Characterization of uptake and metabolism of very long-chain fatty acids in peroxisome-deficient CHO cells

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Abstract

Fatty acids (FAs) longer than C20 are classified as very long-chain fatty acids (VLCFAs). Although biosynthesis and degradation of VLCFAs are important for the development and integrity of the myelin sheath, knowledge on the incorporation of extracellular VLCFAs into the cells is limited due to the experimental difficulty of solubilizing them. In this study, we found that a small amount of isopropanol solubilized VLCFAs in aqueous medium by facilitating the formation of the VLCFA/albumin complex. Using this solubilizing technique, we examined the role of the peroxisome in the uptake and metabolism of VLCFAs in Chinese hamster ovary (CHO) cells. When wild-type CHO cells were incubated with saturated VLCFAs (S-VLCFAs), such as C23:0 FA, C24:0 FA, and C26:0 FA, extensive uptake was observed. Most of the incorporated S-VLCFAs were oxidatively degraded without acylation into cellular lipids. In contrast, in peroxisome-deficient CHO cells uptake of S-VLCFAs was marginal and oxidative metabolism was not observed. Extensive uptake and acylation of monounsaturated (MU)-VLCFAs, such as C24:1 FA and C22:1 FA, were observed in both types of CHO cells. However, oxidative metabolism was evident only in wild-type cells. Similar manners of uptake and metabolism of S-VLCFAs and MU-VLCFAs were observed in IFRS1, a Schwan cell-derived cell line. These results indicate that peroxisome-deficient cells limit intracellular S-VLCFAs at a low level by halting uptake, and as a result, peroxisome-deficient cells almost completely lose the clearance ability of S-VLCFAs accumulated outside of the cells.

Keywords:

Peroxisome

Very long-chain fatty acids

Beta-oxidation

Peroxisome disease

Abbreviations: α-CD, α-cyclodextrin; CHO, Chinese hamster ovary; CNS, central nervous system; ELOVL1, elongation of very long-chain fatty acids protein 1; FAs, fatty acids; FAMEs, fatty acid methyl esters; FATP, Fatty acid transporter; FFA, free fatty acids; GC, gas chromatography; IFRS1, immortalized adult Fischer rat Schwann cell; IP, isopropanol; LCFA, long-chain fatty acid; MU-VLCFAs, monounsaturated very long-chain fatty acids; Na, NaOH; PC, phosphatidylcholine; PE, Phosphatidylethanolamine; S-VLCFAs, saturated very long-chain fatty acids; SM, sphingomyelin; SLs, sphingolipids; TFA; total fatty acid, TAG, triacylglycerol; VLCFAs, very long-chain fatty acids; X-ALD, X-linked adrenoleukodystrophy.

1. Introduction

Fatty acids (FAs) longer than C20 are classified as very long-chain fatty acids (VLCFAs). Lignoceric acid (C24:0) and nervonic acid (C24:1) are typical VLCFAs in animal cells. These VLCFAs are exclusively found in sphingolipids (SLs) as the major N-acyl moieties in mammalian tissues [1-3]. In fact, nearly one-third of the fatty acids consist of C24:0 FA or C24:1 FA in the sphingomyelin (SM) of the white matter of the human brain [3]. Abundance of VLCFAs in white matter is also notable in other SLs, such as brain cerebroside or ganglioside [3]. It has been demonstrated that the expression of elongation of very long-chain fatty acids protein 1 (ELOVL1), a key enzyme for the synthesis of VLCFAs [4], increases with the development of myelination in the central nervous system (CNS) in humans and mice, and that deficiency in the synthesis of VLCFA-containing SLs result in myelin-sheath defects [5]. These observations indicate that VLCFA-containing SLs play an important role in myelin sheath formation.

VLCFAs breakdown takes place only in peroxisomes. This fact is evident from that patients of certain types of peroxisome disease, which show inability of peroxisomal β-oxidation, accumulate VLCFAs in the body [6-15]. One of the major symptoms of peroxisome-associated diseases is demyelination of the CNS and peripheral nerve tissue [6,16,17]. The abnormal accumulation of VLCFAs, such as C26:0 FA, in oligodendrocytes is thought to be a causative event to induce demyelination, where accumulated VLCFAs causes disruptive effect on protein-protein interaction of the myelin structure [18] or induces oxidative stress by impairment of mitochondria function [19,20]. However, exact mechanisms by which VLCFAs triggers these events have not fully elucidated yet.

We have studied the peroxisomal metabolism of FAs. Our recent findings showed peroxisome-dependent metabolic conversion of sciadonic acid [20:3 Δ-5,8,11] to linoleic acid [18:2 Δ-9, 12]

[21,22], and peroxisome-dependent clearance of hydroxy FAs of gut microbiome origin [23]. In these studies, the comparison of peroxisome-deficient cells and their wild-type cells was highly useful for characterizing peroxisome-intrinsic metabolism [21-23]. However, such experiments can only be performed with FAs solubilized in culture medium. Experiments with VLCFAs, especially in saturated (S)-VLCFAs, have been hampered by the difficulty of their solubilization.

In this study, we found that a small amount of isopropanol (IP) solubilized VLCFAs in aqueous medium by facilitating the formation of VLCFA/albumin complex. Using this solubilizing technique, we demonstrated that a significant proportion of exogenous VLCFAs were taken up and oxidatively degraded in peroxisomes without acylation to cellular lipids in Chinese hamster ovary (CHO) cells and IFRS1cells, a Schwann cell-derived cell line. We also showed for the first time that peroxisome-deficient cells are almost totally lost clearance ability of S-VLCFAs accumulated outside of the cells.

2. Materials & Methods

2.1. Materials

Fatty acid-free bovine serum albumin (BSA), heptadecanoic acid (C17:0 FA), tricosanoic acid (C23:0 FA), nervonic acid (C24:1 FA), pentacosanoic acid (C25:0 FA), and hexacosanoic acid (C26:0 FA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1,2-Diheptadecanoyl-*sn*-glycero-3-phosphocholine and 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (DAPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Erucic acid (C22:1 FA) and lignoceric acid (C24:0 FA) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). α-Cyclodextrin (α-CD) was purchased from Nacalai Tesque (Kyoto, Japan). TLC plates coated with silica gel (Merck Art 5721, Darmstadt, Germany) were used. All other reagents were of reagent grade.

2.2. Cell culture

The Chinese hamster ovary (CHO-K1) cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). CHO-zp102 cells were constructed by deletion of *Pex5*, which encodes a peroxisome-targeting signal-1 receptor as described previously [24,25]. The immortalized adult rat Schwann cell line (IFRS1) was kindly provided by Professor K. Watabe (Kyorin University). CHO cells were cultured in Ham's F-12 medium (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) (Biowest, Noaillé, France) and 1% penicillin-streptomycin (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA). IFRS1 cells were maintained in Iscove's modified Dulbecco's medium with GlutaMAX™ Supplement (Gibco BRL) containing 5% FBS, 20 ng/mL recombinant human heregulin-β (EMD Millipore, Billerica, MA, USA), 5 μM forskolin (Sigma-Aldrich), and antibiotic-antimycotic solution containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL

amphotericin (Sigma-Aldrich). All cells used in this study were between passages 3 and 10, and the experiments were performed after the cells reached confluence.

2.3. Preparation of C23:0 FA samples

Various amounts of C23:0 FA (60, 120, and 300 nmol) were placed in a glass tube. After drying the solvent by N_2 flow, 100 mM NaOH and hot water were added. The sodium salt of the FA (FA/Na) suspension (0.5 mL) was heated at 70 °C in a water bath and mixed with 1.5 mL of culture medium which contained 6.6 mg BSA at 37 °C. The solutions were then sonicated in an ultrasonic bath at 37 °C for 5 min. The mixture was further incubated in a water bath at 37 °C for 60 min. The molar ratios of FA/NaOH/BSA were 0.6:6:1, 1.2:6:1 or 3:6:1. Preparation of the α-CD complex of C23:0 FA was conducted using the method reported in [26] with slight modifications. Various amounts of C23:0 FA (60, 120, and 300 nmol) were dissolved in 50 μL IP and mixed with 2 mL of 3 mM α-CD in culture medium at 37 °C. The mixtures were then sonicated in an ultrasonic bath at 37 °C for 5 min. The mixture was further incubated in a water bath at 37 °C for 60 min. Various amounts of C23:0 FA (60, 120, and 300 nmol) were dissolved in 50 μL of IP at 37 °C, and mixed with 2 mL of culture medium containing 6.6 mg BSA at 37 °C. The mixture of FA/IP/BSA was then sonicated in an ultrasonic bath at 37 °C for 5 min, and further incubated in a water bath at 37 °C for 60 min. The molar ratios of FA/BSA were 0.6:1, 1.2:1 or 3:1. These C23:0 FA samples were filtered through a 0.22 μm filter, and the resulting filtrates were subjected to lipid extraction to determine the amount of C23:0 FA by gas chromatography (GC). For examining the effect of temperature on the formation of FA/BSA complex, 300 nmol of C23:0 FA dissolved in 50 μL IP was added to the 2 mL of culture medium which contained 6.6 mg BSA at 4 °C, 15 °C, 30 °C, or 37 °C. The mixture of FA/IP/BSA

was then sonicated in an ultrasonic bath at 4 °C, 15 °C, 30 °C, or 37 °C for 5 min. Then, they were filtered through a 0.22 μm filter to remove the undissolved C23:0 FA. The amount of C23:0 FA in filtrate was extracted and determined using gas chromatography (GC).

2.4. Cytotoxicity assay

The CHO-K1 cells were seeded in 35-mm plastic dishes at a density of $2x10^5$ cells/dish at 37 °C. After exposure to the various concentration of IP for 48 h in CO_2 incubator, adherent cells were harvested by trypsin/EDTA treatment. The collected cells were washed and subjected to a trypan blue dye exclusion assay for assessing numbers of viable cells.

2.5. Preparation of FA/IP/BSA for culture experiments

Three hundred nmol of FA dissolved in 50 μL IP, and FBS-free culture medium containing BSA (6.6 mg/2 mL) were warmed at 37 °C. They were mixed and sonicated in an ultrasonic bath (150 W) at 37 °C for 1-5 min. The mixture was further incubated in a water bath at 37 °C for 60 min. Resulting FA/IP/BSA (2 mL of medium) were added to the cell culture (8 mL of medium). The molar ratio of FA to BSA was 3:1, and the maximum concentration of IP in the culture medium was 0.5% (v/v). Control cells were treated with vehicle (BSA plus IP) only.

2.6. FA uptake assay

CHO cells or IFRS1 cells were seeded into 100 mm petri dishes with 10 mL of FBS-containing medium at $1x10^6$ cells/dish for 24 h at 37 °C in a CO_2 incubator. The culture medium was then replaced with 10 mL serum-free medium for 24 h. After 24 h, cells were supplemented with VLCFAs

(C22:1, C23:0, C24:0, C24:1, C25:0, and C26:0 FA) or LCFA (C17:0 FA) as FA/BSA complex prepared by the IP method. The final concentrations of IP and FA were 0.5% and 30 μM, respectively, in most experiments. At the end of incubation, the conditioned medium was removed, and the cells were washed with 2 mL of FBS-free medium containing 1% BSA. The conditioned medium and the BSA-containing medium used for washing were combined and subjected to lipid extraction. Lipids recovered from the medium and harvested cells were extracted according to the method of Bligh and Dyer [27] after addition of a known amount of synthetic DAPC or 17:0/17:0 PC as an internal standard. Aliquots of the lipid extract were treated with 5% HCl-methanol at 100 °C for 60 min to prepare fatty acid methyl esters (FAMEs). FAMEs were purified by TLC with a developing solvent system of petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) and analyzed by GC equipped with a capillary column (DB-225, 0.25 μm film thickness, 30 m length, 0.25 mm ID; Agilent Technologies, Santa Clara, CA, USA). The oven temperature was maintained at 100 °C for 0.5 min and raised to 195 °C at a rate of 25 °C /min. It was then increased to 205 °C at a rate of 3 °C /min followed by 240 °C at 8 °C/ min, and kept constant for 10 min. The oven temperature was then decreased to 100 °C before the injection of the next sample. The amount of each fatty acid was calculated based on the ratio of the peak area between the objective peak and 17:0/17:0 PC or 20:0/20:0 PC as the internal standard. When examining the distribution of FAs among lipid classes, cellular lipids were separated by TLC. To isolate sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipid extracts were subjected to two-dimensional TLC. The solvent systems used for the first and second chromatography were chloroform/acetone/methanol/acetic acid/water (50:20:10:13:5, v/v/v/v/v) and chloroform/methanol/28% ammonia (60:35:8, v/v/v), respectively. To isolate triacylglycerol (TAG) and free fatty acids (FFA), petroleum ether:diethyl

ether:acetic acid (80:20:1, v/v/v) were used. Isolated lipids were recovered from the silica gel by the method of Bligh and Dyer [27], and then subjected to methanolysis and analyzed using GC as described above.

2.7. Time-dependent changes in the amounts of FAs incorporated in CHO cells

Serum starved cells were incubated with 30 μM VLCFAs (C23:0, C24:0, and C24:1 FA) or LCFA (C17:0 FA) as the described above. After 3 h, the culture medium was replaced with serum-free medium which did not contain FAs, and further incubated. The cells were washed and harvested at the indicated times as described above, and cellular lipids were extracted using the method of Bligh and Dyer [27]. An aliquot of the cellular lipid was subjected to methanolysis and analyzed by GC as described above. The amount of the VLCFAs or LCFA in the cellular lipid during the initial 3 h incubation was set to 100%. The disappearance of FAs was expressed as a percentage of the initial amount of FAs.

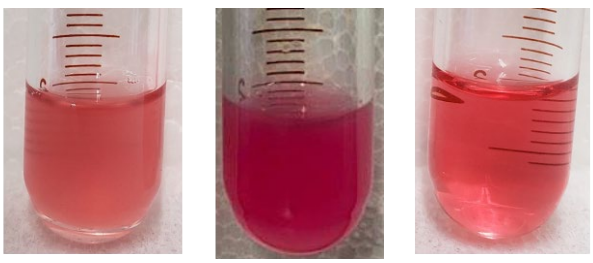
2.8. Statistical analysis

Student's t test was performed for experiments with 2 groups. For multiple comparisons, one-way or two-way ANOVA followed by a Tukey *post-hoc* test was performed.

3. Results

3.1. IP facilitates formation of VLCFA/BSA complex and enables VLCFA uptake into the cells

Converting FAs into their dissociation form by NaOH treatment followed by complexing with albumin is a popular method for FA solubilization in aqueous media. We successfully solubilized various LCFAs using this method and characterized their metabolism in peroxisome [21-23]. However, C23:0 FA was not solubilized by this method. In fact, C23:0 FA did not pass through the 0.22 μm filter when its sodium salt was mixed with BSA (Fig. 1A), indicating that C23:0 FA does not form albumin complex by this conventional method. Next, the solubilization method of VLCFAs using α-CD [26] was examined. We found that this method can solubilize low concentrations of C23:0 FA to some extent, but it did not solubilize higher concentrations of C23:0 FA (Fig. 1A). The extent of solubilization of VLCFAs by this method was consistent with that reported previously [26]. Here, we found that mixing an IP solution of C23:0 FA with a BSA solution greatly enhanced the solubility of C23:0 FA. As shown in Supplementary Fig. 1, the C23:0 FA/IP/BSA mixture (150 μM) in the medium showed clear color and completely passed through the 0.22 μm filter, indicating that most of the C23:0 FA forms albumin complex. We found all preparation process, including mixing IP solution of C23:0 FA with BSA-containing medium, and sonication of the resulting mixture, should be performed over 30 °C to dissolve the C23:0 FA by complexing with BSA (Fig. 1B). The key to successful solubilization seems to be the avoidance of aggregation of VLCFAs in aqueous solution.



Supplementary Fig. 1. Photographs of the C23:0 FA solution before filtration.

Three hundred nmol of C23:0 FA were treated with NaOH solution followed by hot water, and mixed with BSA solution (FA/Na/BSA). Three hundred nmol of C23:0 FA dissolved in IP were mixed with 3 mM α-cyclodextrin solution (FA/IP/α-CD) or BSA solution (FA/IP/BSA).

We examined the cellular uptake of C23:0 FA (30 μM, 300 nmol/dish) using CHO-K1 cells. When the cells were supplemented with C23:0 FA/IP/BSA, the disappearance of C23:0 FA from the culture medium was approximately100 nmol/dish during 24 h incubation. In contrast, C23:0 FA detected in cellular lipids was only 8 nmol. They were distributed in SM and PC, but not FFA (Fig. 1C). The large difference between the amount of C23:0 FA that disappeared from the medium and the amount that appeared in the cellular lipid was explained by oxidative degradation of C23:0 FA by peroxisomes as described below. We conducted similar experiments with C23:0 FA prepared using the conventional method (FA/Na/BSA), and found that approximately 45 nmol of the supplemented C23:0 FA disappeared from the medium (Fig. 1C). In this experiment, most of this decrease was found in the FFA form in the cellular fraction (35 nmol), indicating that the C23:0 FA that disappeared from the medium was merely cell-associating but not esterified to cellar lipids (Fig. 1C).

We found that up to 2% of IP did not affect the viability of the CHO cells (Fig. 1D). Based on these experiments, we conducted the following experiments using the FA/IP/BSA mixture. The final concentration of IP in the medium did not exceed 0.5% in all experiments.

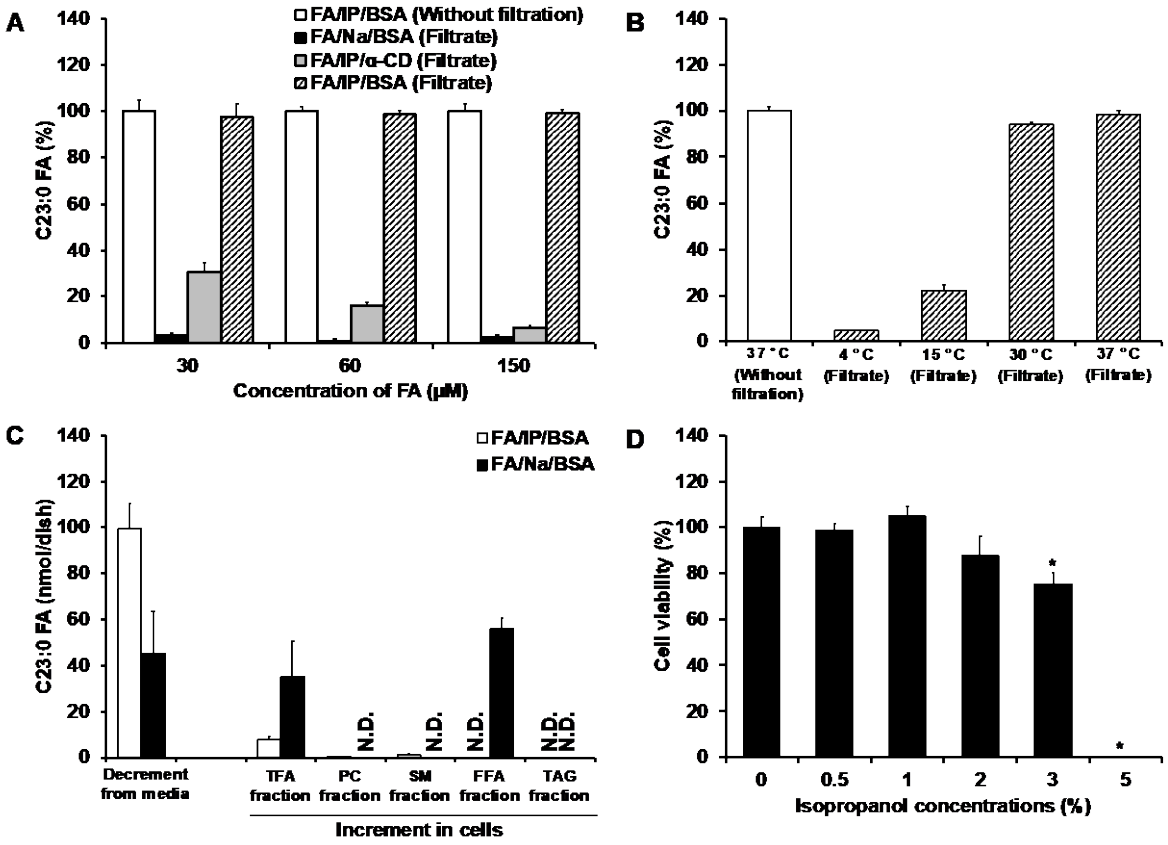


Fig. 1. IP facilitates formation of VLCFA/BSA complex and enables uptake of VLCFA into the cells.

(A) Different amounts of C23:0 FA (30, 60, and 150 μM) were treated with NaOH solution followed by hot water, and mixed with BSA solution (FA/Na/BSA). Different amounts of C23:0 FA dissolved in IP were mixed with 3 mM α-cyclodextrin solution (FA/IP/α-CD) or BSA solution (FA/IP/BSA). The mixtures were filtered through a 0.22 μm filter, and the filtrates were subjected to GC analysis. The amount of C23:0 FA in FA/IP/BSA solution (without filtration) was simultaneously determined, and used as 100% control. Values are means ± S.D. of three independent experiments. (B) Amount of C23:0 FA (150 μM) dissolved in IP were mixed with BSA solution at 4 °C, 15 °C, 30 °C, and 37 °C. The mixtures were filtered through a 0.22 μm filter. The FA dissolved in the filtrate was extracted and subjected to GC analysis after methyl esterification. Values are means ± S.D. of three independent experiments. (C) CHO-K1 cells were incubated with 30 μM of C23:0 FA/IP/BSA mixture or C23:0 FA/Na/BSA mixture in serum-free medium for 24 h. The cells and media were collected separately and subjected to lipid extraction. The amounts of C23:0 FA in the PC, SM, TAG, and FFA fractions were determined after separation of each lipid class by TLC. (D) CHO-K1 cells were incubated in serum-free medium with the indicated concentration of IP for 48 h. Viable cells were counted by the trypan blue dye exclusion assay.

Values are presented as means ± S.D. of three independent experiments and survival is expressed as percentage of vehicle control. Significance was analyzed by one-way ANOVA followed by the Tukey *post-hoc* test. *$p <$ 0.05, significantly different from control cells. TFA: total fatty acid, N.D.: not detected, IP: isopropanol, Na: NaOH, α-CD: α-cyclodextrin.

3.2. Cellular uptake of C23:0 FA in CHO-K1 (wild-type) and CHO-zp102 cells (peroxisome-deficient cells)

Uptake and metabolism of C23:0 FA was examined in CHO-K1 and CHO-zp102 using the improved FA-solubilizing technique. The initial concentration of the FA in the culture medium was set to 30 μM (300 nmol/dish). We found that the C23:0 FA recovered from the cultured medium of cells following incubation for 0.5 min was 283 nmol/dish. After 24 h incubation, the C23:0 FA remaining in the medium was found to be 155 nmol/dish in the wild-type cells, indicating that 128 nmol/dish of C23:0 FA disappeared from the culture medium during the incubation (Fig. 2A left). The same experiment was conducted using peroxisome-deficient cells. We found that the amount of C23:0 FA that disappeared from the culture medium was only 10 nmol/dish after 24 h of incubation (Fig. 2A left). We also examined extent of degradation of C23:0 FA in the medium under the absence of cells for 24 h at 37 °C in a CO_2 incubator, and found that decomposition of C23:0 FA during the incubation was under detectable level (Fig. 2A right). We found time-dependent accumulation of odd-numbered FAs (C19:0 FA, C17:0 FA, C17:1 FA and C15:0 FA) in cellular lipids in wild-type cells (Fig. 2B left, Fig. 2C), indicating that chain-shortening metabolism of C23:0 FA by peroxisomal β-oxidation was performed in wild-type cells. In peroxisome-deficient cells, the amount of C23:0 FA detected in the cellular lipids was 10 nmol/dish with no detectable odd number FAs other than C23:0 FA (Fig. 2B right). These results indicated that a very small amount of C23:0 FA was taken up by the cells and that neither elongation, desaturation, nor oxidative degradation occurred to incorporate

C23:0 FA in peroxisome-deficient cells. From these results, it was concluded that the large imbalance of C23:0 FA between disappearance from the medium (128 nmol) and appearance in cellular lipids (8 nmol) observed in wild-type cells was due to peroxisome-dependent degradation of C23:0 FA. These results indicate that the capacity for uptake of extracellular C23:0 FA is largely dependent on the activity of peroxisomal β-oxidation. In addition, 94% of the incorporated C23:0 FA was metabolized via β-oxidation in peroxisomes in wild-type cells.

We also investigated the distribution of C23:0 FA incorporated into the cellular lipids of wild-type and peroxisome-deficient cells. In both CHO cells, C23:0 FA was incorporated into the PC and SM fractions. The chain-shortened metabolic products of C23:0 FA (C19:0 FA, C17:0 FA, C17:1 FA and C15:0 FA) were also acylated into PC and SM fractions in wild-type cells, but no detectable chain-shortened metabolites were detected in peroxisome-deficient cells (Fig. 2D). The C17:1 FA appeared in wild-type cells was considered to be formed from C17:0 FA by FA desaturase. Because, a part of uptaken C17:0 FA was desaturated to C17:1 FA in our experimental condition as shown below.

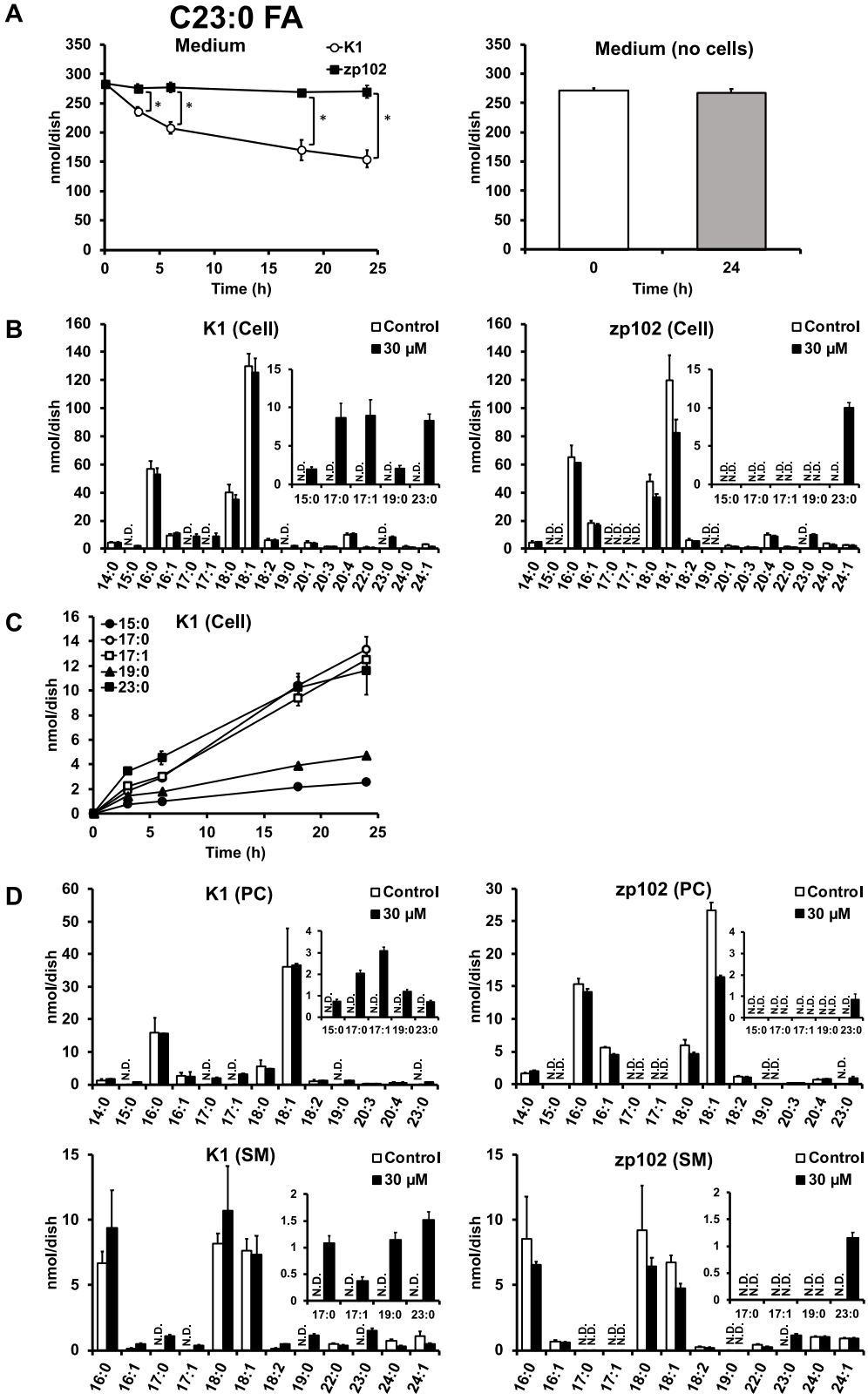

A
C23:0 FA
Medium
K1
zp102
nmol/dish
Time (h)
Medium (no cells)
B
K1 (Cell)
zp102 (Cell)
Control
30 μM
C
K1 (Cell)
15:0
17:0
17:1
19:0
23:0
D
K1 (PC)
zp102 (PC)
K1 (SM)
zp102 (SM)
N.D.

Fig. 2. Cellular uptake of C23:0 FA in CHO-K1 and CHO-zp102 cells.

CHO-K1 (wild-type) cells and CHO-zp102 (peroxisome-deficient) cells were incubated with 30 μM C23:0 FA in serum-free medium for the indicated time periods. Medium (A) and cells (B-D) were collected separately at the respective time points and subjected to lipid extraction. The fatty acid methyl esters prepared from total lipids (B, C), and PC and SM fractions of the cells (D) were analyzed after separation of each lipid class by TLC. The fatty acid profiles shown in B and D were obtained from cells incubated for 24 h. Values are means ± S.D. of three independent experiments at each time point. PC: phosphatidylcholine, SM: sphingomyelin. Significance was analyzed by two-way ANOVA followed by the Tukey *post-hoc* test. $*p < 0.05$ indicates the significant difference between indicated groups. N.D.: not detected.

3.3. Cellular uptake of C17:0 FA in CHO-K1 and CHO-zp102 cells

Cellular uptake and metabolism of C17:0 FA was examined following incubation of cells with 30 μM of C17:0 FA (300 nmol/dish). The initial amount of the C17:0 FA recovered from the culture medium of wild-type (CHO-K1) cells was approximately 255 nmol/dish. The C17:0 FA remaining in the medium decreased to 65 nmol/dish at the end of incubation in wild-type cells (Fig. 3A). The decrement and decreasing time-course of the C17:0 FA from the medium of peroxisome-deficient cells were essentially the same as those observed in wild-type cells (Fig. 3A). Regarding cellular lipids, C17:1 FA, C19:0 FA, and C19:1 FA in addition to C17:0 FA were found in both wild-type and peroxisome-deficient cells at similar levels (Fig. 3B). These FAs increased along with an increase in the uptake of C17:0 FA into the cells of wild-type cells (Fig. 3C), indicating that they are desaturation or elongation metabolites of C17:0 FA as shown in previous report [28]. A small amount of C15:0 FA was detected only in the wild-type cells. However, the amount of C15:0 FA was marginal compared to other metabolites of C17:0 FA. These results indicate that peroxisomal metabolism does not contribute to the uptake and metabolism of extracellular C17:0 FA. The incorporated C17:0 FA and its metabolites were mainly distributed in the PC and SM fractions in wild-type cells. The pattern of

distribution of C17:0 FA and its metabolites in peroxisome-deficient cells was similar to that of wild-type cells (Fig. 3D).

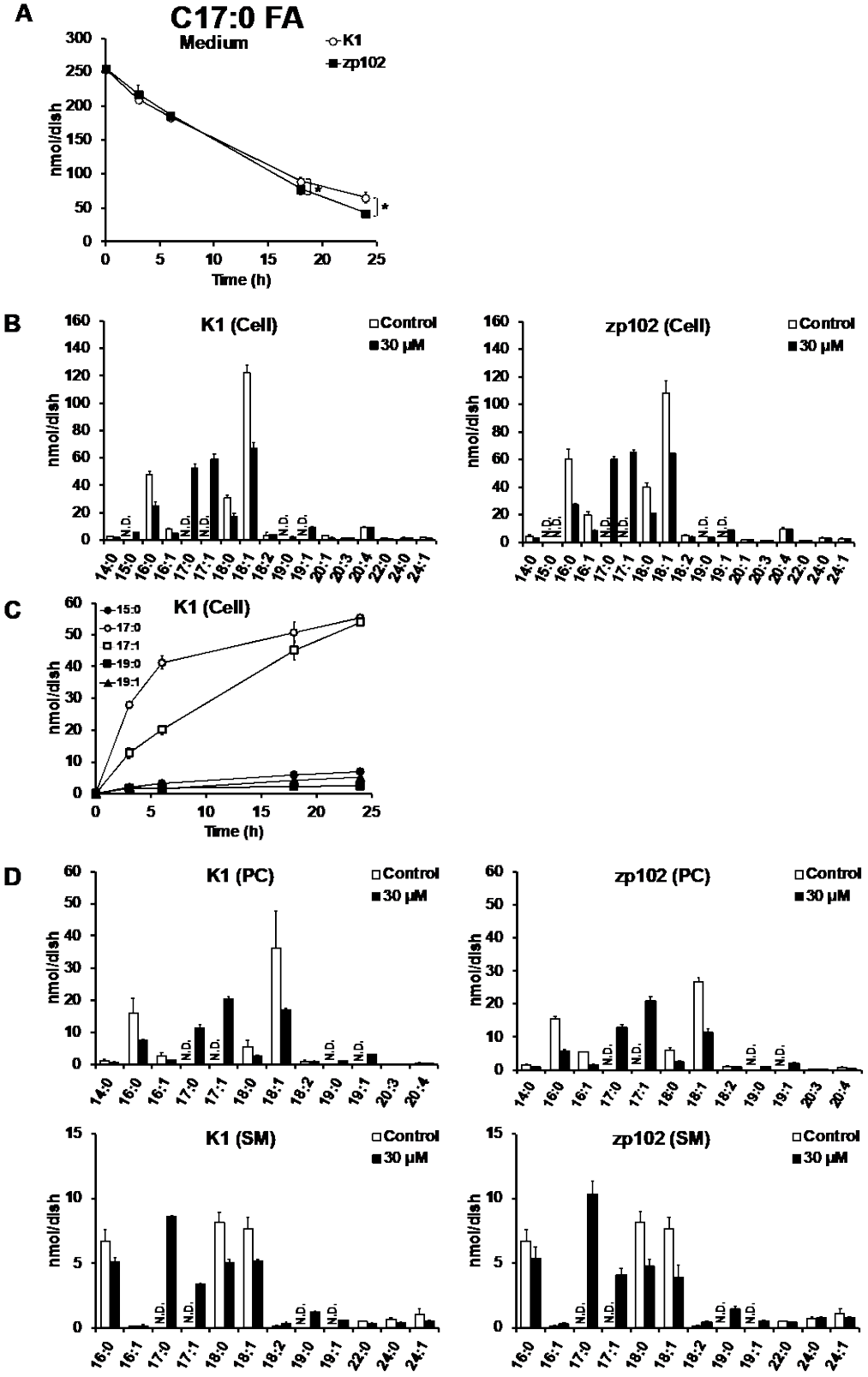

A
C17:0 FA
Medium
K1
zp102
nmol/dish
Time (h)
B
K1 (Cell)
zp102 (Cell)
Control
30 μM
N.D.
14:0
15:0
16:0
16:1
17:0
17:1
18:0
18:1
18:2
19:0
19:1
20:1
20:3
20:4
22:0
24:0
24:1
C
K1 (Cell)
15:0
17:0
17:1
19:0
19:1
D
K1 (PC)
zp102 (PC)
K1 (SM)
zp102 (SM)

Fig. 3. Cellular uptake of C17:0 FA in CHO-K1 and CHO-zp102 cells.

CHO-K1 (wild-type) cells and CHO-zp102 (peroxisome-deficient) cells were incubated with 30 μM C17:0 FA in serum-free medium for the indicated time periods. Medium (A) and cells (B-D) were collected separately at the respective time points and subjected to lipid extraction. The fatty acid methyl esters prepared from total lipids (B, C), and PC and SM fractions of the cells (D) were analyzed after separation of each lipid class by TLC. The fatty acid profiles shown in B and D were obtained from cells incubated for 24 h. Values are means ± S.D of three independent experiments at each time point. PC: phosphatidylcholine, SM: sphingomyelin. Significance was analyzed by two-way ANOVA followed by the Tukey *post-hoc* test. $*p < 0.05$ indicates the significant difference between indicated groups. N.D.: not detected.

3.4. Peroxisome-dependent disappearance of VLCFAs in CHO cells

Pulse/chase experiments with C23:0 FA, C24:0 FA, C24:1 FA, and C17:0 FA were performed using CHO cells. When the cells were incubated with 30 μM C23:0 FA (300 nmol/dish) for 3 h, the levels of C23:0 FA accumulated to the cellular lipids of wild-type and peroxisome-deficient CHO cells were approximately 2.9 and 2.2 nmol/dish, respectively. Approximately 50% of the incorporated C23:0 FA disappeared during an additional 6 h of incubation with FA-free medium in wild-type cells. In contrast, peroxisome-deficient cells retained 95% of the incorporated C23:0 FA in their cellular lipids after an additional 24 h of incubation (Fig. 4A). Initial levels of C17:0 FA acylated to cellular lipids of wild type and peroxisome-deficient CHO cells were approximately 25 and 35 nmol/dish, respectively, and the time-courses of disappearance of C17:0 FA from the cellular lipids were almost the same between wild-type and peroxisome-deficient CHO cells (Fig. 4B). Most of this disappearance was compensated by an increase in the amount of its metabolites (C17:1 FA, C19:0 FA, and C19:1 FA) in both cells (data not shown). Experiments conducted with C24:0 FA and C24:1 FA in both types of CHO cells showed that 70% of the initial levels of VLCFAs were degraded after an additional incubation for 24 h in wild-type cells, whereas this degradation was almost abolished

in peroxisome-deficient cells (Fig. 4C, D). These results clearly demonstrate that the incorporated VLCFAs were metabolized in a peroxisome-dependent fashion, whereas most of the C17:0 FA (LCFA) was metabolized in a peroxisome-independent manner.

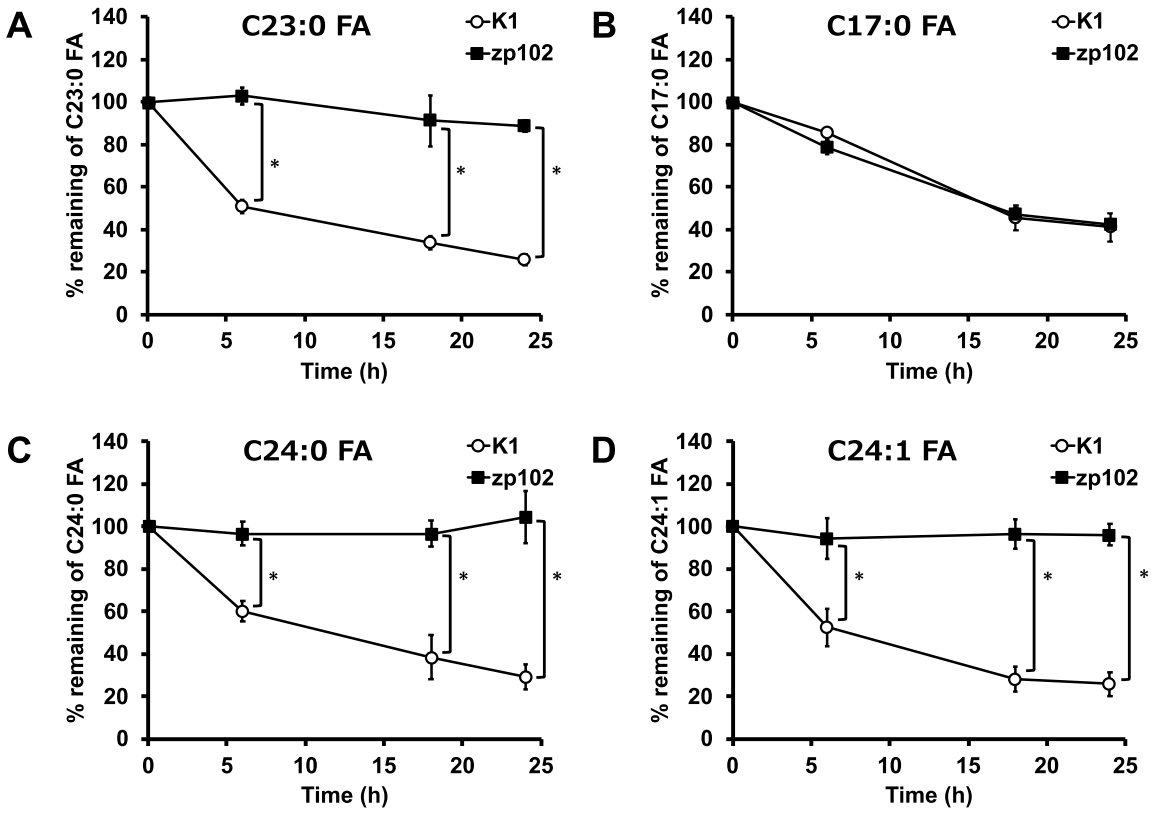


Fig. 4. Time-dependent change in the incorporated FA in CHO-K1 and CHO-zp102 cells.

CHO-K1 (wild-type) cells and CHO-zp102 (peroxisome-deficient) cells were incubated with 30 μM C23:0 FA (A), C17:0 FA (B), C24:0 FA (C) or C24:1 FA (D) in serum-free medium for 3 h. Then, the medium was replaced with serum-free medium without the FA. After subsequent cultivation for the indicated times, the cells were harvested and subjected to lipid extraction. The amount of each fatty acid was measured and expressed as a percentage of that during the initial 3 h of incubation. The initial amounts of exogenous C23:0 FA in wild-type and peroxisome-deficient CHO cells were 2.9 ± 1.0 and 2.2 ± 0.1 nmol/dish, respectively. The initial amounts of exogenous C17:0 FA in wild-type and peroxisome-deficient CHO cells were 25.7 ± 2.3 and 35.6 ± 3.8 nmol/dish, respectively. The initial amounts of exogenous C24:0 FA in wild-type and peroxisome-deficient CHO cells were 4.8 ± 0.2 and 4.1 ± 1.0 nmol/dish, respectively. The initial amounts of exogenous C24:1 FA in

wild-type and peroxisome-deficient CHO cells were 7.3 ± 1.3 and 9.4 ± 0.6 nmol/dish, respectively. Values are means ± S.D. of three independent experiments at each time point. Significance was analyzed by two-way ANOVA followed by the Tukey *post-hoc* test. $*p < 0.05$ indicates the significant difference between indicated groups.

3.5. Metabolic difference between S-VLCFAs and MU-VLCFAs in peroxisome-deficient CHO cells

The incorporation and metabolism of S-VLCFAs was characterized using wild-type and peroxisome-deficient CHO cells. A large decrease (100 nmol/dish) of the supplemented C24:0 FA from the medium of wild-type cells was observed during 24 h incubation, whereas the decrements in C24:0 FA in the medium of peroxisome-deficient cells was marginal (Fig. 5A). Despite the large decrease in C24:0 FA in the medium, the amount of the FA detected in the cellular lipids of wild-type cells was around 8 nmol/dish, which was comparable to that in peroxisome-deficient cells (Fig. 5B). The accumulated exogenous C24:0 FA was mainly acylated into SM, but not in PC, PE or TAG fraction in both CHO cells (Fig. 5C). These results are essentially the same as those observed for C23:0 FA. We also confirmed that extensive incorporation followed by peroxisome-dependent degradation was performed for C26:0 FA in wild-type cells, and that peroxisome-deficient cells showed virtually no incorporation of the extracellular C26:0 FA (Fig. 5D, E). The C26:0 FA was under detection level in PC, PE, SM and TAG in both cell types (Fig. 5F).

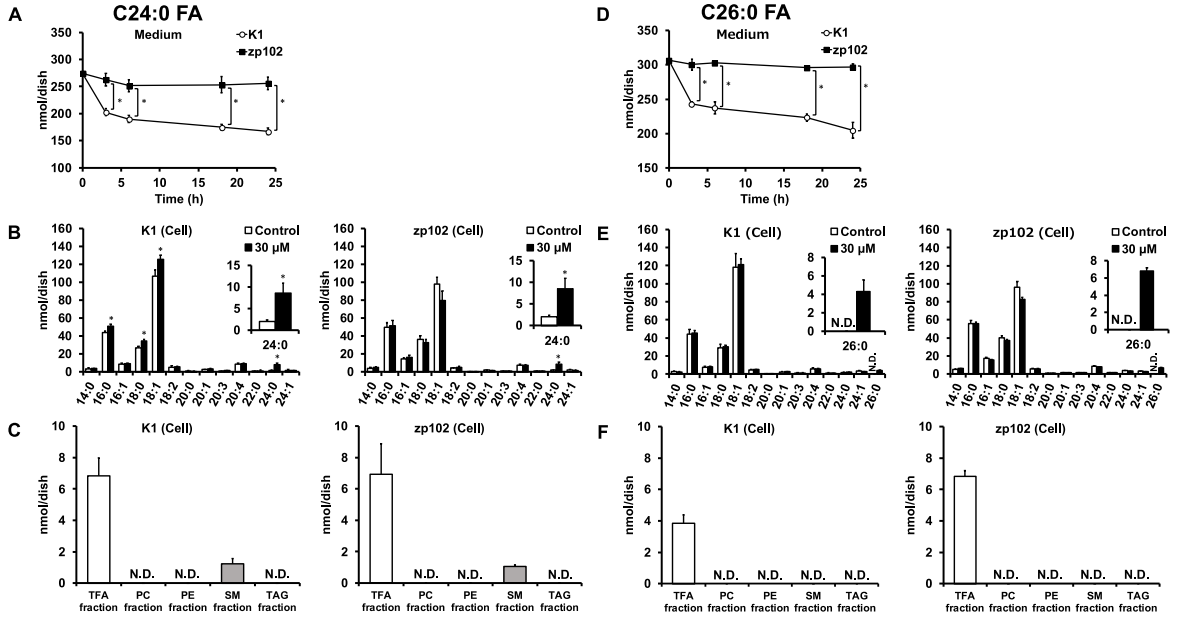


Fig. 5. Cellular uptake of saturated VLCFAs in CHO-K1 and CHO-zp102 cells.

CHO-K1 (wild-type) cells and CHO-zp102 (peroxisome-deficient) cells were incubated with 30 μM C24:0 FA and C26:0 FA in serum-free medium for the indicated time periods. The medium (A, D) and cells (B, E) were collected separately at the respective time points and subjected to lipid extraction. The fatty acid methyl esters prepared from total lipids (B, E), and PC, PE, SM and TAG fractions of the cells (C, F) were analyzed after separation of each lipid class by TLC. The fatty acid profiles shown in B and E were obtained from cells incubated for 24 h. Values are means ± S.D. of three independent experiments at each time point. TFA: total fatty acid, PC: phosphatidylcholine, PE: Phosphatidylethanolamine, SM: sphingomyelin, TAG: triacylglycerol. Significance was analyzed by two-way ANOVA followed by the Tukey *post-hoc* test (A, D) or Student's *t* test (B). $*p < 0.05$ indicates the significant difference between indicated groups. N.D.: not detected.

The uptake capacity of extracellular MU-VLCFAs was larger than that of S-VLCFAs in peroxisome-deficient cells. As shown in Fig. 6A and B, approximately 40 nmol/dish of C24:1 FA was decreased from the medium, and equivalent amounts of C24:1 FA were present in cellular lipids in peroxisome-deficient cells. This was four times that in the experiments with C24:0 FA in peroxisome-deficient cells. The acylation level of C24:1 FA in peroxisome-deficient cells exceeded that observed

in wild-type cells. Extensive incorporation and acylation were also observed in the experiments with C22:1 FA. As shown in Fig. 6D, approximately 120 nmol/dish of C22:1 FA were decreased from the medium, and equivalent amounts of C22:1 FA were present in cellular lipids in peroxisome-deficient cells. The acylation levels of C22:1 FA in peroxisome-deficient cells were 2.5 times that in wild-type cells. These results indicated that the capacities of incorporation and acylation of MU-VLCFAs were higher than those of S-VLCFAs in peroxisome-deficient cells. The extensive acylation of C24:1 FA or C22:1 FA in cellular lipids seemed to be compensated by a decrease in C18:1 FA (Fig. 6B right, Fig. 6E right). In wild-type CHO cells, C24:1 FA was acylated to PC, SM, and TAG, but not to PE fraction. In contrast, C24:1 FA was acylated to PC, PE, SM and TAG fraction in peroxisome-deficient cells (Fig. 6C). The incorporated C22:1 FA in peroxisome-deficient cells were found to be distributed to PC, PE, SM and TAG at higher level compared to those of wild-type cells (Fig. 6F). Decreased levels of C24:1 FA and C22:1 FA in the medium of wild-type cells were higher than those in peroxisome-deficient cells. Despite the large decrease in the medium, the amounts of C24:1 FA and C22:1 FA present in cellular lipids of wild-type cells were very low and lower than those present in cellular lipids of peroxisome-deficient cells, indicating that peroxisome-dependent oxidative degradation operates for these MU-VLCFAs, which facilitates extensive incorporation (Fig. 6A-F).

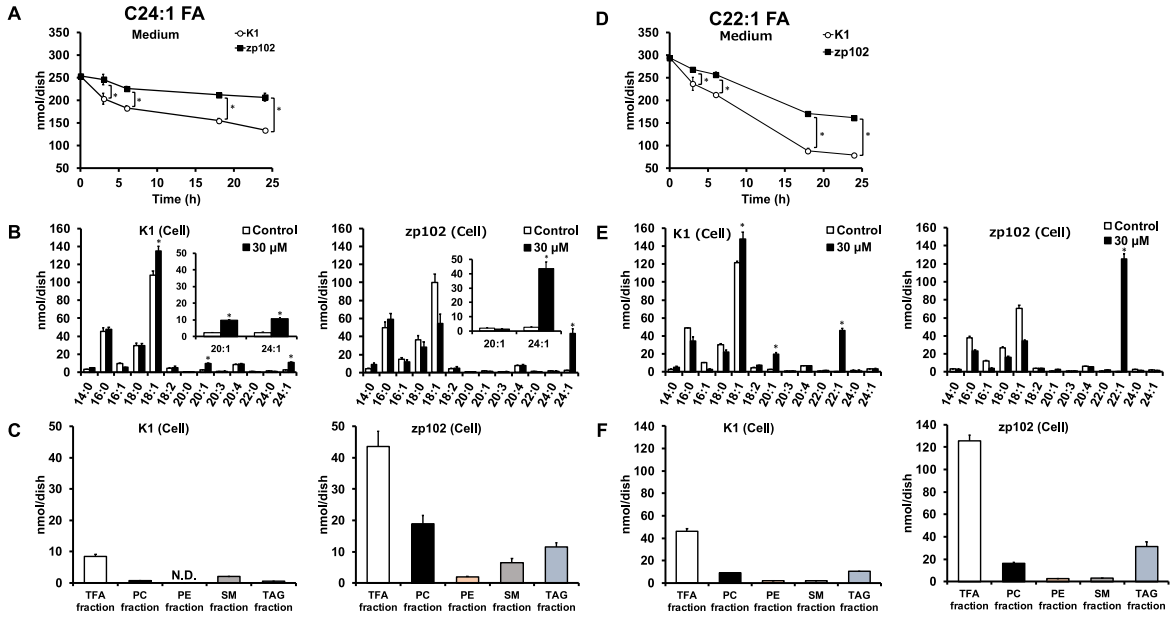


Fig. 6. Cellular uptake of mono-unsaturated VLCFAs in CHO-K1 and CHO-zp102 cells.

CHO-K1 (wild-type) cells and CHO-zp102 (peroxisome-deficient) cells were incubated with 30 μM C24:1 FA and C22:1 FA in serum-free medium for the indicated time periods. The medium (A, D) and cells (B, E) were collected separately at the respective time points and subjected to lipid extraction. The fatty acid methyl esters prepared from total lipids (B, E), and PC, PE, SM, and TAG fractions of the cells (C, F) were analyzed after separation of each lipid class by TLC. The fatty acid profiles shown in B and E were obtained from cells incubated for 24 h. Values are means ± S.D. of three independent experiments at each time point. TFA: total fatty acid, PC: phosphatidylcholine, PE: Phosphatidylethanolamine, SM: sphingomyelin, TAG: triacylglycerol. Significance was analyzed by two-way ANOVA followed by the Tukey *post-hoc* test (A, D) or Student's *t* test (B, E). $*p < 0.05$ indicates the significant difference between indicated groups. N.D.: not detected.

3.6. Incorporation and metabolism of extracellular VLCFAs in IFRS1 cells

Immortalized adult Fischer rat Schwann cells (IFRS1) are a Schwann cell line derived from the peripheral nerve tissue of Fischer 344 rats. We investigated the cellular uptake and metabolism of exogenous VLCFAs in IFRS1 cells (Fig. 7). We found that all S-VLCFAs (C23:0, C24:0 FA, C25:0 FA, and C26:0 FA) were removed from the culture medium to a similar extent during 24 h of incubation. The amounts of decrements of MU-VLCFAs (C24:1 FA and C22:1 FA) were higher than

those of S-VLCFAs. The decrease in the amount of C22:1 FA from the medium was comparable to that of C17:0 FA (Fig. 7A). Despite the large amount of decrease of these VLCFAs from the medium (70-250 nmol/dish), the increments of these VLCFAs in the cellular lipids were very low (1-60 nmol/dish) (Fig. 7B-H). We found chain-shortened odd numbered FAs in the cellular lipids in the experiments with C23:0 FA and C25:0 FA, suggesting that the operation of oxidative degradation of VLCFAs in peroxisomes in IFRS1 cells (Fig. 7C, E). These results suggest that VLCFAs were taken up and oxidatively degraded in peroxisomes without acylation of cellular lipids in IFRS1 cells, as observed in CHO cells. Analysis of the distribution of acylated VLCFAs in IFRS1 cells revealed that, the incorporated C24:0 FA was mainly acylated to SM but not to PC or TAG fraction (Fig. 7I). On the other hand, C22:1 FA and C24:1 FA were acylated to PC, SM and TAG fraction (Fig. 7J, K).

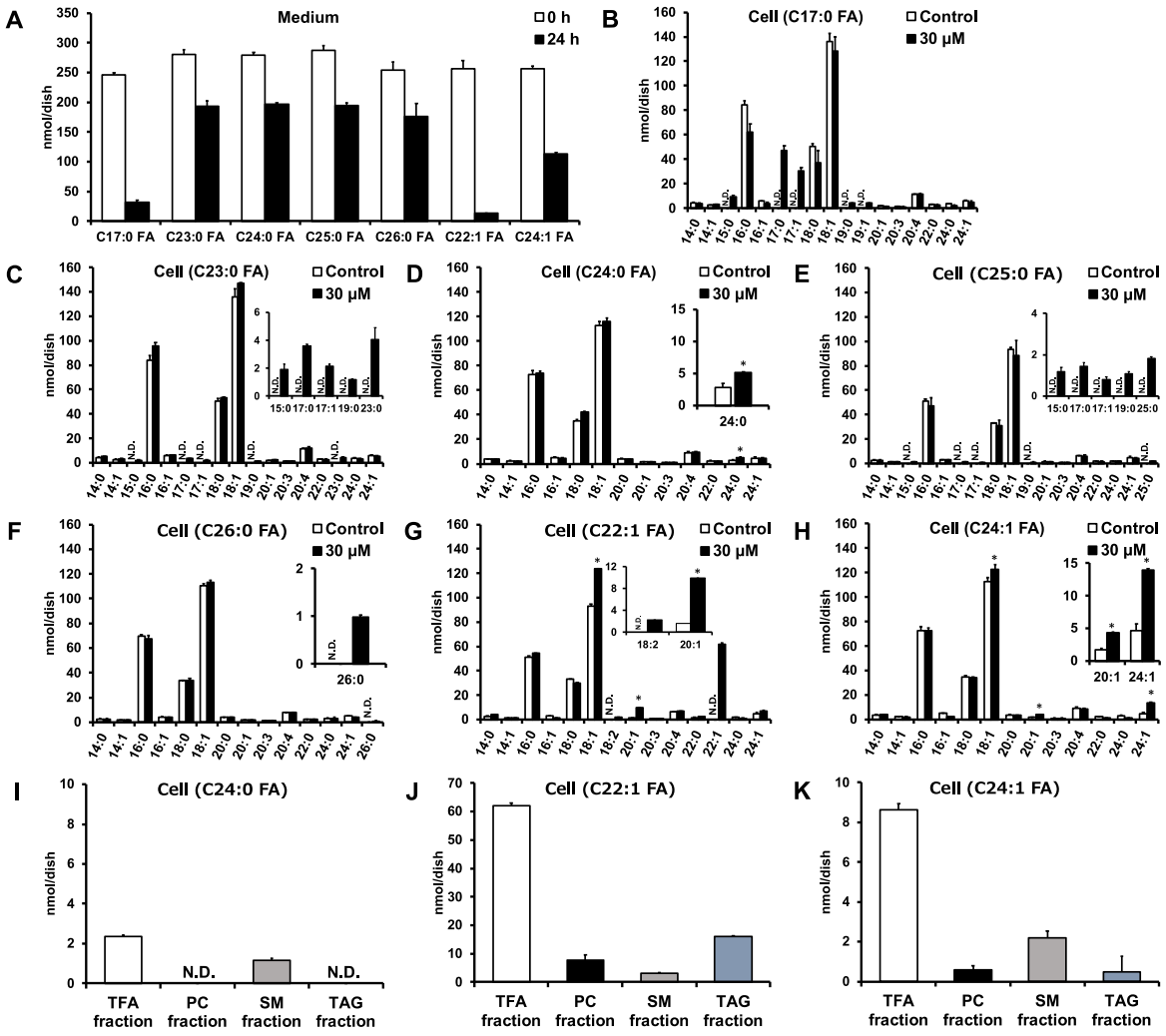


Fig. 7. Cellular uptake of FAs by IFRS1 cells.

IFRS1 cells were incubated with 30 μM C17:0 FA, C23:0 FA, C24:0 FA, C25:0 FA, C26:0 FA, C22:1 FA, or C24:1 FA in serum-free medium for 24 h. Medium (A) and cells (B-H) were collected separately at the respective time point and subjected to lipid extraction. The fatty acid methyl esters prepared from total lipids (B-H), and PC, SM, and TAG fractions of the cells (I-K) were analyzed after separation of each lipid class by TLC. The fatty acid profiles shown in B-H were obtained from cells incubated for 24 h. Values are means ± S.D. of three independent experiments. TFA: total fatty acid, PC: phosphatidylcholine, SM: sphingomyelin, TAG: triacylglycerol. Significance was analyzed by Student's *t* test. **p* < 0.05 indicates the significant difference between indicated groups. N.D.: not detected.

4. Discussion

The development of myelination has been shown to be accompanied by VLCFAs synthesis. However, abnormal accumulation of VLCFAs seems to associate with impaired myelination as observed in several types of peroxisome diseases [6,16,17]. Despite the importance of VLCFAs in the integrity of neuronal tissue, cellular events triggered by the accumulation of VLCFAs are not fully understood. One of the difficulties in the biological study of VLCFAs is that they do not dissolve in aqueous medium, especially S-VLCFAs. In the present study, we developed a simple method to solubilize VLCFAs in an aqueous medium using IP with BSA. An important point in the preparation of VLCFA/BSA complex is the avoidance of the aggregation of VLCFAs. Once it occurs in an aqueous solution, complexing with albumin is difficult. Adding well-dispersed VLCFAs into a well-dispersed BSA solution is a key technique. This is suggested by the fact that mixing these two solutions at room temperature (~20 °C) does not completely solubilize VLCFAs. Warming (37 °C) both solutions followed by sonication and rewarming, was essential. Alfa-CD has been utilized to deliver VLCFAs into cells [29-32]. However, higher concentrations of VLCFAs do not seem to favour the formation of water-soluble complexes in the presence of α-CD; therefore, α-CD provides only a limited solution for delivery of VLCFAs, as we observed.

Using the improved solubilization method of VLCFAs, we characterized the effect of peroxisome-deficiency on the uptake and metabolism of VLCFAs in CHO cells. We found that extracellular S-VLCFAs were incorporated and immediately degraded by peroxisomes in wild-type cells. As a result, the levels of S-VLCFAs were quite low in cellular lipids despite extensive uptake into CHO cells. Peroxisome-deficient cells also maintained cellular S-VLCFAs but at very low levels by inhibiting extracellular S-VLCFAs uptake. The difference in the capacity of cellular uptake between wild-type

and peroxisome-deficient cells resulted in a remarkable difference in the extracellular concentration of VLCFAs. In wild-type cells, the levels of extracellular S-VLCFAs decreased with the incubation time, whereas they were unchanged in the peroxisome-deficient CHO cells. We designated the uptake and metabolism of exogenous S-VLCFAs in wild-type cells and peroxisome-deficient cells to "uptake and peroxisome-oxidation" and "non-uptake", respectively, as shown in Fig. 8. In contrast to these S-VLCFAs, the capacity to incorporate MU-VLCFAs (C24:1 FA and C22:1 FA) in peroxisome-deficient cells was high. Most of the incorporated MU-VLCFAs was found to be acylated in the peroxisome-deficient cells. From these results, we designated the uptake and metabolism of MU-VLCFAs exogenous in peroxisome-deficient cells as "limited uptake and acylation" (Fig. 8). We also found that the amounts of LCFA (C17:0 FA) disappeared from the culture medium and the metabolic profile of cellular lipids in peroxisome-deficient CHO cells were almost the same as those of wild-type CHO cells (Fig. 3), indicating that peroxisomes do not contribute to the incorporation and metabolism of LCFA.

Numerous studies on lipids of fibroblasts of Zellweger syndrome patients, patients of peroxisomal β-oxidation disorders and their model mice showed elevated level of VLCFAs in both glycerolipids and sphingolipids [7-15]. Considering exogenous S-VLCFA's inability to enter into the cells, free form of the exogenous S-VLCFAs may not be main source of elevated S-VLCFAs of body cells. One of possible sources of the S-VLCFAs is endogenous biosynthesis. This notion is consistent with the fact that gene silencing of ELOVL1 effectively reduce VLCFAs in fibroblasts from peroxisomal β-oxidation disorders [10]. Another possible source of the S-VLCFAs is VLCFA-containing glycerolipid or sphingolipid associated with lipoprotein in blood. In this regard, we observed significantly elevated level of C24- and C26-ceramide in plasma of X-linked adrenoleukodystrophy

(X-ALD) patients (unpublished data).

In this study, we showed that a large portion of VLCFAs taken up into the cells was degraded in a peroxisome-dependent manner in CHO cells (Fig. 4). We also detected the chain-shortened metabolites of the VLCFAs added to the cells (Fig. 2, 5, 6). These results are consistent with the facts that peroxisome is essential organelle for degradation of VLCFAs [33-37] and that chain-shortening metabolites produced in peroxisomes are used as cellular lipids as observed in the metabolism of C22:1 FA [38-41]. The physiological role of chain-shortening of VLCFAs in peroxisome had been considered as conversion to LCFA that is available for mitochondrial oxidation. However, peroxisomal metabolite-derived carbons seems to be preferentially used for *de novo* synthesis of FA and cholesterol [40-43]. This fact indicates that providing building block of lipid synthesis is also physiological significance of peroxisomal degradation of FA as proposed in [42]. This notion is consistent with our previous observation that gymnosperm-derived C20 polymethylene-interrupted FAs are converted to C18 essential fatty acids by sequential metabolism of peroxisomal oxidation followed by microsomal FA chain-elongation [21].

It should be noted that the analysis of the remaining FA in the medium is important to determine the extent of uptake in our assay system, in which labelled FA was not used. We could not realize the extensive incorporation of VLCFAs in wild-type cells until examining the FA in the medium, because the change in the level of VLCFAs, as well as the FA profile of cellular lipids, was almost similar to that of non-supplemented cells.

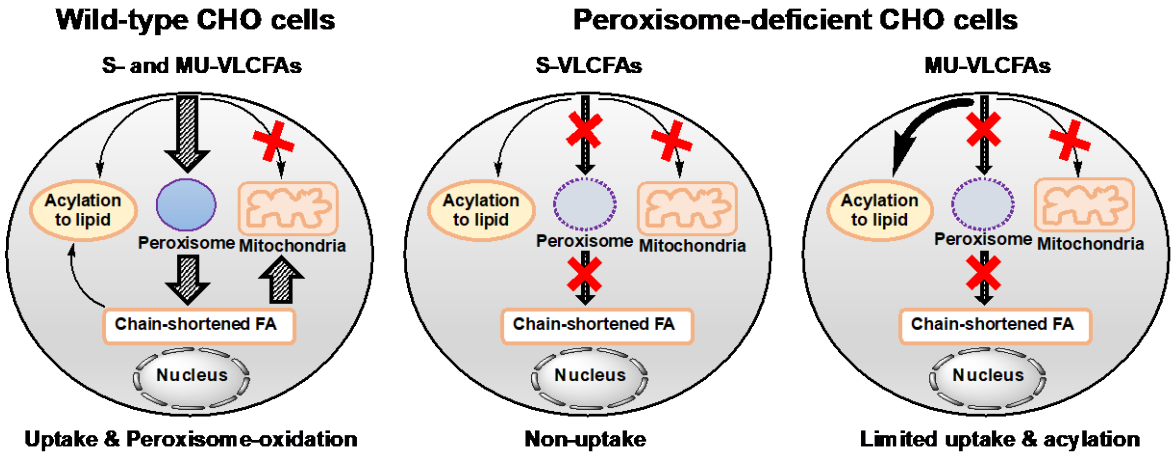


Fig. 8. Schematic presentation of metabolism of S- and MU-VLCFAs in peroxisome-deficient cells.

Given the uptake of LCFA or MU-VLCFAs into the peroxisome-deficient cells, inability of peroxisome-deficient cells to uptake extracellular S-VLCFAs is striking. This can be explained by the lacking of driving forces required for cellular uptake. The difference in the decrement of S-VLCFAs from the medium of wild-type cells and that of peroxisome-deficient cells suggests that peroxisomal oxidation is a strong driving force for uptaking of exogenous S-VLCFAs. Similarly, difference in the decrements of S-VLCFAs from the medium of the peroxisome-deficient cells and that of MU-VLCFAs from the medium of the cells suggest that acylation to cellular lipids is also working as a driving force for VLCFAs entry. The S-VLCFA's inability to enter into the cells as observed in the peroxisome-deficient cells may be attributable to the lack of these driving forces. Another possible mechanism for S-VLCFA's entry is regulatory action of membrane-associated FA-binding proteins (FABP) or FA transporters (FATP). Recent investigation revealed that protein-mediated mechanism operates for FA entry [44,45]. FAT/CD36 have been shown to be present in a variety of mammalian cell types and involved in the entry of various FAs including VLCFAs [46]. It has been reported that

the cellular FA uptake rate is governed primarily by the presence of CD36 at the cell surface, and that the subcellular recycling of the CD36 is mediated by AMP-activated kinase and insulin signaling cascades [47]. FA transporter 1 (FATP1) is also known to transport VLCFAs. The mobilization of FATP1 is shown to be regulated by cellular signaling just like the case of glucose transporter 4 [48]. Although volume of intracellular pool of S-VLCFAs may be very low, the intracellular pool of S-VLCFAs may affect the signaling systems to halt uptake of it.

In this study, we showed that VLCFAs are taken up by IFRS1 cells, Schwann cell-derived cell line. The amounts of S-VLCFAs and MU-VLCFAs taken up from the medium and the levels of their acylation in IFRS1 cells were comparable to those of CHO cells. These results indicate the possibility that similar regulation of the uptake and intracellular metabolism of VLCFAs operates in Schwann cells. This notion is consistent with other reports showing that myelinating cells have the ability to uptake FAs via CD36, FATP1, and FATP4 [49,50].

The peroxisome-deficient cell line used in this study was generated by deletion of *Pex5*, a causative gene of Zellweger syndrome. Here, we showed that the *Pex5*-deficient cells cannot uptake extracellular S-VLCFAs, suggesting that Zellweger syndrome patients cannot eliminate extracellular S-VLCFAs. The major neurological symptoms of Zellweger syndrome are weak hypotonia, hearing and vision loss, and seizures. These symptoms are considered to be caused by the under-development of myelin [51-53]. It is worthwhile to examine the effect of high concentrations of extracellular VLCFAs on myelination by oligodendrocytes.

X-ALD is the most common peroxisome disease, with several types of symptoms. The causative gene is *Abcd1*, which encodes a VLCFA-specific transporter localized in peroxisomes [54,55]. Because of the functional defect of oxidation of VLCFAs in peroxisome, X-ALD patients accumulate

VLCFAs in the body [56]. Childhood cerebrum, the most common clinical type of X-ALD (45% of total X-ALD), is characterized by progressive demyelination of the CNS [6,16,17]. At present, the effective treatment of X-ALD is the transplantation of healthy bone marrow before the onset of demyelination. Considering our results, it may be possible to consider that microglial cells recruited from healthy bone marrow at certain conditions would effectively eliminate the accumulated VLCFAs by their normal peroxisome activity in neuronal tissue.

In summary, we developed a method for solubilizing VLCFAs in aqueous medium by facilitating formation of VLCFA/BSA complex. Using this approach, we showed capacities of entry of exogenous VLCFAs in wild type cells and peroxisome-deficient cells. We also demonstrated for the first time that peroxisome-deficient CHO cells almost lost the ability for clearance of S-VLCFAs accumulated outside of the cells. Our results indicate that peroxisomes play important role in the clearance of S-VLCFAs accumulated inside and outside of the cells.

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