Both the mitochondrial and the ABCD1-independent pathways are estimated to be ca. 30% of overall VLCFA β-oxidation by the experiment using POCA in wild type and Abcd1-deficient cells (Figs. 2D, 5). Recently, Fourcade et al. have demonstrated that in cortical brain slices from an Abcd1-deficient mouse, the C26:0 β-oxidation activities were reduced to 50% of that in the wild-type mouse.31) This data are consistent with our findings.

On the other hand, ABCD1-independent and mitochondrial pathways also contribute significantly to VLCFA β-oxidation in astrocytic cells. In terms of ABCD1-independent peroxisomal VLCFA β-oxidation, the potential candidates are ABCD2 and ABCD3. Overexpression of ABCD2 has been shown to compensate for ABCD1 deficiency in abcd1-deficient mouse or X-ALD fibroblasts, suggesting that ABCD2 may be involved in the ABCD1-independent peroxisomal β-oxidation pathway. Recently, Singh et al. showed that silencing of the Abcd2 gene decreased the ratio of the C24:0:C16:0 β-oxidation activity, as the silencing of the Abcd1 gene had done, in mouse primary astrocytes.32) Fourcade et al. demonstrated that C26:0 β-oxidation activity in the cortical brain slices of Abcd2-deficient mice decreased to approximately 70% of the control.31) It is thus possible that Abcd2 contributes to the abcd1-independent

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**Fig. 3. ABCD1-Knockdown in U87 Cells**

(A) U87 cells were transfected with ABCD1-specific shRNA or scramble shRNA by Nucleofector and cultured in a 6-well dish. After 24-h-inubation, an aliquot of the cells was transferred to new 6-well dish and incubated for 5d. Cells were fixed by formaldehyde solution at 2, 3, 4, 5 and 6 d posttransfection. ABCD1 protein in the fixed cells was detected by immunofluorescence using an anti-ABCD1 antibody. (B) U87 cells were transfected with ABCD1-specific shRNA as above. At 4 d post-transfection, cells were harvested and solubilized in sample buffer. Cellular proteins were separated by SDS-PAGE and subjected to immunoblot analysis. (C) Silencing effects of shRNAs on VLCFA β-oxidation in U87 cells. Scramble, ABCD1 or ACOX1-specific shRNA was transfected into U87 cells as above. At 4 d post-transfection, cells were harvested for β-oxidation. C24:0 β-oxidation activity was measured in the presence of 25 µM POCA. Results are presented as the % relative to the activities in cells transfected with scramble shRNA (0.44 nmol/mg/h). Statistically significant variations are indicated by asterisks (*p<0.05).
pathway in the case of mouse astrocytes. In contrast, there is no report about expression of ABCD2 in human astrocytes. The expression of \textit{ABCD2} gene was reported to be not detected in human glioblastoma cell line (T98G).\textsuperscript{33) We also could not detect ABCD2 protein in U87 cells by immunoblot analysis. Thus, in human astrocytes, the contribution seems to be little compared to that in mouse astrocytes. In addition, no decrease in the C24:0 β-oxidation activity was observed when ABCD3 was silenced by ABCD3-specific shRNA (data not shown) in ABCD1-knockdown U87 cells, suggesting that ABCD3 is little involved in ABCD1-independent C24:0 β-oxidation. This data are consistent with the fact that overexpression of ABCD3 in CHO cells stimulated C16:0 β-oxidation activity, but slightly inhibited 24:0 β-oxidation activity.\textsuperscript{10) Fatty acid β-oxidation in peroxisomes is thought to depend on the active transport of acyl-CoA across membranes and passive diffusion of free fatty acids within membranes. In the latter case, almost free VLCFA in cytosol is bound to fatty acid-binding proteins (FABPs) and transferred to peroxisomal membranes. The VLCFA is then activated to VLCFA-CoA by very long chain acyl-CoA synthetases, such as ACSVL1 and ACSVL5, on the luminal side of peroxisomes for β-oxidation.\textsuperscript{34) It has been reported that VLCFA β-oxidation was reduced by the knockout of ACSVL1 or ACSVL5.\textsuperscript{34) Recently, Pillai \textit{et al.} reported that VLCFA diffuses rapidly across phospholipids bilayers and biological membranes.\textsuperscript{35) In a previous study we also reported that baicalein 5,6,7-trimethyl ether stimulated peroxisomal VLCFA β-oxidation in X-ALD fibroblasts by the up-regulation of \textit{ACSVL1} mRNA, but not \textit{ABCD2}.\textsuperscript{36) Therefore, it is likely that the free VLCFA transport in peroxisomes also contributes partly to ABCD1-independent VLCFA β-oxidation in astrocytes.

In conclusion, we have demonstrated that three pathways are involved in VLCFA β-oxidation in astrocytic cells, the ABCD1-dependent and independent peroxisomal pathways and the mitochondrial pathway. Effective restoration of VLCFA β-oxidation in ABCD1-deficient astrocytes is an important strategy in X-ALD therapy. Activation of the ABCD1-independent pathway for the restoration of VLCFA β-oxidation in peroxisomes as well as mitochondria is a novel target for the treatment of X-ALD.
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