# Study on metabolism of very long-chain fatty acids in peroxisomes and their related disease

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## Chapter 2

**Peroxisomes attenuate lipotoxicity of intermediate very long-chain fatty acids and monounsaturated very long-chain fatty acids**

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# Abbreviations

| | |
|---|---|
| α-CD | α-cyclodextrin |
| ACOXs | Acyl-CoA oxidase |
| C | Cellular lipids |
| 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 |
| LCFAs | Long-chain fatty acids |
| M | Medium |
| MU-VLCFAs | Monounsaturated very long-chain fatty acids |
| Na | NaOH |
| PC | Phosphatidylcholine |
| PE | Phosphatidylethanolamine |
| ROS | Reactive oxygen species |
| S-VLCFAs | Saturated very long-chain fatty acids |
| SM | Sphingomyelin |
| SLs | Sphingolipids |
| TDYA | 10,12-tricosadiynoic acid |
| TFA | Total fatty acid |
| TAG | Triacylglycerol |
| VLCFAs | Very long-chain fatty acids |
| X-ALD | X-linked adrenoleukodystrophy |
| ZS | Zellweger syndrome |

# General introduction

# General introduction

The predominant cellular fatty acids (FAs) in animals are long-chain fatty acids (LCFAs) with chain-length of C16–C18 and polyunsaturated FA with chain-length of C18-C22. FAs longer than C20 are grouped as very long-chain fatty acids (VLCFAs). The most abundant VLCFAs in animals are lignoceric acid (C24:0) and nervonic acid (C24:1) which are found exclusively as major N-acyl residues of sphingolipids [1-3]. VLCFAs are synthesized through a four-step elongation process by endoplasmic reticulum membrane embedded enzymes. The first condensation reaction catalyzed by the enzyme ELOVL is the rate-limiting step [4]. To date, seven mammalian ELOVL enzymes (ELOVL1-7) with distinct substrate specificities are identified. Among them, ELOVL1 is mainly responsible for the assembly of VLCFAs (C24-C26) [4].

Peroxisome is a single membrane organelle with a size ranging from 0.1 to 1 μm in diameter existing in virtually all eukaryotic cells [5]. The peroxisomes were first discovered by Rhodin in 1954 under an electron microscopy in mouse renal cells as functionally uncharacterized organelle, called microbodies [6]. Since then, many oxidative metabolisms intrinsic to this organelle have been identified, and now they are called peroxisomes. They play crucial roles in a variety of metabolic processes such as lipid metabolism. One of them is a β-oxidation of FAs, especially VLCFAs (C≥20) and branched-chain FAs [7].

Metabolic breakdown of VLCFAs takes place only in peroxisomes. Peroxisomal β-oxidation is accomplished through four consecutive phases, including desaturation, hydration, dehydrogenation and thiolytic cleavage. Acyl-CoA oxidase (ACOXs), namely ACOX1 and ACOX2, L- and D-bifunctional protein (LBP and DBP), and the thiolase 3-oxoacyl-CoA thiolase (pTH1) and sterol carrier protein X (pTH2) are required for the above-mentioned steps [8]. ACOX1 reacts with the CoA ester of VLCFAs, while ACOX2 degrades branched-chain FAs. DBP is found to catalyze the hydration and subsequent dehydrogenation of peroxisomal β-oxidation substrates, including the enoyl-CoA esters of VLCFAs. pTH1 is known to be involved in thiolytic cleavage of CoA ester of straight-chain FAs, whereas pTH2 acts on that of branched-chain FAs and bile acid precursors [9,10].

Peroxisomes play an essential role in normal human development and maintenance of specific organ functions including neurons, adrenal, liver and kidney. This is evident from the fact that absence of functional peroxisomes leads to heterogeneous and devastating genetic disorders [11]. These include single peroxisomal enzyme deficiency and peroxisome biogenesis disorder. Zellweger syndrome (ZS) is a peroxisome biogenesis disorder caused by impaired peroxisomal functions accompanied with accumulation of VLCFAs as biochemical characteristics [11]. The most common phenotypes of ZS are profound neurological abnormalities such as neural migration defects and dysmyelination [11]. Acyl-CoA oxidase 1 and D-bifunctional protein are peroxisomal enzymes that are required for the peroxisomal β-oxidation. These proteins deficiency results in excessive accumulation of VLCFAs, and show severe neurodegeneration [11]. X-linked adrenoleukodystrophy (X-ALD) is a metabolic disorder characterized by defects in the ABCD1 genes, which encodes peroxisomal membrane protein ALDP [12,13]. ALDP is involved to transport VLCFAs (as VLCFA-CoA) into the peroxisome [12,13], and the loss of its function results in elevated levels of VLCFAs in the plasma and tissues [14]. The abnormal accumulation of saturated VLCFAs, specifically C24:0 FA and C26:0 FA, is thought to directly contribute to the pathogenesis of X-ALD such as demyelination with inflammation and oxidative damage [15-18]. However, the relation of abnormal accumulation of VLCFAs to progression of symptoms of the peroxisome diseases, such as demyelination/neurodegeneration remains largely unclear.

To understand the relationship between VLCFAs accumulation and the clinical symptoms of X-ALD, it is important to know the impact of VLCFAs accumulation in different cells, including neural cells. However, metabolic regulation of endogenous or exogenous VLCFAs, and cellular events induced by abnormally accumulated VLCFAs have not fully understood yet. One of difficulties of biological experiments on VLCFAs is extremely low aqueous solubility of VLCFAs. In fact, dissolving them into aqueous medium is a major challenge.

In this study, I developed a method for solubilizing VLCFAs in aqueous medium. Using this improved method, I characterized the uptake and metabolism of VLCFAs in peroxisome-deficient Chinese Hamster Ovary (CHO) cells, its wild-type cells and IFRS1 cells, Schwann cell-derived cell line. I also examined the effect of VLCFAs on viability of cultured cells. Results showed that elongated VLCFAs (C24:0 FA and C26:0 FA) are not toxic but rather anti-apoptotic to all the cells

examined. On the other hand, intermediate VLCFAs, such as C20:0 FA, or monounsaturated-VLCFAs, such as C24:1 FA, accumulated in peroxisome-deficient CHO cells and induce apoptosis of the cells. From these observations, it is concluded that peroxisomal β-oxidation plays an important role in prevention of accumulation of apoptotic VLCFAs.

# References


[1] A. Poulos, Very long chain fatty acids in higher animals--a review, Lipids 30 (1995) 1-14. https://doi.org/10.1007/BF02537036.

[2] A. Kihara, Very long-chain fatty acids: elongation, physiology and related disorders, J. Biochem. 152 (2012) 387-395. https://doi.org/10.1093/jb/mvs105.

[3] J.S. O'Brien, E.L. Sampson, Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter, and myelin, J. Lipid Res. 6 (1965) 545-551. https://doi.org/10.1016/S0022-2275(20)39620-6.

[4] Y. Ohno, S. Suto, M. Yamanaka, Y. Mizutani, S. Mitsutake, Y. Igarashi, T. Sassa, A. Kihara, ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis, Proc. Natl. Acad. Sci. USA 107 (2010) 18439-18444. https://doi.org/10.1073/pnas.1005572107.

[5] W.H. Kunau, V. Dommes, H. Schulz, beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress, Prog. Lipid Res. 34 (1995) 267-342. https://doi.org/10.1016/0163-7827(95)00011-9.

[6] C. De Duve, P. Baudhuin, Peroxisomes (microbodies and related particles), Physiol. Rev. 46 (1966) 323-357. https://doi.org/10.1152/physrev.1966.46.2.323.

[7] P.P.V. Veldhoven, Biochemistry and genetics of inherited disorders of peroxisomal fatty acid metabolism, J. Lipid Res. 51 (2010) 2863–2895. https://doi.org/10.1194/jlr.R005959.

[8] R.J. Wanders, B.T. Poll-The, Role of peroxisomes in human lipid metabolism and its importance for neurological development, Neurosci. Lett. 637 (2017) 11–17. https://doi.org/10.1016/j. neulet.2015.06.018.

[9] R.J. Wanders, Peroxisomes, lipid metabolism, and peroxisomal disorders, Mol. Genet. Metab. 83 (2004) 16–27. https://doi.org/10.1016/j.ymgme.2004.08.016.

[10] R.J. Wanders, P. Vreken, S. Ferdinandusse, G.A. Jansen, H.R. Waterham, C.W. van Roermund, E.G. Van Grunsven, Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. Biochem. Soc. Trans. 29 (2001) 250-267. https://doi.org/10.1042/0300-5127:0290250.

[11] P.P.V. Veldhoven, Biochemistry and genetics of inherited disorders of peroxisomal fatty acid metabolism, J. Lipid Res. 51 (2010) 2863–2895. https://doi.org/10.1194/jlr.R005959.

[12] C. Wiesinger, M. Kunze, G. Regelsberger, S. Forss-Petter, J. Berger, Impaired very long-chain acyl-CoA β-oxidation in human X-linked adrenoleukodystrophy fibroblasts is a direct consequence of ABCD1 transporter dysfunction, J. Biol. Chem. 288 (2013) 19269-19279. https://doi.org/10.1074/jbc.M112.445445

[13] M. Morita, N. Shimozawa, Y. Kashiwayama, Y. Suzuki, T. Imanaka, ABC subfamily D proteins and very long chain fatty acid metabolism as novel targets in adrenoleukodystrophy, Curr. Drug Targets 12 (2011) 694-706. https://doi.org/10.2174/138945011795378577.

[14] S. Kemp, J. Berger, P. Aubourg, X-linked adrenoleukodystrophy: Clinical, metabolic, genetic and pathophysiological aspects, Biochim. Biophys. Acta 1822 (2012) 1465-1474. https://doi.org/10.1016/j.bbadis.2012.03.012.

[15] M. Engelen, S. Kemp, M. de Visser, B.M. van Geel, R.J. Wanders, P. Aubourg, B.T. Poll-The, X-linked adrenoleukodystrophy (X-ALD): clinical presentation and guidelines for diagnosis, follow-up and management, Orphanet J. Rare Dis. 13 (2012) 7:51. https://doi.org/10.1186/1750-1172-7-51.

[16] I. Ferrer, J.P. Aubourg, A. Pujol, General aspects and neuropathology of X-linked adrenoleukodystrophy, Brain Pathol. 20 (2010) 817-830. https://doi.org/10.1111/j.1750-3639.2010.00390.x.

[17] A.S. Paintlia, A.G. Gilg, M. Khan, A.K. Singh, E. Barbosa, I. Singh, Correlation of very long chain fatty acid accumulation and inflammatory disease progression in childhood X-ALD: implications for potential therapies, Neurobiol. Dis. 14 (2003) 425-439. https://doi.org/10.1016/j.nbd.2003.08.013.

[18] C.R. Vargas, M. Wajner, L.R. Sirtori, L. Goulart, M. Chiochetta, D. Coelho, A. Latini, S. Llesuy, A. Bello-Klein, R. Giugliani, M. Deon, C.F. Mello, Evidence that oxidative stress is increased in patients with X-linked adrenoleukodystrophy, Biochim. Biophys. Acta. 1688 (2004) 26-32. https://doi.org/10.1016/j.bbadis.2003.10.004.

# *CHAPTER-1*

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

# 1.1 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, I 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, I 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. I also showed for the first time that peroxisome-deficient cells are almost totally lost clearance ability of S-VLCFAs accumulated outside of the cells.

# 1.2 Materials and methods

### 1.2.1 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.

### 1.2.2 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.

### 1.2.3 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 isopropanol 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).

### 1.2.4 Cytotoxicity assay

The CHO-K1 cells were seeded in 35-mm plastic dishes at a density of 2 x $10^5$ 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.

### 1.2.5 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.

### 1.2.6 FA uptake assay

CHO cells or IFRS1 cells were seeded into 100 mm petri dishes with 10 mL of FBS-containing medium at 1 x $10^6$ 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.

### 1.2.7 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.

### 1.2.8 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.

# 1.3 Results

### 1.3.1 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 medium. 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. I 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, I 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 Fig. 2, 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. I 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.

I 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 approximately 100 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. I 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).

I found that up to 2% of IP did not affect the viability of the CHO cells (Fig. 1D). Based on these experiments, I 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.

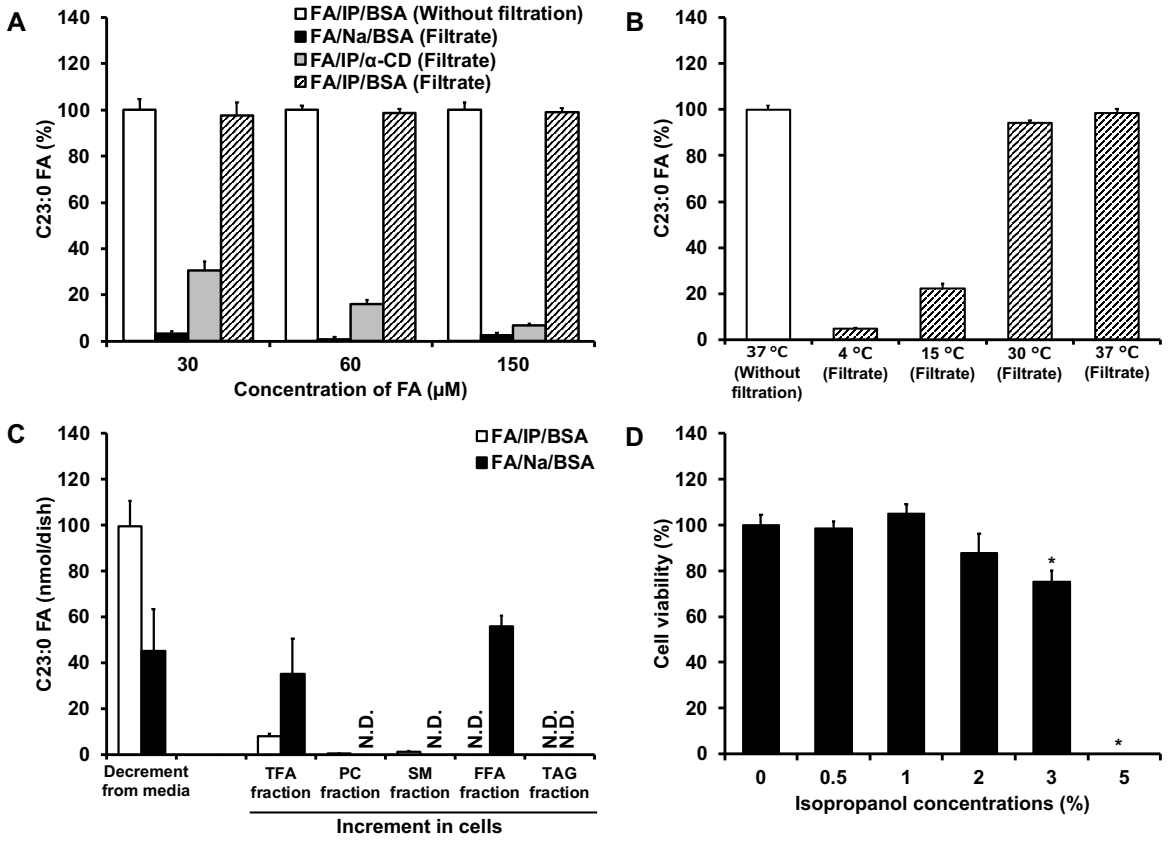



**Fig. 1 IP facilitates formation of VLCFA/BSA complex and enables uptake of VLCFA into the cells.**

(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 isopropanol 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 isopropanol 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.

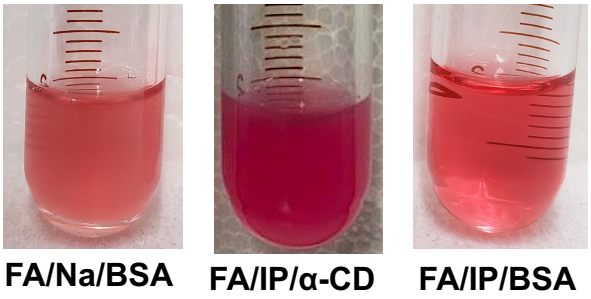



**Fig. 2 Photographs of the C23:0 FA solution before filtration.**

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 isopropanol were mixed with 3 mM α-cyclodextrin solution (FA/IP/α-CD) or BSA solution (FA/IP/BSA).

### 1.3.2 Cellular uptake of C23:0 FA in CHO-K1 and CHO-zp102 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). I 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 media 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. 3A left). The same experiment was conducted using peroxisome-deficient cells. I 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. 3A left). I 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 the C23:0 FA was under detectable level during the incubation (Fig. 3A right). I 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. 3B left, Fig. 3C), 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. 3B 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.

I 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. 3D). 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.

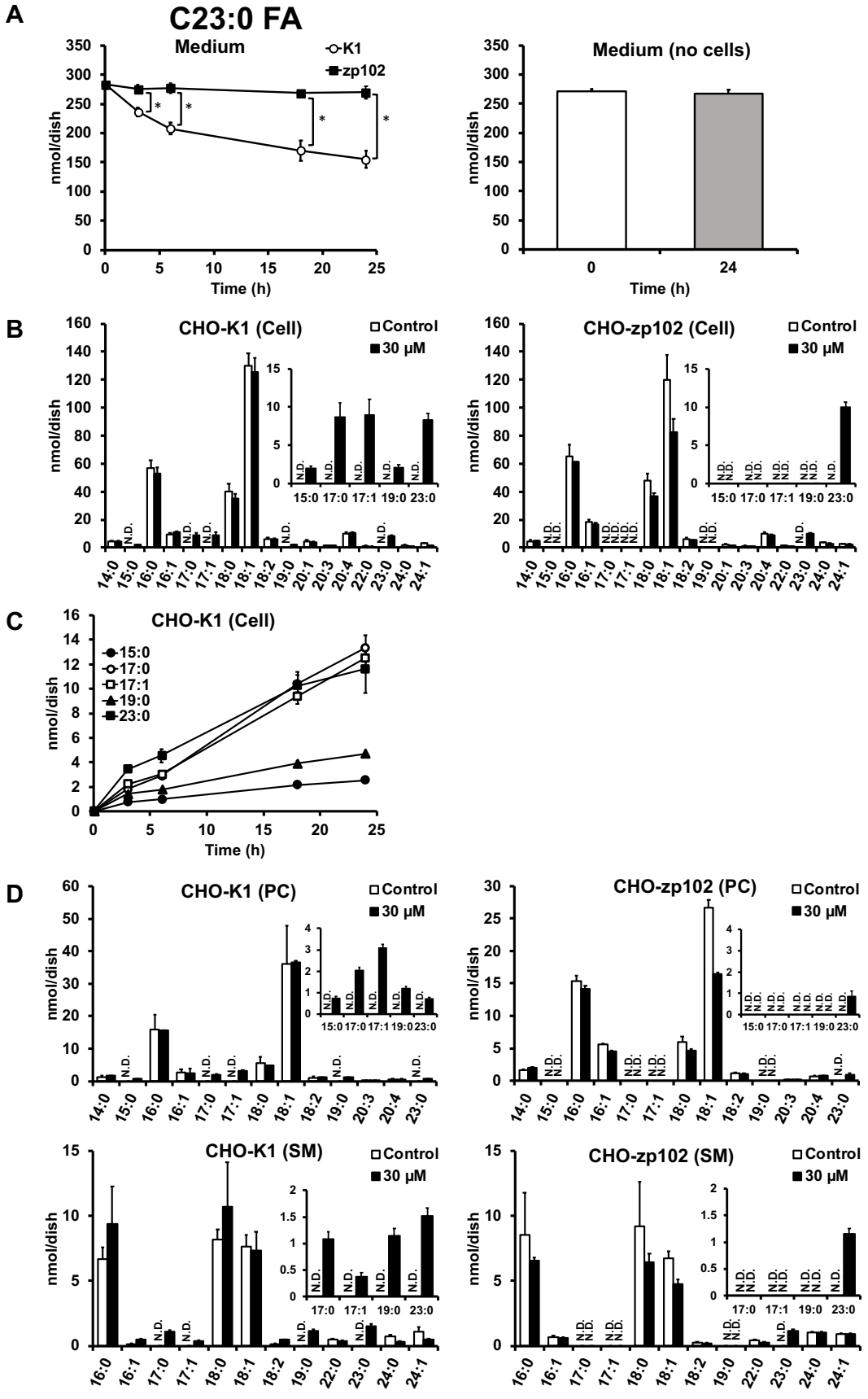

A
C23:0 FA
Medium
K1
zp102
nmol/dish
Time (h)
Medium (no cells)
B
CHO-K1 (Cell)
CHO-zp102 (Cell)
Control
30 μM
15:0 17:0 17:1 19:0 23:0
C
CHO-K1 (Cell)
15:0
17:0
17:1
19:0
23:0
D
CHO-K1 (PC)
CHO-zp102 (PC)
CHO-K1 (SM)
CHO-zp102 (SM)

**Fig. 3 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.

### 1.3.3 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. 4A). 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. 4A). 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. 4B). These FAs increased along with an increase in the uptake of C17:0 FA into the cells of wild-type cells (Fig. 4C), 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. 4D).

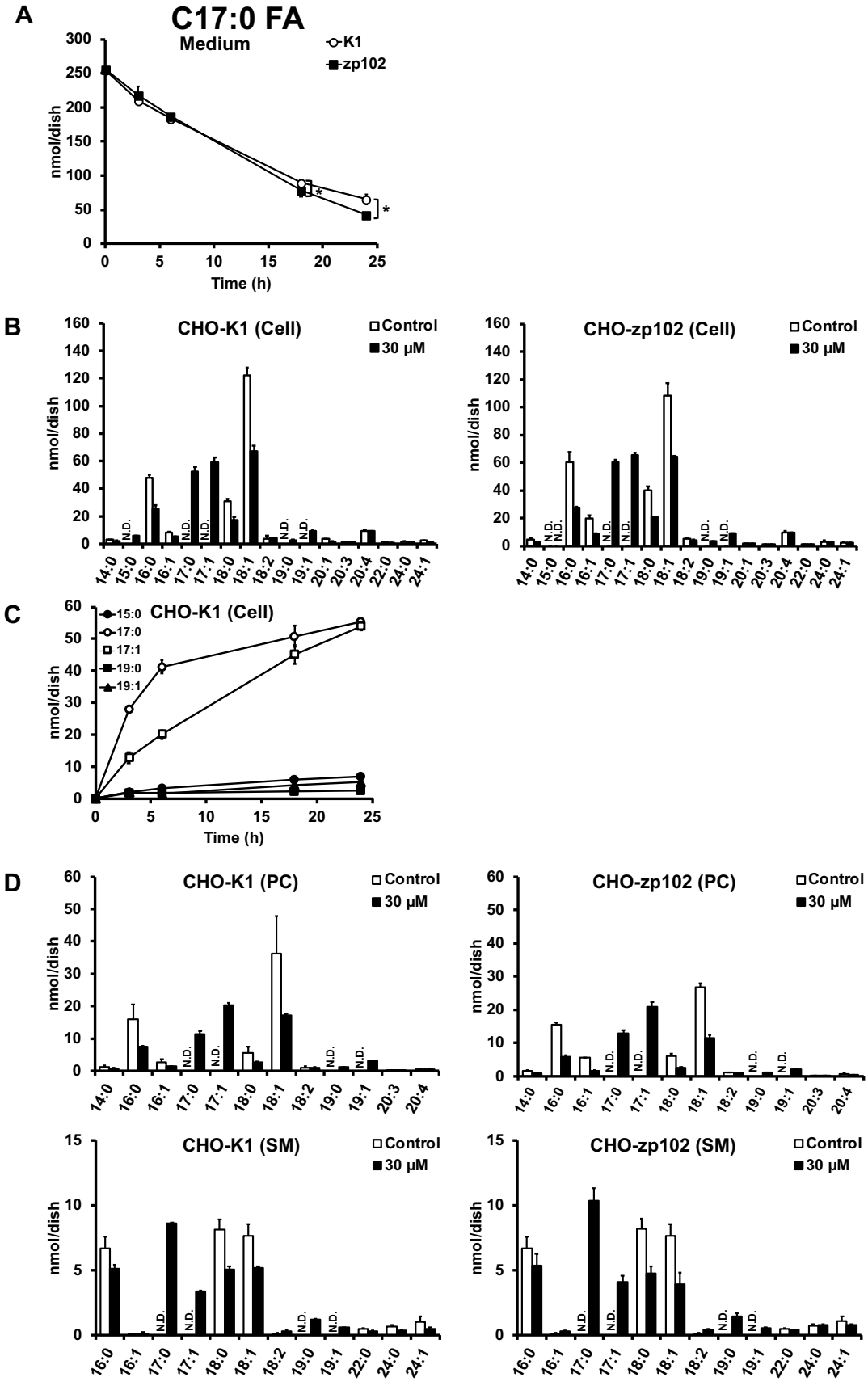

A
C17:0 FA
Medium
K1
zp102
nmol/dish
Time (h)
B
CHO-K1 (Cell)
CHO-zp102 (Cell)
Control
30 μM
C
CHO-K1 (Cell)
15:0
17:0
17:1
19:0
19:1
D
CHO-K1 (PC)
CHO-zp102 (PC)
CHO-K1 (SM)
CHO-zp102 (SM)
N.D.

**Fig. 4 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.

### 1.3.4 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. 5A). 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. 5B). 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. 5C, 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.

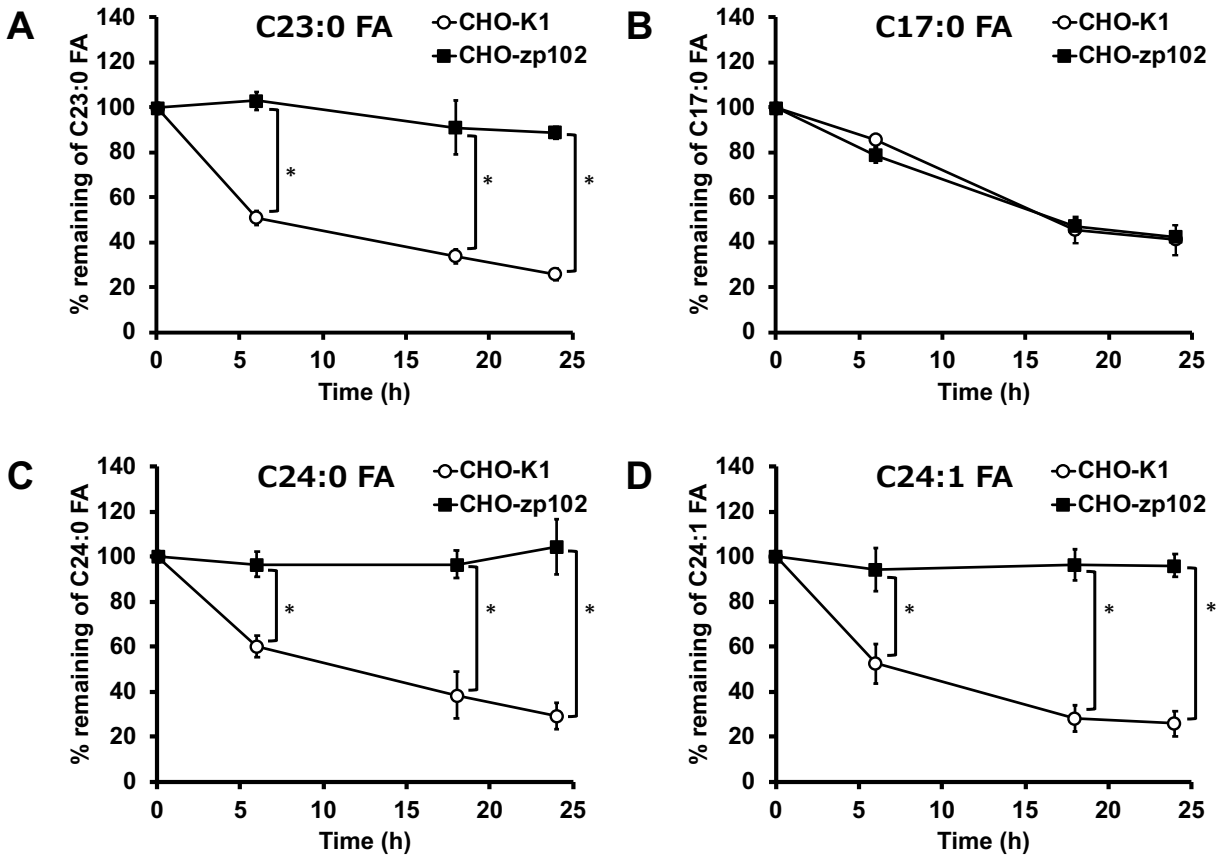



**Fig. 5 Time-dependent change in the incorporated FA in CHO-K1 and CHO-zp102 cells.**

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.

### 1.3.5 Metabolic difference between S-VLCFAs and MU-VLCFAs in CHO-zp102 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. 6A). 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. 6B). The accumulated exogenous C24:0 FA was mainly acylated into SM, but not in PC, PE or TAG fraction in both CHO cells (Fig. 6C). These results are essentially the same as those observed for C23:0 FA. I 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. 6D, E). The C26:0 FA was under detection level in PC, PE, SM, and TAG in both cell types (Fig. 6F).

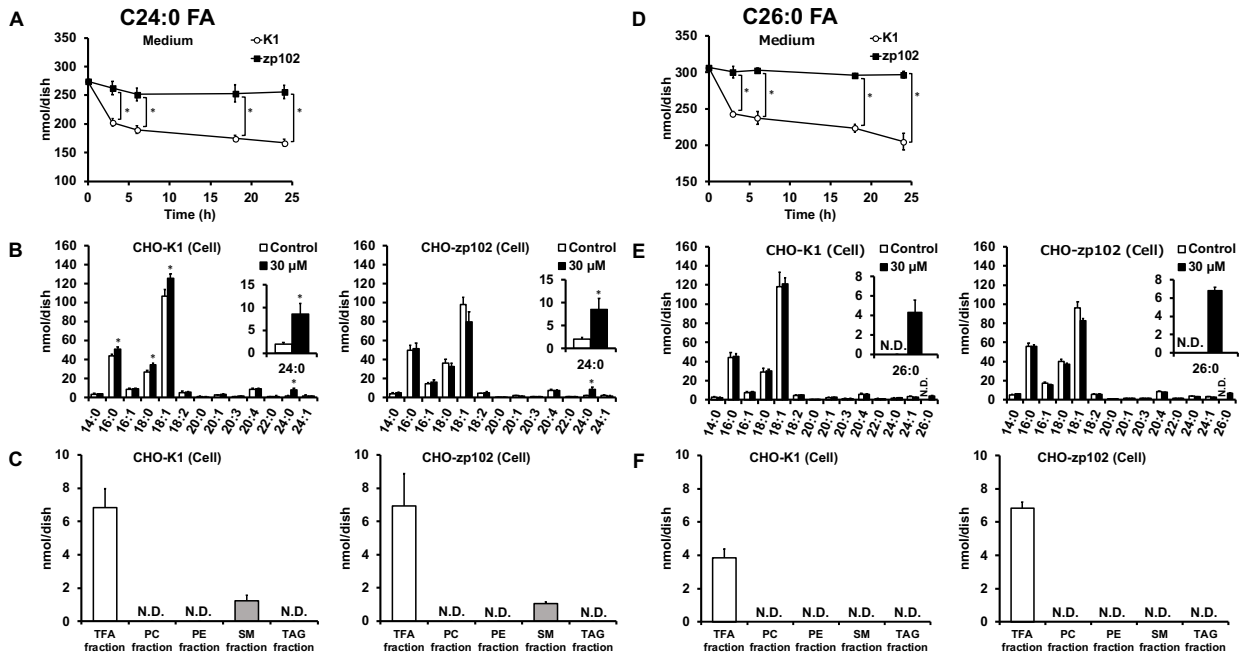



**Fig. 6 Cellular uptake of S-VLCFAs in CHO-K1 and CHO-zp102 cells.**

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. 7A 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. 7D, 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. 7B right, Fig. 7E right). In wild-type CHO cells, C24:1 FA was acylated to PC, SM, and TAG, but not in PE fraction. In contrast, C24:1 FA was acylated to PC, PE, SM, and TAG fraction in peroxisome-deficient cells (Fig. 7C). 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. 7F). 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. 7A-F).

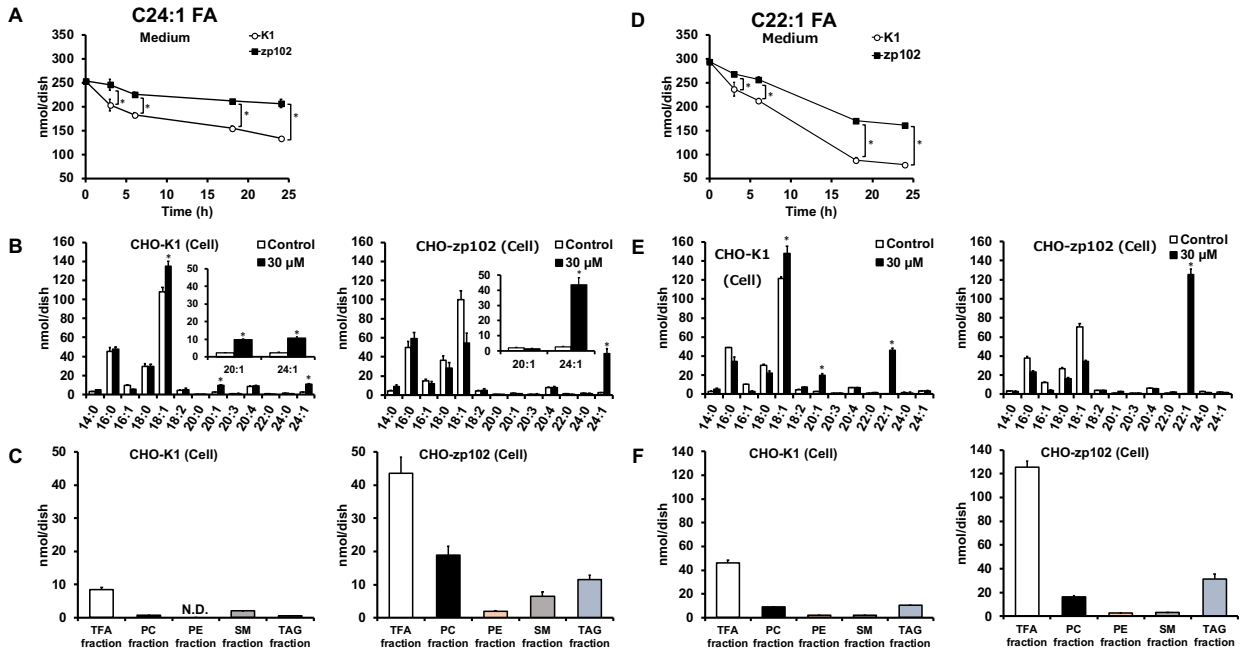



**Fig. 7 Cellular uptake of MU-VLCFAs in CHO-K1 and CHO-zp102 cells.**

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.

### 1.3.6 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. I investigated the cellular uptake and metabolism of exogenous VLCFAs in IFRS1 cells (Fig. 8). I 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. 8A). 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. 8B-H). I 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. 8C, 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. 8I). On the other hand, C22:1 FA and C24:1 FA were acylated to PC, SM and TAG fraction (Fig. 8J, K).

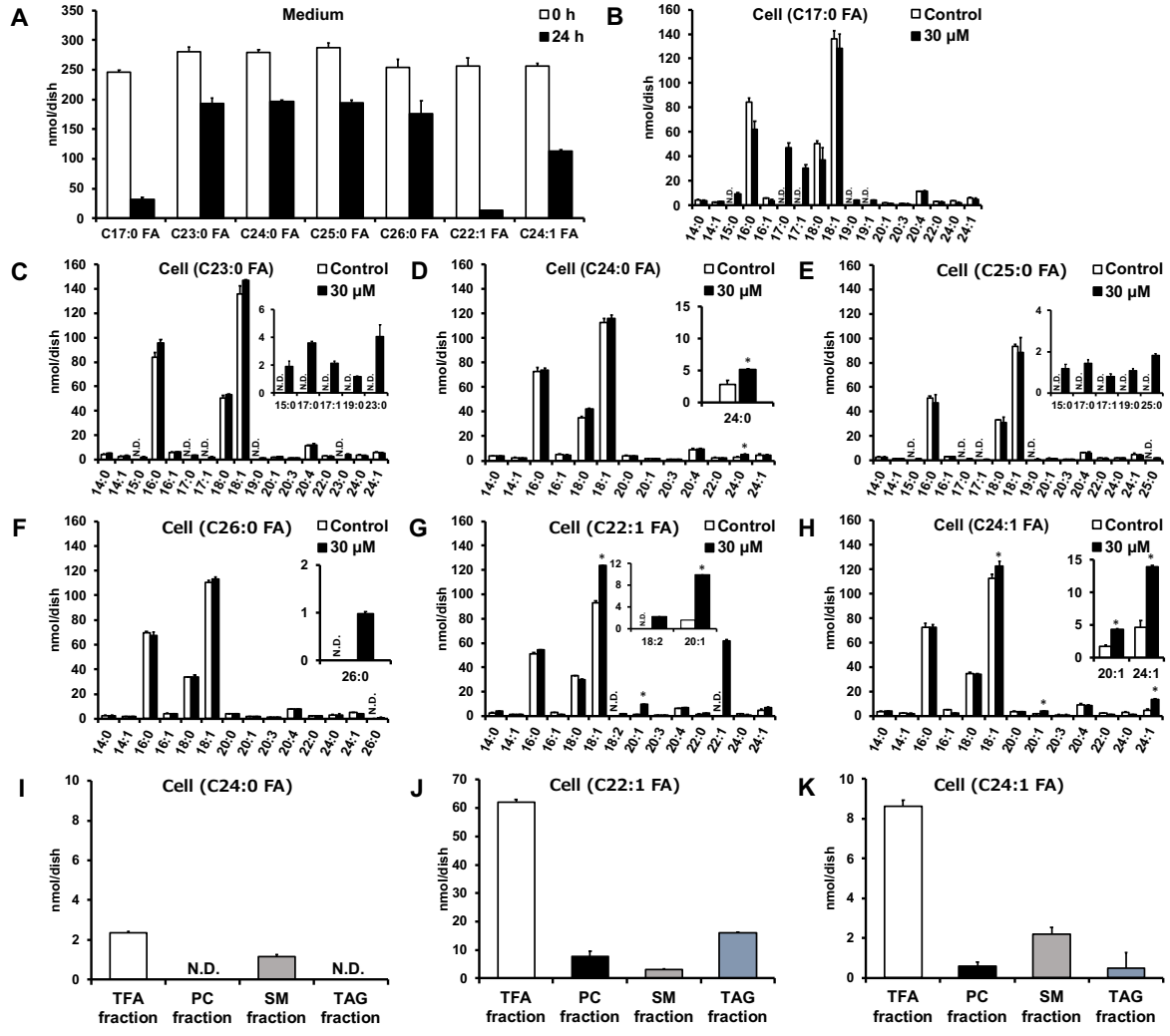



**Fig. 8 Cellular uptake of FAs by IFRS1 cells.**

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.

## 1.4 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, I 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 I observed.

Using the improved solubilization method of VLCFAs, I characterized the effect of peroxisome-deficiency on the uptake and metabolism of VLCFAs in CHO cells. I 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. I 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. 9. 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, I designated the uptake and metabolism of MU-VLCFAs exogenous in peroxisome-deficient cells as “limited uptake and acylation” (Fig. 9). I 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. 4), 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 the exogenous S-VLCFA’s inability to enter into the cells, free form of 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, I showed that a large portion of VLCFAs taken up into the cells was degraded in a peroxisome-dependent manner in CHO cells (Fig. 5). I also detected the chain-shortened metabolites of the VLCFAs added to the cells (Fig. 3, 6, 7). 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. I 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.

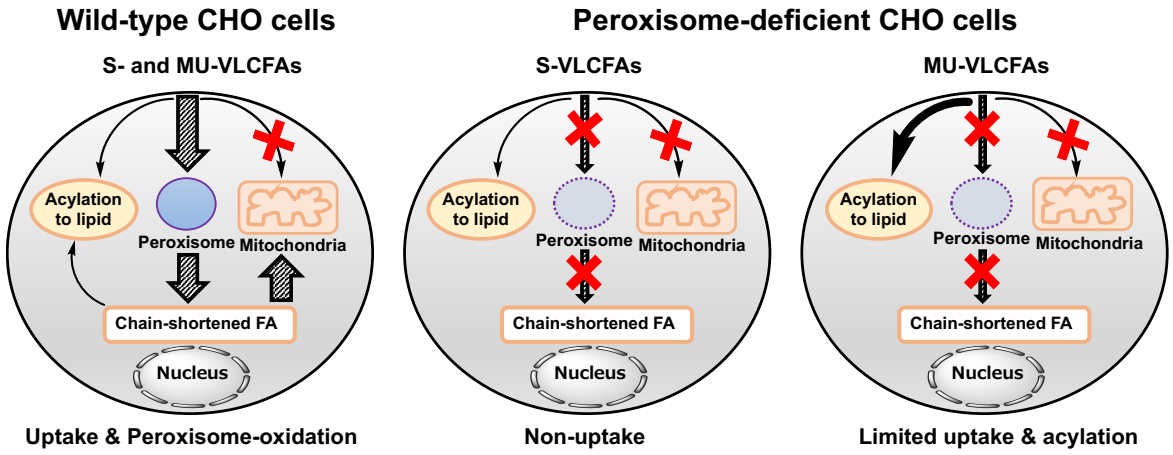



**Fig. 9 Schematic presentation of metabolism of S- and MU-VLCFAs in CHO-zp102 cells.**

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 lack 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 in 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 system to halt uptake of it.

In this study, I 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 peroxisomes 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 peroxisomes, 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 peroxisomes activity in neuronal tissue.

In summary, I developed a method for solubilizing VLCFAs in aqueous medium by facilitating formation of VLCFA/BSA complex. Using this approach, I showed capacities of entry of exogenous VLCFAs in wild-type cells and peroxisome-deficient cells. I 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.

# 1.5 References


[1] A. Poulos, Very long chain fatty acids in higher animals--a review, Lipids 30 (1995) 1-14. https://doi.org/10.1007/BF02537036.

[2] A. Kihara, Very long-chain fatty acids: elongation, physiology and related disorders, J. Biochem. 152 (2012) 387-395. https://doi.org/10.1093/jb/mvs105.

[3] J.S. O'Brien, E.L. Sampson, Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter, and myelin, J. Lipid Res. 6 (1965) 545-551. https://doi.org/10.1016/S0022-2275(20)39620-6.

[4] Y. Ohno, S. Suto, M. Yamanaka, Y. Mizutani, S. Mitsutake, Y. Igarashi, T. Sassa, A. Kihara, ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis, Proc. Natl. Acad. Sci. USA 107 (2010) 18439-18444. https://doi.org/10.1073/pnas.1005572107.

[5] M. Isokawa, T. Sassa, S. Hattori, T. Miyakawa, A. Kihara, Reduced chain length in myelin sphingolipids and poorer motor coordination in mice deficient in the fatty acid elongase Elovl1, FASEB Bioadv. 1 (2019) 747-759. https://doi.org/10.1096/fba.2019-00067.

[6] P.P.V. Veldhoven, Biochemistry and genetics of inherited disorders of peroxisomal fatty acid metabolism, J. Lipid Res. 51 (2010) 2863–2895. https://doi.org/10.1194/jlr.R005959.

[7] B.J. Pettus, M. Baes, M. Busman, Y.A. Hannun, P.P.V. Veldhoven, Mass spectrometric analysis of ceramide perturbations in brain and fibroblasts of mice and human patients with peroxisomal disorders, Rapid Commun. Mass Spectrom. 18 (2004) 1569-1574. https://doi.org/10.1002/rcm.1520.

[8] K. Hama, T. Nagai, C. Nishizawa C, K. Ikeda, M. Morita, N. Satoh, H. Nakanishi, T. Imanaka, N. Shimozawa, R. Taguchi, K. Inoue, K. Yokoyama, Molecular species of phospholipids with very long chain fatty acids in skin fibroblasts of Zellweger syndrome, Lipids 48 (2013) 1253-1267. https://doi.org/10.1007/s11745-013-3848-5.

[9] Y. Fujiwara, K. Hama, N. Shimozawa, K. Yokoyama, Glycosphingolipids with very long-chain fatty acids accumulate in fibroblasts from adrenoleukodystrophy patients, Int. J. Mol. Sci. 22 (2021) 8645. https://doi.org/10.3390/ijms22168645.

[10] Y. Abe, M. Honsho, H. Nakanishi, R. Taguchi, Y. Fujiki, Very-long-chain polyunsaturated fatty acids accumulate in phosphatidylcholine of fibroblasts from patients with Zellweger

syndrome and acyl-CoA oxidase1 deficiency, Biochim. Biophys. Acta 1841 (2014) 610-619. https://doi.org/10.1016/j.bbalip.2014.01.001.

[11] T. Kobayashi, N. Shinnoh, A. Kondo, T. Yamada, Adrenoleukodystrophy protein-deficient mice represent abnormality of very long chain fatty acid metabolism, Biochem. Biophys. Res. Commun. 232 (1997) 631-636. https://doi.org/10.1006/bbrc.1997.6340.

[12] S. Takashima, K. Toyoshi, T. Itoh, N. Kajiwara, A. Honda, A. Ohba, S. Takemoto, S. Yoshida, N. Shimozawa, Detection of unusual very-long-chain fatty acid and ether lipid derivatives in the fibroblasts and plasma of patients with peroxisomal diseases using liquid chromatography-mass spectrometry, Mol. Genet. Metab. 120 (2017) 255-268. https://doi.org/10.1016/j.ymgme.2016.12.013.

[13] A.B. Moser, N. Kreiter, L. Bezman, S. Lu, G.V. Raymond, S. Naidu, H.W. Moser, Plasma very long chain fatty acids in 3,000 peroxisome disease patients and 29,000 controls, Ann. Neurol. 45 (1999) 100-110. https://doi.org/10.1002/1531-8249(199901)45:1<100::aid-art16>3.0.co;2-u.

[14] W.C. Hubbard, A.B. Moser, A.C. Liu, R.O. Jones, S.J. Steinberg, F. Lorey, S.R. Panny, R.F. Vogt, D. Macaya, C.T. Turgeon, S. Tortorelli, G.V. Raymond, Newborn screening for X-linked adrenoleukodystrophy (X-ALD): validation of a combined liquid chromatography-tandem mass spectrometric (LC-MS/MS) method, Mol. Genet. Metab. 97 (2009) 212-220. https://doi.org/10.1016/j.ymgme.2009.03.010.

[15] K. Herzog, M.L. Pras-Raves, M.A. Vervaart, A.C. Luyf, A.C. van Kampen, R.J. Wanders, H.R. Waterham, F.M. Vaz, Lipidomic analysis of fibroblasts from Zellweger spectrum disorder patients identifies disease-specific phospholipid ratios, J. Lipid Res. 57 (2016) 1447-1454. https://doi.org/10.1194/jlr.M067470.

[16] M. Engelen, S. Kemp, M. de Visser, B.M. van Geel, R.J. Wanders, P. Aubourg, B.T. Poll-The, X-linked adrenoleukodystrophy (X-ALD): clinical presentation and guidelines for diagnosis, follow-up and management, Orphanet J. Rare Dis. 13 (2012) 7:51. https://doi.org/10.1186/1750-1172-7-51.

[17] I. Ferrer, J.P. Aubourg, A. Pujol, General aspects and neuropathology of X-linked adrenoleukodystrophy, Brain Pathol. 20 (2010) 817-830. https://doi.org/10.1111/j.1750-3639.2010.00390.x.

[18] Y. Poitelon, A.M. Kopec, S. Belin, Myelin fat facts: an overview of lipids and fatty acid metabolism, Cells 9 (2020) 812. https://doi.org/10.3390/cells9040812.

[19] J. López-Erauskin, J. Galino, M. Ruiz, J.M. Cuezva, I. Fabregat, D. Cacabelos, J. Boada, J. Martínez, I. Ferrer, R. Pamplona, F. Villarroya, M. Portero-Otín, S. Fourcade, A. Pujol, Impaired mitochondrial oxidative phosphorylation in the peroxisomal disease X-linked adrenoleukodystrophy, Hum. Mol. Genet. 22 (2013) 3296-3305. https://doi.org/10.1093/hmg/ddt186.

[20] M.C. van de Beek, R. Ofman, I. Dijkstra, F. Wijburg, M. Engelen, R. Wanders, S. Kemp, Lipid-induced endoplasmic reticulum stress in X-linked adrenoleukodystrophy, Biochim. Biophys. Acta Mol. Basis Dis. 1863 (2017) 2255-2265. https://doi.org/10.1016/j.bbadis.2017.06.003.

[21] T. Tanaka, J. Morishige, D. Iwawaki, T. Fukuhara, N. Hamamura, K. Hirano, T. Osumi, K. Satouchi, Metabolic pathway that produces essential fatty acids from polymethylene-interrupted polyunsaturated fatty acids in animal cells, FEBS J. 274 (2007) 2728-2737. https://doi.org/10.1111/j.1742-4658.2007.05807.x.

[22] T. Tanaka, S. Uozumi, K. Morito, T. Osumi, A. Tokumura, Metabolic conversion of C20 polymethylene-interrupted polyunsaturated fatty acids to essential fatty acids, Lipids 49 (2014) 423-429. https://doi.org/10.1007/s11745-014-3896-5.

[23] K. Morito, R. Shimizu, N. Kitamura, S.B. Park, S. Kishino, J. Ogawa, T. Fukuta, K. Kogure, T. Tanaka, Gut microbial metabolites of linoleic acid are metabolized by accelerated peroxisomal β-oxidation in mammalian cells, Biochim. Biophys. Acta 1864 (2019) 1619-1628. https://doi.org/10.1016/j.bbalip.2019.07.010.

[24] T. Tsukamoto, A. Bogaki, K. Okumoto, K. Tateishi, Y. Fujiki, N. Shimozawa, Y. Suzuki, N. Kondo, T. Osumi, Isolation of a new peroxisome-deficient CHO cell mutant defective in peroxisome targeting signal-1 receptor, Biochem. Biophys. Res. Commun. 230 (1997) 402–406. https://doi.org/10.1006/bbrc.1996.5971.

[25] M. Yamasaki, N. Hashiguchi, C. Fujiwara, T. Imanaka, T. Tsukamoto, T. Osumi, Formation of peroxisomes from peroxisomal ghosts in a peroxisome-deficient mammalian cell mutant upon complementation by protein microinjection, J. Biol. Chem. 274 (1999) 35293-35296. https://doi.org/10.1074/jbc.274.50.35293.

[26] I. Singh, Y. Kishimoto, Effect of cyclodextrins on the solubilization of lignoceric acid, ceramide, and cerebroside, and on the enzymatic reactions involving these compounds, J. Lipid Res. 24 (1983) 662–665. https://doi.org/10.1016/S0022-2275(20)37973-6.

[27] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917. https://doi.org/10.1139/o59-099.

[28] J.R. Paulsrud, S. Stewart, G. Graff, R.T. Holman, Desaturation of saturated fatty acids by rat liver microsomes, Lipids 5 (1970) 611-616. https://doi.org/10.1007/BF02531339.

[29] S. Hein, P. Schönfeld, S. Kahlert, G. Reiser, Toxic effects of X-linked adrenoleukodystrophy-associated, very long chain fatty acids on glial cells and neurons from rat hippocampus in culture, Hum. Mol. Genet. 17 (2008) 1750–1761. https://doi.org/10.1093/hmg/ddn066.

[30] M. Doria, T. Nury, D. Delmas, T. Moreau, G. Lizard, A. Vejux, Protective function of autophagy during VLCFA-induced cytotoxicity in a neurodegenerative cell model, Free Radic. Biol. Med. 137 (2019) 46-58. https://doi.org/10.1016/j.freeradbiomed.2019.04.016.

[31] T. Nury, M. Doria, G. Lizard, A. Vejux, Docosahexaenoic acid attenuates mitochondrial alterations and oxidative stress leading to cell death induced by very long-chain fatty acids in a mouse oligodendrocyte model, Int. J. Mol. Sci. 21 (2020) 641. https://doi.org/10.3390/ijms21020641.

[32] M. Baarine, K. Ragot, A. Athias, T. Nury, Z. Kattan, E.C. Genin, P. Andreoletti, F. Ménétrier, J.M. Riedinger, M. Bardou, G. Lizard, Incidence of Abcd1 level on the induction of cell death and organelle dysfunctions triggered by very long chain fatty acids and TNF-α on oligodendrocytes and astrocytes, Neurotoxicology 33 (2012) 212-228. https://doi.org/10.1016/j.neuro.2011.10.007.

[33] I. Singh, A.E. Moser, S. Goldfischer, H.W. Moser, Lignoceric acid is oxidized in the peroxisome: implications for the Zellweger cerebro-hepato-renal syndrome and adrenoleukodystrophy, Proc. Natl. Acad. Sci. USA 81 (1984) 4203-4207. https://doi.org/10.1073/pnas.81.13.4203.

[34] R. Sandhir, M. Khan, A. Chahal, I. Singh, Localization of nervonic acid beta-oxidation in human and rodent peroxisomes: impaired oxidation in Zellweger syndrome and X-linked adrenoleukodystrophy, J. Lipid Res. 39 (1998) 2161-2171. https://doi.org/10.1016/S0022-2275(20)32471-8

[35] M. Baes, S. Huyghe, P. Carmeliet, P.E. Declercq, D. Collen, G.P. Mannaerts, P.P.V. Veldhoven, Inactivation of the peroxisomal multifunctional protein-2 in mice impedes the degradation of not only 2-methyl-branched fatty acids and bile acid intermediates but also of very long chain fatty acids, J. Biol. Chem. 275 (2000) 16329-16336. https://doi.org/10.1074/jbc.M001994200.

[36] J.F. Lu, A.M. Lawler, P.A. Watkins, J.M. Powers, A.B. Moser, H.W. Moser, K.D. Smith, A mouse model for X-linked adrenoleukodystrophy, Proc. Natl. Acad. Sci. USA 94 (1997) 9366-9371. https://doi.org/10.1073/pnas.94.17.9366.

[37] M. Baes, P. Gressens, S. Huyghe, N.K. De, C. Qi, Y. Jia, G.P. Mannaerts, P. Evrard, V.P. Van, P.E. Declercq, J.K. Reddy, The neuronal migration defect in mice with Zellweger syndrome (Pex5 knockout) is not caused by the inactivity of peroxisomal beta-oxidation, J. Neuropathol. Exp. Neurol. 61 (2002) 368-374. https://doi.org/10.1093/jnen/61.4.368.

[38] N. Ong, J. Bezard, J. Lecerf, Incorporation and metabolic conversion of erucic acid in various tissues of the rat in short term experiments, Lipids 12 (1977) 563-569. https://doi.org/10.1007/BF02533382.

[39] E.N. Christiansen, M.S. Thomassen, R.Z. Christiansen, H. Osmundsen, K.R. Norum, Metabolism of erucic acid in perfused rat liver: increased chain shortening after feeding partially hydrogenated marine oil and rapeseed oil, Lipids 14 (1979) 829-835. https://doi.org/10.1007/BF02534124.

[40] M.Y. Golovko, E.J. Murphy, Uptake and metabolism of plasma-derived erucic acid by rat brain, J. Lipid Res. 47 (2006) 1289-1297. https://doi.org/10.1194/jlr.M600029-JLR200.

[41] C.C. Murphy, E.J. Murphy, M.Y. Golovko, Erucic acid is differentially taken up and metabolized in rat liver and heart, Lipids 43 (2008) 391-400. https://doi.org/10.1007/s11745-008-3168-3.

[42] A.E. Reszko, T. Kasumov, F. David, K.A. Jobbins, K.R. Thomas, C.L. Hoppel, H. Brunengraber, C.D. Rosiers, Peroxisomal fatty acid oxidation is a substantial source of the acetyl moiety of malonyl-CoA in rat heart, J. Biol. Chem. 279 (2004) 19574-9. https://doi.org/10.1074/jbc.M400162200.

[43] J. Kondrup, P.B. Lazarow, Flux of palmitate through the peroxisomal and mitochondrial beta-oxidation systems in isolated rat hepatocytes, Biochim. Biophys. Acta 835 (1985) 147-153. https://doi.org/10.1016/0005-2760(85)90041-4.

[44] R.W. Schwenk, G.P. Holloway, J.J. Luiken, A. Bonen, J.F. Glatz, Fatty acid transport across the cell membrane: regulation by fatty acid transporters, Prostaglandins Leukot. Essent. Fatty Acids 82 (2010) 149-154. https://doi.org/10.1016/j.plefa.2010.02.029.

[45] J.J. Luiken, A. Bonen, J.F. Glatz, Cellular fatty acid uptake is acutely regulated by membrane-associated fatty acid-binding proteins, Prostaglandins Leukot. Essent. Fatty Acids 67 (2002) 73-78. https://doi.org/10.1054/plef.2002.0401.

[46] V.A. Drover, D.V. Nguyen, C.C. Bastie, Y.F. Darlington, N.A. Abumrad, J.E. Pessin, E. London, D. Sahoo, M.C. Phillips, CD36 mediates both cellular uptake of very long chain fatty acids and their intestinal absorption in mice, J. Biol. Chem. 283 (2008) 13108-13115. https://doi.org/10.1074/jbc.M708086200.

[47] J.F.C. Glatz, J.J.F.P. Luiken, Dynamic role of the transmembrane glycoprotein CD36 (SR-B2) in cellular fatty acid uptake and utilization, J. Lipid Res. 59 (2018) 1084-1093. https://doi.org/10.1194/jlr.R082933.

[48] Q. Wu, A.M. Ortegon, B. Tsang, H. Doege, K.R. Feingold, A. Stahl, FATP1 is an insulin-sensitive fatty acid transporter involved in diet-induced obesity, Mol. Cell Biol. 26 (2006) 3455-3467. https://doi.org/10.1128/MCB.26.9.3455-3467.2006.

[49] J.A. Hamilton, K. Brunaldi, A model for fatty acid transport into the brain, J. Mol. Neurosci. 33 (2007) 12-17. https://doi.org/10.1007/s12031-007-0050-3.

[50] W. Zhang, R. Chen, T. Yang, N. Xu, J. Chen, Y. Gao, R.A. Stetler, Fatty acid transporting proteins: Roles in brain development, aging, and stroke, Prostaglandins Leukot. Essent. Fatty Acids 136 (2018) 35-45. https://doi.org/10.1016/j.plefa.2017.04.004.

[51] C. Argyriou, M.D. D'Agostino, N. Braverman, Peroxisome biogenesis disorders, Transl. Sci. Rare Dis. 1 (2016) 111-144. https://doi.org/10.3233/TRD-160003.

[52] B.T. Poll-The, J.M. Saudubray, H.A. Ogier, M. Odievre, J.M. Scotto, L. Monnens, L.C. Govaerts, F. Roels, A. Cornelis, R.B.H. Schutgens, et al, Infantile Refsum disease: an inherited peroxisomal disorder. Comparison with Zellweger syndrome and neonatal adrenoleukodystrophy, Eur. J. Pediatr. 146 (1987) 477-483. https://doi.org/10.1007/BF00441598.

[53] R.I. Kelley, N.S. Datta, W.B. Dobyns, A.K. Hajra, A.B. Moser, M.J. Noetzel, E.H. Zackai, H.W. Moser, Neonatal adrenoleukodystrophy: new cases, biochemical studies, and

differentiation from Zellweger and related peroxisomal polydystrophy syndromes, Am. J. Med. Genet. 23 (1986) 869-901. https://doi.org/10.1002/ajmg.1320230404.

[54] R.J.A. Wanders, W.F. Visser, C.W. van Roermund, S. Kemp, H.R. Waterham, The peroxisomal ABC transporter family, Pflugers Arch. 453 (2006) 719-734. https://doi.org/10.1007/s00424-006-0142-x.

[55] M. Morita, N. Shimozawa, Y. Kashiwayama, Y. Suzuki, T. Imanaka, ABC subfamily D proteins and very long chain fatty acid metabolism as novel targets in adrenoleukodystrophy, Curr. Drug Targets 12 (2011) 694-706. https://doi.org/10.2174/138945011795378577.

[56] S. Kemp, J. Berger, P. Aubourg, X-linked adrenoleukodystrophy: Clinical, metabolic, genetic and pathophysiological aspects, Biochim. Biophys. Acta 1822 (2012) 1465-1474. https://doi.org/10.1016/j.bbadis.2012.03.012.

# *CHAPTER-2*

## Peroxisomes attenuate lipotoxicity of intermediate very long-chain fatty acids and monounsaturated very long-chain fatty acids

# 2.1 Introduction

Peroxisomes are multifunctional organelles found in virtually all eukaryotic cells and play essential roles both in degradation and synthesis of lipid molecules [1]. A central function of peroxisomes in mammals is oxidation of fatty acids that are not oxidized in mitochondria. These include very long-chain fatty acids (VLCFAs, C≥20), long-chain dicarboxylic acids, branched-chain fatty acids, and certain xenobiotics [2]. Intact peroxisomal function is crucial for normal human development and integrity of organic functions including neurons, adrenal, liver and kidney. This is evident from the fact that genetic defects in the peroxisomal function lead to a variety of health problems called peroxisomal disorders [2-11]. The peroxisome disease is classified into two groups, these are peroxisome biogenesis disorder and single peroxisomal enzyme deficiency. Zellweger syndrome (ZS), a typical peroxisome biogenesis disorder, is the most severe form of peroxisome disease. The characteristic clinical features of ZS are neuronal migration defects, dysmyelination and neural heterotopia [2] with being no treatments for recovery. Because of defect of peroxisome-assembly proteins, functional peroxisomes were absent in the cells of ZS patients. As a result, abnormally accumulated VLCFAs are characteristically observed in ZS patients [2]. Acyl-CoA oxidase 1 and D-bifunctional protein are enzymes involved in peroxisomal β-oxidation of FA. The peroxisome disease caused by the functional deficiency of these enzymes are classified as single peroxisomal enzyme deficiency. The resulting inability of peroxisomal β-oxidation leads to accumulation of VLCFAs, and exhibit severe neurodegeneration [2]. X-linked adrenoleukodystrophy (X-ALD) is the most frequent peroxisomal disease, caused by defects in the ABCD1 genes, which encodes peroxisomal membrane protein ALDP [12,13]. The function of ALDP is to transport VLCFAs (as VLCFA-CoA) into the peroxisome [12,13], and the loss of function of ALDP leads to elevated levels of VLCFAs in the plasma and tissues [14]. The accumulation of saturated (S)-VLCFAs, specifically C24:0 FA and C26:0 FA is characteristic feature of this disease, and typical symptoms of the X-ALD are also neurodegenerations, such as demyelination with inflammation and oxidative damage [15-18].

Excess of VLCFAs (C24:0 FA and C26:0 FA) is found to induce several types of cell toxicity on neural cell types. Those include plasma membrane changes, oxidative stress, lysosomal dysfunction, and mitochondrial dysfunction [19-24]. From these facts, it seems that accumulation

of VLCFAs is causative events leading to neurodegeneration. However, this assumption is too simplistic, because there are X-ALD patients with mild or no neuronal symptoms with being elevated VLCFAs in plasma. At present, the relation of accumulation of VLCFAs to progression of symptoms of the peroxisome diseases, such as demyelination/neurodegeneration remains obscure.

To clarify the relationship between accumulation of VLCFAs and symptoms of peroxisome disease, it is highly important to know the effect of VLCFAs on functions of different cells, including myelinating cells. However, biological experiments using VLCFAs are limited because of the extremely low aqueous solubility of VLCFAs. Recently, I developed a method to disperse VLCFAs in aqueous medium. The method was based on the formation of VLCFA/albumin complex in the presence of a small amount of alcohol in the albumin solution under the warmed (37 $^{o}$C) condition [25]. Our method enables to solubilize C16 to C26 saturated FAs in culture medium and perform experiments for determination of effect of these FAs on cultured cells including peroxisome-deficient CHO cells.

In this study, I showed that intermediate VLCFAs (such as C20:0 FA and C20:1 FA) exert apoptosis, whereas elongated VLCFAs (such as C24:0 FA and C26:0 FA) shows anti-apoptotic activities in peroxisome-deficient cells. The apoptotic activity was found to be related with the extent of accumulation of the FA in cellular lipids. I also demonstrated that peroxisomes play a pivotal role in detoxification of the apoptotic VLCFAs.

# 2.2 Materials and methods

### 2.2.1 Materials

Fatty acid-free bovine serum albumin (BSA), 10,12-tricosadiynoic acid (TDYA), heptadecanoic acid (C17:0 FA), arachidic acid (C20:0 FA), paullinic acid (C20:1 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). Palmitic acid (C16:0 FA) and stearic acid (C18:0 FA) were from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Oleic acid (C18:1 FA) was Nacalai Tesque (Kyoto, Japan) and 4',6'-diamidino-2-phenylindole (DAPI) solution were Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 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). Behenic acid (C22:0 FA), erucic acid (C22:1 FA) and lignoceric acid (C24:0 FA) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Proteinase K, RNase, 100bp DNA ladder, and BlueJuice$^{TM}$ Gel Loading Buffer were from Invitrogen (Carlsbad, CA, USA). TLC plates coated with silica gel (Merck Art 5721, Darmstadt, Germany) were used. All other reagents were of reagent grade, HPLC grade.

### 2.2.2 Cell culture

The cell culture method is the same as described in the Materials and Methods section of Chapter 1.

### 2.2.3 Fatty acid preparation

The fatty acid preparation method used for solubility check experiments is the same as described in the Materials and Methods section of Chapter 1.

### 2.2.4 Preparation of FA/IP/BSA for culture experiments

The fatty acid preparation method utilized for culture experiments is the same as described in the Materials and Methods section of Chapter 1.

### 2.2.5 FA uptake assay

The method of fatty acid uptake assay is the same as described in the Materials and Methods section of Chapter 1. For TDYA (peroxisome inhibitor) plus FA treatment, CHO-K1 cells were seeded at 1 x $10^6$ cells petri dish in 100 mm petri dishes with 10 mL of FBS-containing medium for 24 h at 37 °C in a $CO_2$ incubator. The culture medium was replaced with 8 mL serum-free medium for 24 h, followed by treatment with 30 μM of TDYA as a pretreatment. Following incubation for 6 h, FA was added and incubated for 24 h in the presence or absence of TDYA. After the incubation period, lipids of the cells and medium were extracted separately after addition of appropriate internal standard, and subjected to methanolysis for determination of the FA by GC, as described in the Materials and Methods section of Chapter 1.

### 2.2.6 Cytotoxicity assay

Cells were seeded onto 35-mm dishes at 2 × $10^5$ cells/dish at 37 °C in a $CO_2$ incubator. Following incubation for 24 h, the culture medium was replaced with serum-free medium and supplemented with different concentrations of FA as FA/BSA complex prepared by the IP method. After 48 h of supplementation, the adherent cells were harvested with the aid of trypsin/EDTA, washed with PBS, and subjected to a trypan blue-exclusion assay for assessment of the number of living cells.

For TDYA (peroxisome inhibitor) plus FA treatment, CHO-K1 cells were seeded onto 35-mm dishes at 1 × $10^5$ cells/dish at 37 °C in a $CO_2$ incubator for 24 h. The culture medium was replaced with fresh serum-free medium, and further incubated for 24 h. After 24 h, cells were incubated with 30 μM of TDYA before addition of FA. Following incubation for 6 h, FA was added for 48 h in the presence or absence of TDYA. After the incubation period, cells were fixed for fluorescence microscopy as described below.

### 2.2.7 Nuclear staining with DAPI (4`,6-diamidino-2-phenylindole)

CHO cells were seeded onto 35-mm dishes at 2 × $10^5$ cells/dish for 24 h. After 24 h, the medium was replaced to serum-free medium, and incubated with FA at indicated concentration for 48 h in the presence or absence of TDYA. FA-treated and non-treated cells were fixed with 4% paraformaldehyde in PBS for 20 min at 37 $^oC$ and permeabilized with 0.1% Triton X-100 in PBS for 5 min at 37 $^oC$ followed by washing with PBS. Cells were incubated with DAPI (10 μg/mL) at

37 ºC for 30 min, after which the cells were washed with PBS and then observed immediately under fluorescence microscope. Cells with a condensed or fragmented nucleus were considered as apoptotic cells, while cells showing intact shape of nucleus were regarded as living. The number of both total and apoptotic cells in each microscopic field were counted. The percentage of living cells were calculated based on these values.

### 2.2.8 DNA fragmentation assay

CHO cells were seeded onto 35-mm dishes at $2 \times 10^5$ cells/dish for 24 h. After 24 h, the medium was replaced to serum-free medium, and incubated with FA at indicated concentration for 48 h. The medium and adherent cells were collected and centrifuged at 2,100 rpm for 5 min at 4 °C. The supernatant was gently withdrawn, and the resultant pellet was lysed in 341 μL of 100 mM Tris-HCl (pH 8.5) containing 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/mL proteinase K at 37 °C for 60 min. Afterwards, 10 μL of 10 mg/mL proteinase K was added to the samples and allowed to stand for 30 min at 50 °C. Subsequently, 141 μL of 5 M NaCl and 471 μL of ethanol were added to the lysate, and kept at -80 °C for 60 min. Following centrifugation for 30 min at 15,000 rpm at 4 °C, the DNA pellets were dissolved in 20 μL of 10 mM Tris-HCl (pH 7.2) and 2 μL of RNase at 37 °C for 30 min. The DNA samples were loaded onto 1.5% agarose gel, separated with electrophoresis, and analyzed their extent of fragmentation under the UV light.

### 2.2.9 Statistical analysis

Statistical analyses were performed by Student's t-test (to compare two groups) and one-way ANOVA with post-hoc Tukey test (to compare more than two groups).

## 2.3 Results

### 2.3.1 FA/IP/BSA improve solubility of VLCFAs

Previously, I reported that adding VLCFAs as isopropanol solution into aqueous BSA at warmed condition (~37 ºC) facilitates formation of FA/albumin complex, and that VLCFAs prepared by this method (FA/IP/BSA) is efficiently introduced into cultured cells [25]. In this study, I applied our method to C16 to C26 FAs with saturated or unsaturated carbon chains. As shown in Fig. 10A, B, all saturated and unsaturated FAs prepared as FA/IP/BSA passed through the 0.22 μm filter, indicating that most FAs prepared by this method completely form albumin complex. In contrast, saturated FAs prepared by the conventional method that use FA sodium salt and albumin (FA/Na/BSA) did not completely form albumin complex except for C16:0 FA. This conventional method seems to be applicable to unsaturated VLCFAs, but not saturated FA especially in C≥20 VLCFAs (Fig. 10C, D). I applied the FA/IP/BSA method in all cultured cell experiments in this study. It should be mentioned that final IP concentration in the medium was not exceeded 0.5% in all experiments and that IP up to 2% in the medium did not affect viability of the cells as described in our previous study [25].

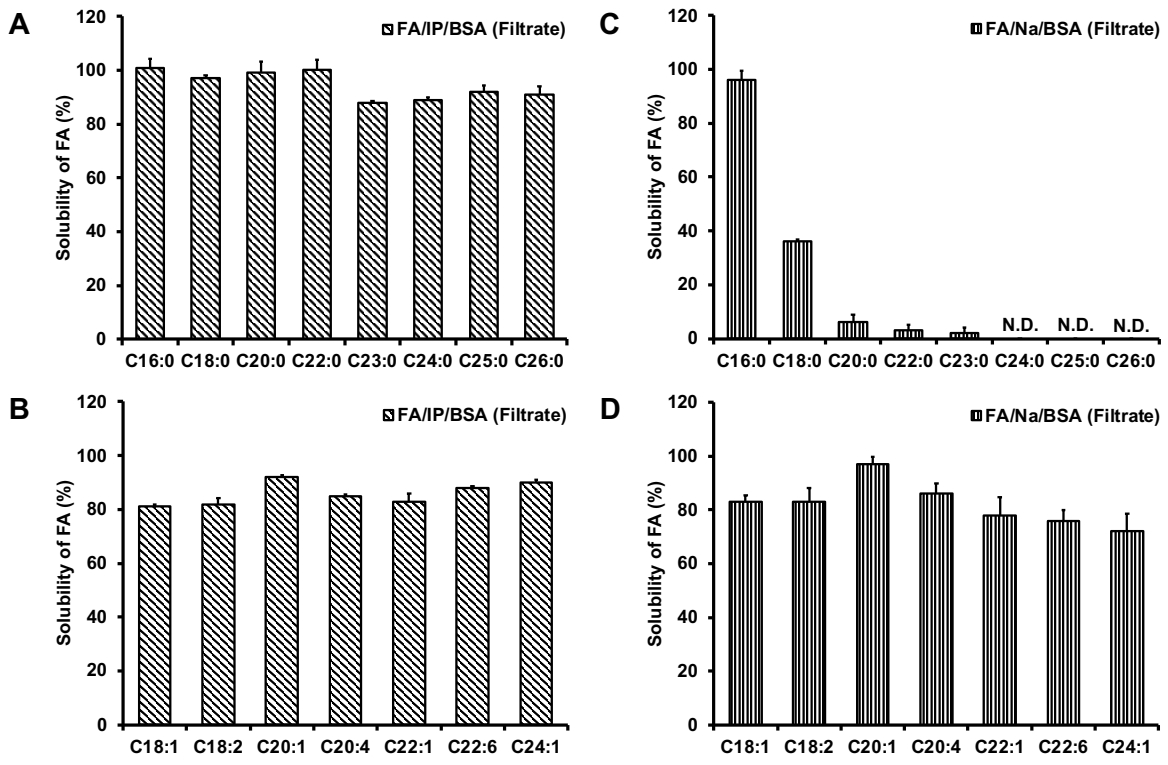



**Fig. 10 IP facilitates formation of VLCFA/BSA complex**

Three hundred nanomole of FAs dissolved in IP were mixed with BSA solution (FA/IP/BSA) (A, B). Three hundred nanomole of FAs were treated with NaOH solution followed by heating at 65 $^{o}$C, and mixed with BSA solution (FA/Na/BSA) (C, D). The complexed solutions were filtered through a 0.22 μm membrane filter. The FA dissolved in the filtrate was extracted and subjected to GC analysis after methyl esterification as described in the Materials and Methods section of Chapter 1. Values are means ± S.D. of three independent experiments. N.D.: not detected.

### 2.3.2 Clearance of exogenous FAs from the culture medium and incorporation of FAs into the cellular lipids in CHO cells

I examined the uptake of the saturated (S) or monounsaturated (MU) LCFAs or VLCFAs into peroxisome-deficient (CHO-zp102) cells and its wild-type (CH0-K1) cells using the complexation approach (FA/IP/BSA) [25]. After incubating 30 μM (300 nmol/dish) FAs in serum-free medium for 24h, FA uptake was measured by determination of decrement of FAs in the culture medium and increment of the FA in the cells (Fig. 11A, B). It should be mentioned that most parts of the FA detected in the cells were acylated form, but not free form as reported in our previous study [25].

**C20:1~C26:0 → Peroxisome substrate**

There were large differences between decrements of added VLCFAs (C20:1, C22:0, C22:1, C23:0. C24:0, C24:1, C25:0, and C26:0 FA) in the medium and increment of corresponding VLCFAs in the cellular lipids of wild-type CHO cells, indicating that a large portion of these up-taken VLCFAs were degraded in the cells during a 24 h of incubation (Fig. 11A). In contrast, most of the decrements of these added VLCFAs in the medium was compensated by an increase of corresponding VLCFAs in the cellular lipids of the peroxisome-deficient cells (Fig. 11B). These results suggested that degradation of up-taken FA was not so operated in peroxisome-deficient cells during a 24 h of incubation. These differences in the metabolism of VLCFAs in the peroxisome-deficient cells and its wild-type cells indicated that a large portion of up-taken VLCFAs (C20:1~C26:0) is degraded exclusively by peroxisomal β-oxidation if peroxisome is working. It should be noted that considerable amounts of S-VLCFAs (C22:0, C23:0, C24:0, C25:0, C26:0) were introduced into the wild-type cells, but they were not so accumulated in the cellular lipids by the clearance by peroxisomes. Whereas, S-VLCFAs up-taken by peroxisome-deficient cells was marginal. As a result, levels of cellular S-VLCFAs were very low in both type of CHO cells as shown in our previous study [25].

**C16:0 FA, C18:0 FA, and C18:1 FA → Mitochondrial substrate**

Decrement of exogenous C16:0 FA from the culture medium was comparable between wild-type and peroxisome-deficient cells during a 24 h of incubation. The cellular levels of C16:0 FA were also similar levels in these cells (Fig. 11A, B). This was also the case for C18:0 FA (Fig. 11A, B). These results indicated that C16:0 FA and C18:0 FA are metabolized peroxisome-independent manner. Thus, these LCFAs are considered to be degraded exclusively in mitochondrial β-oxidation. Although accumulation level of added C18:1 FA was higher in peroxisome-deficient cells, the differences between decrement of C18:1 FA in the medium and increment of C18:1 FA in the peroxisome-deficient cells were comparable to that observed in wild-type cells (around 100 nmol/dish each) (Fig. 11A, B). This result indicated that mitochondria play a pivotal role in oxidative metabolism of C18:1 FA.

**C20:0 FA → Peroxisome (75%) and Mitochondrial (25%) substrate**

The difference between decrement of C20:0 FA in the medium and increment of C20:0 FA in the peroxisome-deficient cells was around 50 nmol/dish, indicating that a significant portion of up-taken C20:0 FA was degraded in mitochondria (Fig. 11B). On the other hand, the difference

between decrement of C20:0 FA in the medium and increment of C20:0 FA in the wild-type cells was around 200 nmol, indicating that peroxisomal β-oxidation has larger potential for its oxidative degradation (Fig. 11A). From these results, around 75% and 25% of disappeared C20:0 FA is estimated to be degraded in peroxisome and mitochondria, respectively, in wild-type cells.

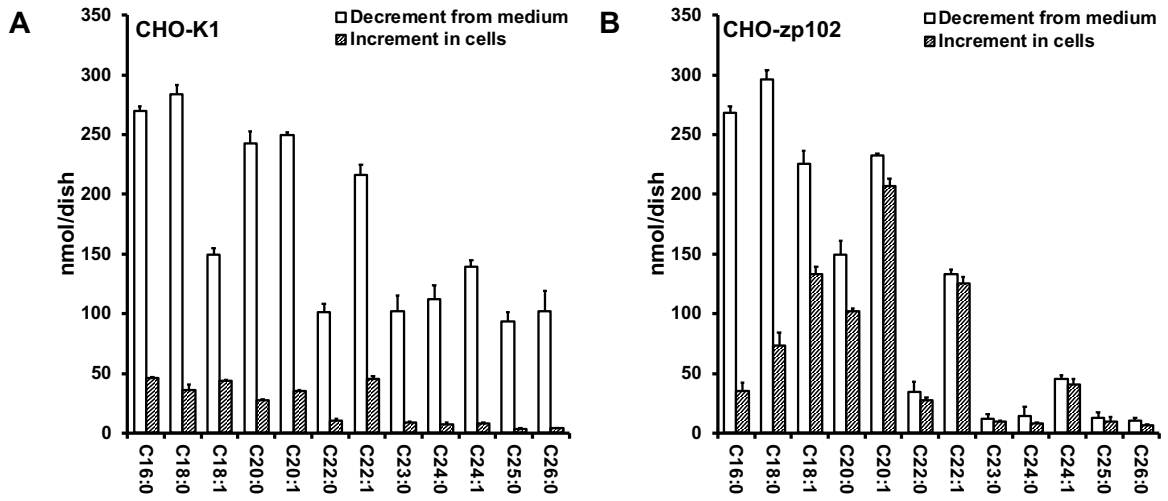



**Fig. 11 Cellular uptake of FA in CHO-K1 and CHO-zp102 cells.**

CHO-K1 (wild-type) cells and CHO-zp102 (peroxisome-deficient) cells were incubated with C16:0 FA, C18:0 FA, C18:1 FA, C20:0 FA, C20:1 FA, C22:0 FA, C22:1 FA, C23:0 FA, C24:0 FA, C24:1 FA C25:0 FA, and C26:0 FA in serum-free medium for 24 h at 30 μM (A, B). The medium and cells were collected separately at 24 h and subjected to lipid extraction. The fatty acid methyl esters were analyzed by GC as mentioned in the Materials and Methods section of Chapter 1. Values are means ± S.D. of three independent experiments.

### 2.3.3 Cytotoxicity of extracellular VLCFAs in CHO-zp102 cells

Effect of VLCFAs on peroxisome-deficient CHO cells was examined. I found that cytotoxic effects of C20:0 FA, C20:1 FA, C22:0 FA, C22:1 FA, and C24:1 FA on peroxisome-deficient cells (Fig. 12A). Among these FAs, C20:0 FA showed the highest toxicity, which was observed from 5 μM. These toxic VLCFAs have a property of easily accumulating in cellular lipids of peroxisome-deficient cells as shown in Fig. 11. In contrast, C16:0 FA, C18:0 FA, C18:1, C23:0 FA, and C25:0 FA were found not to promote any loss of cell viability up to 30 μM (Fig. 12A). Interestingly, I found that C24:0 FA and C26:0 FA trends to have cytoprotective effects on peroxisome-deficient cells (Fig. 12A). In contrast to peroxisome-deficient cells, cytotoxic effect was not observed following 48h-incubation with C16:0 FA, C18:0 FA, C18:1 FA, C20:1 FA, C22:0 FA, C22:1 FA, C23:0 FA, C24:1 FA, and C25:0 FA at up to 30 μM (Fig. 12B). Marked cytotoxic effect was

observed only when wild-type cells were incubated with C20:0 FA at 30 μM. Interestingly, cytoprotective effects of C24:0 FA and C26:0 FA in wild-type CHO cells were observed at 30 μM as seen in peroxisome-deficient cells (Fig. 12B).

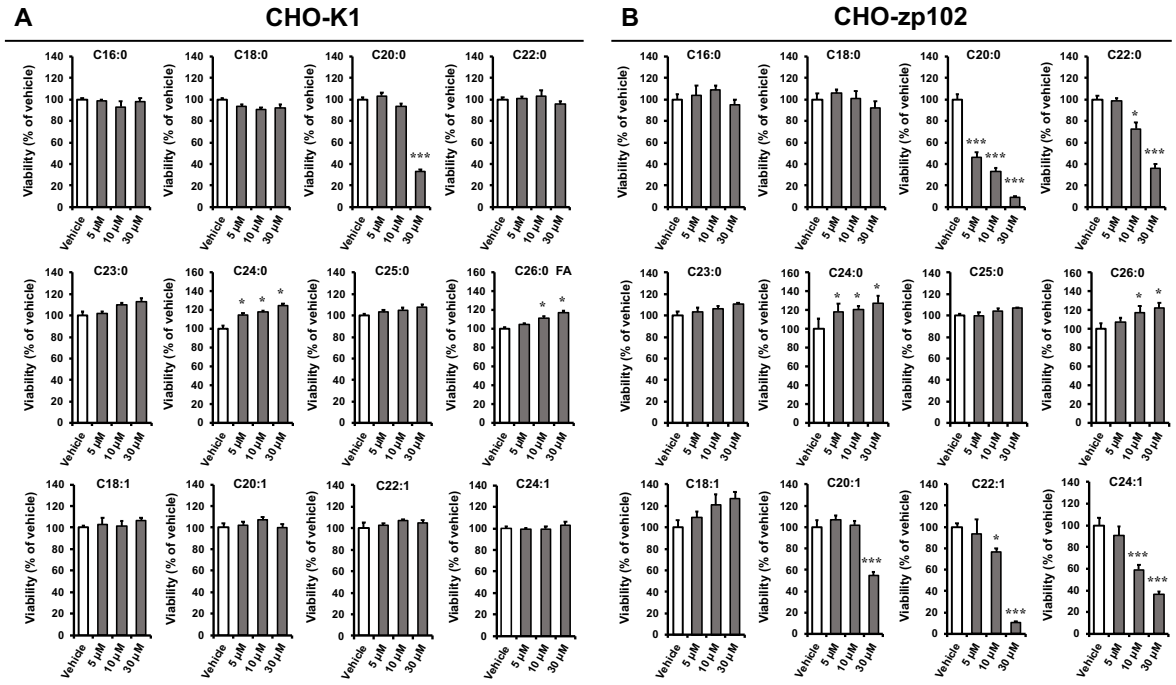



**Fig. 12 VLCFAs induces CHO-zp102 cell death**

CHO-zp102 (peroxisome-deficient) cells and CHO-K1 (wild-type) cells were incubated in serum-free medium with indicated concentrations of C16:0 FA, C18:0 FA, C18:1 FA, C20:0 FA, C20:1 FA, C22:0 FA, C22:1 FA, C23:0 FA, C24:0 FA, C24:1 FA, C25:0 FA, and C26:0 FA for 48 h (A, B). Cells were harvested by trypsin-EDTA treatment, and subjected to trypan blue-exclusion test. The data shown are the percentage of the value of control cells. The results are expressed as means ± S.D. (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey`s multiple comparison test. *$p < 0.05$ vs. vehicle*, and* ***$p < 0.005$ vs. vehicle.

### 2.3.4 VLCFAs induces apoptosis in CHO-zp102 cells

Nuclear morphology and DNA ladder formation were assessed to clarify whether observed FA-toxicity is apoptotic cell death. As shown in Fig. 13A, most of peroxisome-deficient cells incubated with C20:0 FA, C22:0 FA, C22:1 FA, and C24:1 FA (30 μM) showed morphological alterations of nuclei, such as condensed chromatin and fragmented nuclei. Whereas, most of the control cells did not display any changes of nuclei. Living cell ratio was calculated by counting the cells that retained intact nuclei in total cells. The results shown in Fig. 13B were in good agreement with those obtained in the trypan blue exclusion test (Fig. 12). The apoptotic feature of the peroxisome-

deficient cells treated with 30 μM of C20:0 FA, C22:0 FA, C22:1 FA, and C24:1 FA was also confirmed by a DNA fragmentation assay as shown in Fig. 13C. In contrast to those VLCFAs, peroxisome-deficient cells treated with C18:1 FA, C24:0 FA, and C26:0 FA (30 μM) did not display apoptotic feature as judged by both nuclear condensation and DNA fragmentation (Fig. 13A, B, C). The C20:0 FA (30 μM)-induced cell death in wild-type cells were also found to be apoptosis as judged by nuclear shape, whereas treatment with C24:0 FA was not (Fig. 13D, E). Apoptosis of C20:0 FA treated-wild-type cells was also confirmed by a DNA fragmentation assay (Fig. 13F).

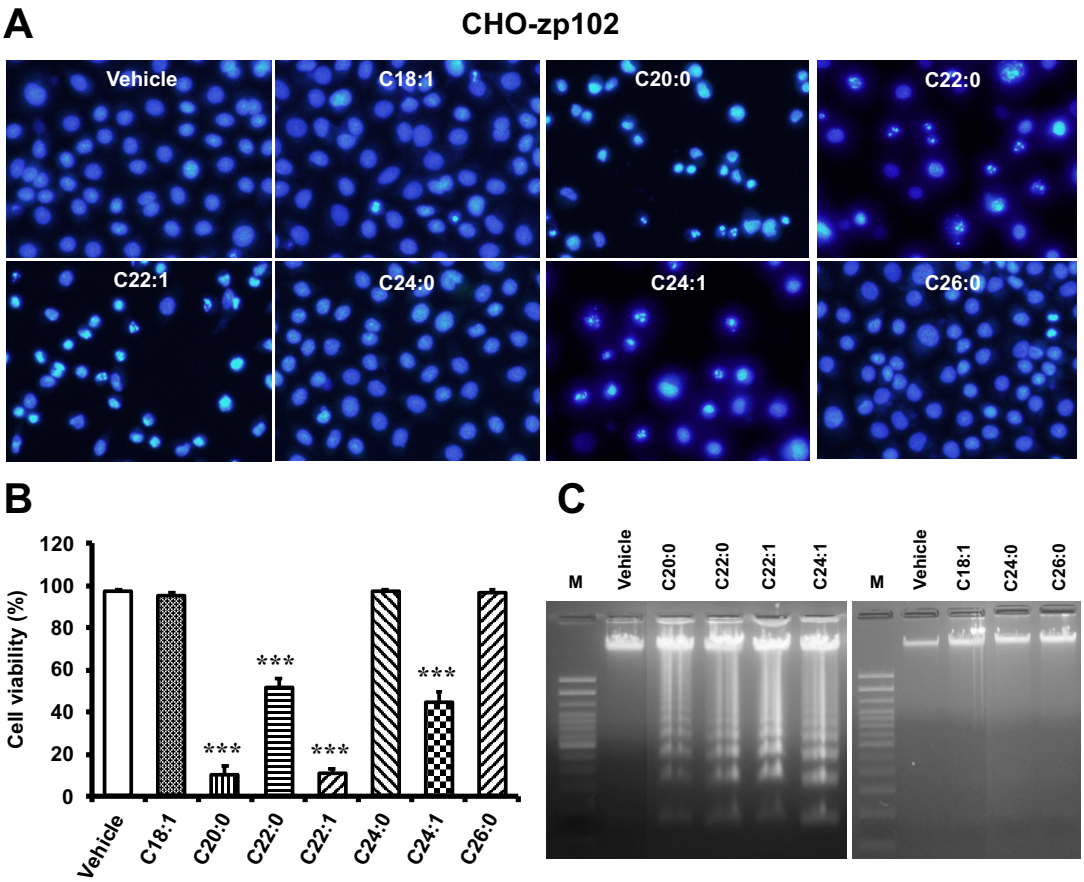

A
CHO-zp102
Vehicle
C18:1
C20:0
C22:0
C22:1
C24:0
C24:1
C26:0
B
Cell viability (%)
120
100
80
60
40
20
0
***
***
***
Vehicle
C18:1
C20:0
C22:0
C22:1
C24:0
C24:1
C26:0
C
M
Vehicle
C20:0
C22:0
C22:1
C24:1
M
Vehicle
C18:1
C24:0
C26:0
D
CHO-K1
Vehicle
C20:0
C24:0
E
Cell viability (%)
120
100
80
60
40
20
0
***
Vehicle
C20:0
C24:0
F
M
Vehicle
C20:0
C24:0

**Fig. 13 VLCFAs induces apoptosis in CHO-zp102 cells**

CHO-zp102 (peroxisome-deficient) cells and CHO-K1 (wild-type) cells were subjected to 30 μM of FA in serum-free medium for 48 h, followed by staining with DAPI and observed by fluorescence microscopy (A, D). The percentage of living cells of each dish was assessed as mentioned in the section 2.7 (B, E). The results are expressed as means ± S.D. (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey`s multiple comparison test. *$*p < 0.05$* vs. vehicle*, and* ***$*p < 0.005$* vs. vehicle. The cells were treated with the indicated concentration of FA for 48 h and DNA fragmentation was analyzed on 1.5% agarose gel electrophoresis. M, indicates the 100-bp DNA ladder size marker (C, F). The data shown are the representatives of three independent experiments.

### 2.3.5 Effect of peroxisome inhibitor on the accumulation and cytotoxicity of C20:0 FA

It seems that the cytotoxicity of VLCFAs has relation to its cellular accumulation, which is preventable by peroxisomal β-oxidation. To obtain further evidence on this hypothesis, I examined whether the peroxisome inhibitor enhances the sensitivity to cytotoxic C20:0 FA by augmentation of its accumulation in wild-type cells. TDYA, a potent inhibitor of acyl-CoA oxidase 1, was utilized to inhibit peroxisomal β-oxidation, a peroxisomal function [26]. When the wild-type cells were supplemented with 5 and 10 μM C20:0 FA in the absence of TDYA, accumulations of C20:0 FA in the cellular lipids were marginal levels with no apparent cytotoxicity (Fig. 14A, D). At 30 μM C20:0 FA, accumulation of C20:0 FA (28 nmol/dish) became apparent, and remarkable cytotoxicity was observed (Fig. 14A, D). When TDYA was added to the culture medium, accumulation of C20:0 FA in cellular lipids in wild-type CHO cells was significantly enhanced from the low concentration of C20:0 FA (5 μM, 15 nmol/dish; 10 μM, 29 nmol/dish; 30 μM, 64 nmol/dish) (Fig. 14B). The levels of C20:0 FA-accumulation in TDYA-treated wild-type cells was comparable to those observed in C20:0 FA-accumulation in peroxisome-deficient CHO cells (5 μM, 16 nmol/dish; 10 μM, 44 nmol/dish; 30 μM, 79 nmol/dish) (Fig. 14C), indicating that peroxisomal β-oxidation was inhibited by the TDYA. I found that the sensitivity to cytotoxic C20:0 FA was enhanced in TDYA-treated wild-type cells. The C20:0 FA-dose dependent cytotoxicity observed in TDYA-treated wild-type cells was similar manner to those observed in peroxisome-deficient cells (Fig. 14D). Relationship between cytotoxicity and accumulation of C20:0 FA in the different experiments is shown Fig. 14E. This figure clearly shows that C20:0 FA-induced cell toxicity correlated with the intracellular accumulation of C20:0 FA.

The inhibiting effect of TDYA on the peroxisomal β-oxidation was also confirmed by experiments with C23:0 FA, where the chain-shortened metabolites of C23:0 FA, such as C17:0

FA and C19:0 FA were completely absent in the C23:0 FA supplemented wild-type cells in the presence but not the absence of 30 μM TDYA (Fig. 15A, B). Similar experiments were conducted with C22:1 FA and C24:1 FA. We found that sensitivity to cytotoxic C22:1 and C24:1 FA was also increased in the presence of TDYA, and that the toxicity was partially correlated with the level of intracellular accumulation of these VLCFAs (Fig. 16A, B).

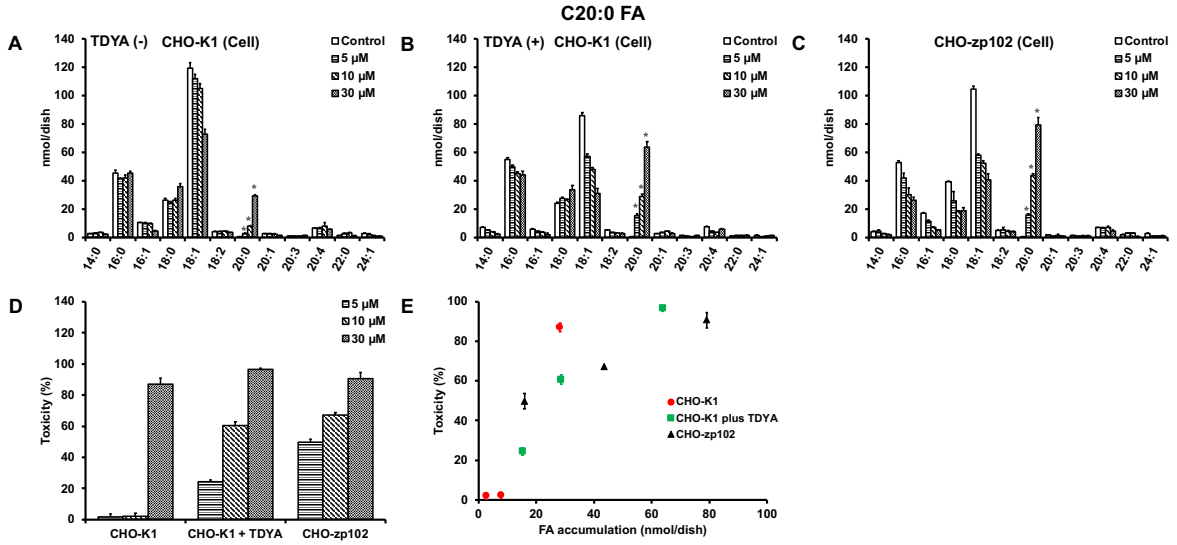
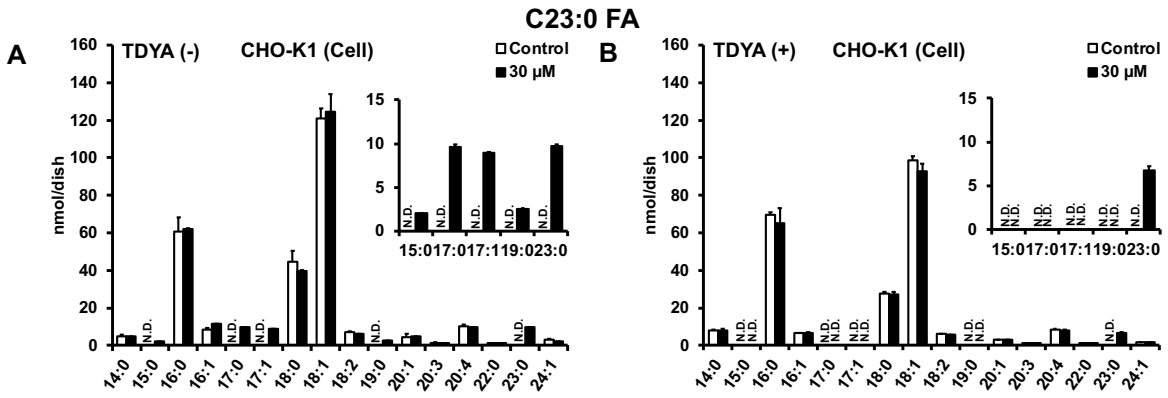


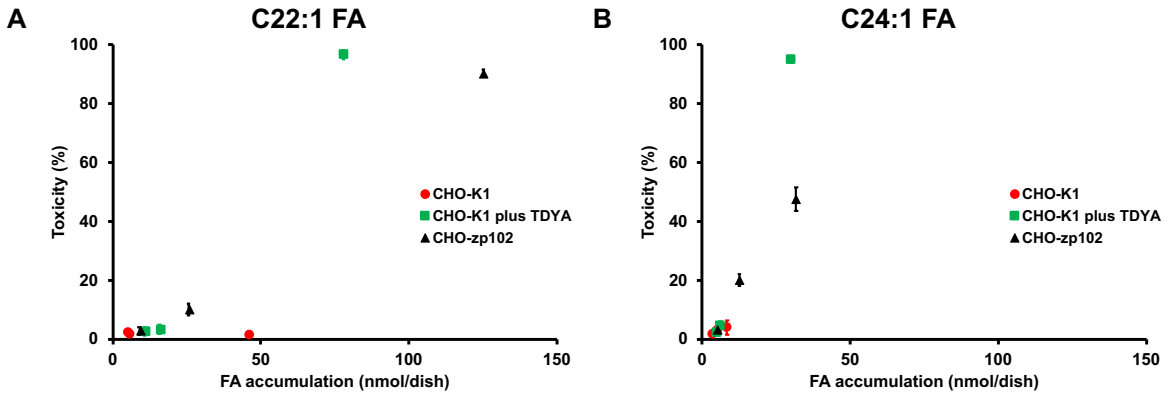

**Fig. 14 Fatty acid composition of cellular lipids of CHO-K1 cells and CHO zp102 cells supplemented with different concentrations of C20:0 FA in the presence or absence of TDYA.**

CHO-K1 (wild-type) cells in the presence or absence of TDYA and CHO-zp102 (peroxisome-deficient) cells were incubated with the indicated concentration of FA in serum-free medium for 24 h. The cells (A-C) were collected at the specific time point and subjected to lipid extraction. The fatty acid methyl esters were analyzed by GC as mentioned in the Materials and Methods section of Chapter 1. Values are means ± S.D. of three independent experiments. *$p < 0.05$, significantly different from control cells (Student's t-test). CHO-K1 (wild-type) cells in the presence or absence of TDYA and CHO-zp102 (peroxisome-deficient) cells were incubated with the indicated concentration of FA in serum-free medium for 48 h, followed by staining with DAPI and observed by fluorescence microscopy. The percentage of apoptotic cells in each experiment are shown in (D). The results are expressed as means ± S.D. (n=3). The percentage obtained in the apoptosis assay was plotted against amount of added FA accumulated in the cellular lipids (E). The results are expressed as means ± S.D. (n=3).




## Fig. 15 Effect of TDYA on C23:0 FA β-oxidation in CHO-K1 cells

CHO-K1 (wild-type) cells was incubated with the indicated concentration of C23:0 FA in serum-free medium for 24 h in the presence or absence of TDYA. The cells (A, B) were collected at 24 h and subjected to lipid extraction. The fatty acid methyl esters were analyzed by GC as mentioned in the Materials and Methods section of Chapter 1. Values are means ± S.D. of three independent experiments.




## Fig. 16 Correlation between accumulation of FA and cellular toxicity

CHO-K1 (wild-type) cells in the presence or absence of TDYA and CHO-zp102 (peroxisome-deficient) cells were incubated with the indicated concentration of FA in serum-free medium for 24 h or 48 h. The fatty acid methyl esters or apoptosis assay were determined by the same method as mentioned in the legend to Fig. 5. The percentage obtained in the apoptosis assay was plotted against amount of added FA accumulated in the cellular lipids (A, B). The results are expressed as means ± S.D. (n=3).

## 2.4 Discussion

The development of myelination is known to be accompanied by enhanced synthesis of VLCFAs, and impaired myelination is believed to be caused by increased levels of VLCFAs, as observed in various types of peroxisomal diseases [2,15,16]. However, it is not yet experimentally proven that abnormal accumulation of VLCFAs leads to demyelination or contributes to the progression of neurodegeneration. One of the difficulties in the VLCFAs study is that VLCFAs cannot dissolve in an aqueous medium. Among them, S-VLCFAs are extremely hydrophobic, and therefore, are not efficiently delivered to cells when dispersed in aqueous culture medium. Recently, I developed a method to disperse VLCFAs in an aqueous medium by using a small amount of isopropanol in the presence of BSA under warmed conditions (37 $^{o}$C) [25], and showed that VLCFAs can be efficiently introduced into cells by the developed method described herein [25]. Here, we confirmed that our method is applicable up to C26:0 FA. I also showed the limitation of the conventional method for formation of FA/albumin complex, in which FA is converted to its sodium salt (FA/Na/BSA). Our results showed that the FA/Na/BSA method is applicable to MU-VLCFAs and saturated FA up to C16, but not to saturated FA above C18.

Using this method, I characterized oxidative metabolism of LCFAs and VLCFAs with different chain-lengths. The characterization was based on the clearance level of the added FA during the incubation, which is calculated as a difference between amounts of FA disappeared from the medium (M) and that appeared in the cellular lipids (C) in CHO cells (M-C value) as shown in Fig. 11. In the case of C16:0 FA and C18:0 FA, the higher M-C values were observed in both peroxisome-deficient cells and wild-type cells at similar levels. Thus, major oxidative clearance operated to these LCFAs in both types of cells is mitochondrial activity. This is consistent with earlier reports showing that 94% of oxidative metabolism of 16:0 FA is performed by mitochondria [27,28].

In the case of FA with chain-length above C22:0, the M-C values were very low in peroxisome-deficient cells. In contrast, corresponding M-C values in the wild-type cells is considerably higher, suggesting the operation of peroxisome-dependent clearance to these VLCFAs in wild-type cells. Thus, these VLCFAs were regarded as peroxisome substrates. This notion is in agreement with

those reported so far [29,30]. It should be emphasized that a large amount of uptake and oxidative clearance are operated to S-VLCFAs in wild-type cells.

Based on the comparison of M-C values of C20:0 FA in wild-type and peroxisome-deficient cells, 75% of oxidative metabolism of C20:0 FA was performed by peroxisomes in wild-type cells as described in the results section.

Metabolism of MU-VLCFAs seemed to be different from that of S-VLCFA in terms of capacity of acylation to cellular lipids. Accumulations of C24:1 FA, C22:1 FA, and C20:1 FA are evident in peroxisome-deficient cells, indicating that MU-VLCFAs are differently metabolized from S-VLCFAs in the peroxisome-deficient cells.

In this study, I found that C20:0 FA exerts the strongest toxic effect among tested C16~C26 FAs. The toxicity of C20:0 FA was observed at 5 μM in peroxisome-deficient cells, whereas, it was 30 μM in wild-type cells, suggesting that peroxisome-deficient cells are sensitive to toxicity of C20:0 FA. I also found that C20:1 FA, C22:0 FA, C22:1 FA, and C24:1 FA were toxic to peroxisome-deficient cells at 30 μM but not toxic to wild-type cells at this concentration. These data are in line with earlier studies showing that C20:0 FA (125 μM) had the highest toxicity compared to polyunsaturated FAs and other saturated FAs [31]. Here, I demonstrated that the mechanism of cytotoxicity of these VLCFAs are induction of apoptosis and that there is a close correlation between cytotoxicity and accumulation level of these VLCFAs in cellular lipids. These observations indicated that increased apoptotic sensitivity to these VLCFAs in peroxisome-deficient cells are attributable to inability of oxidative clearance of these VLCFAs by peroxisomes. This notion is supported by the fact that apoptotic sensitivity of wild-type cells increased in parallel with accumulation level of these VLCFAs when peroxisomal β-oxidation was inhibited by TDYA. In conclusion, our findings suggest that peroxisomes play a crucial role in the detoxification of these apoptotic VLCFAs.

Here, I showed that exogenously added C24:0 FA and C26:0 FA did not exert a cytotoxic effect, but rather a protective effect on wild-type and peroxisome-deficient CHO cells. This protective effect of C24:0 FA and C26:0 FA can be partly explained by the increased cellular level of C24:0 ceramide and C26:0 ceramide, respectively (our unpublished data). There have been studies showing that ceramides with different *N*-acyl chains perform different functions in cells

[32]. Alteration of ceramide composition in cells by overexpression or silencing of ceramide synthases shows that long-chain ceramide is pro-apoptotic, while ceramide with VLCFAs is anti-apoptotic [32]. However, contradictory reports regarding the cytotoxicity of C24:0 FA and C26:0 FA have also been published. VLCFAs have been found to induce necrotic cell death by increased production of reactive oxygen species (ROS) or impaired mitochondrial and lysosomal function [19-24]. This discrepancy associated with biological effect of VLCFAs may be due to the differences in experimental conditions, such as method used for FA preparation and cell types. It is worthwhile to characterize metabolic manner and cellular function, such as proliferation, and apoptotic tendency of C24:0 FA and C26:0 FA in peroxisome-deficient oligodendrocytes and Schwann cells for better understanding of peroxisome diseases.

In this study, I demonstrated that peroxisome-deficient CHO cells characteristically accumulate C20:0 FA, C20:1 FA, C22:0 FA, C22:1 FA and C24:1 FA. I also showed that all of them induce apoptosis when the peroxisome-deficient CHO cells accumulated them beyond threshold level. The C20:0 FA, C20:1 FA, C22:0 FA and C22:1 FA are minor VLCFAs in neuronal cells. They may be produced transiently as intermediates of biosynthesis of major VLCFAs, such as C24:0, C26:0 and C26:1, which have been known to accumulate in several kinds of patients of peroxisome disease. Expression of ELOVL1, a key enzyme for the synthesis of VLCFAs [33], has been reported to increase with the development of myelination in the CNS in humans and that deficiency in the synthesis of VLCFA-containing SLs leads to defects in the myelin-sheath [34]. During the myelination process, if ELOVL1 does not do its job, these intermediate VLCFAs may accumulate in neuronal tissue. Alternatively, these intermediate VLCFAs may accumulate in the cells under a condition that a large amount of C24:0 FA and C26:0 FA are present. It may be important to know the level of the intermediate VLCFAs in tissues of patients of peroxisome disease. Here, we characterized C24:1FA, one of major VLCFA in brain, as an apoptotic VLCFA in peroxisome-deficient cells. Level of C24:1 FA in patients in blood and neuronal region may also need to be checked.

In summary, I demonstrated that peroxisome-deficient CHO cells show increased sensitivity to apoptotic intermediate VLCFAs, such as C20:0 FA, C20:1 FA, C22:0 FA, C22:1 FA and MU-VLCFA, that is C24:1 FA. The increased sensitivity is caused by accumulation of these VLCFAs

in the cells whose peroxisomal β-oxidation system is impaired. Peroxisome-deficient cells are considered to be in dangerous state, especially when cells are in a condition performing enhanced synthesis of VLCFAs without no degradation activity of them. It is important to know whether these VLCFAs accumulate and exert deteriorating action on myelin of certain kinds of peroxisome disease patients.

## 2.5 References


[1] W.H. Kunau, V. Dommes, H. Schulz, beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress, Prog. Lipid Res. 34 (1995) 267-342. https://doi.org/10.1016/0163-7827(95)00011-9.

[2] P.P.V. Veldhoven, Biochemistry and genetics of inherited disorders of peroxisomal fatty acid metabolism, J. Lipid Res. 51 (2010) 2863–2895. https://doi.org/10.1194/jlr.R005959.

[3] B.J. Pettus, M. Baes, M. Busman, Y.A. Hannun, P.P.V. Veldhoven, Mass spectrometric analysis of ceramide perturbations in brain and fibroblasts of mice and human patients with peroxisomal disorders, Rapid Commun. Mass Spectrom. 18 (2004) 1569-1574. https://doi.org/10.1002/rcm.1520.

[4] K. Hama, T. Nagai, C. Nishizawa C, K. Ikeda, M. Morita, N. Satoh, H. Nakanishi, T. Imanaka, N. Shimozawa, R. Taguchi, K. Inoue, K. Yokoyama, Molecular species of phospholipids with very long chain fatty acids in skin fibroblasts of Zellweger syndrome, Lipids 48 (2013) 1253-1267. https://doi.org/10.1007/s11745-013-3848-5.

[5] Y. Fujiwara, K. Hama, N. Shimozawa, K. Yokoyama, Glycosphingolipids with very long-chain fatty acids accumulate in fibroblasts from adrenoleukodystrophy patients, Int. J. Mol. Sci. 22 (2021) 8645. https://doi.org/10.3390/ijms22168645.

[6] Y. Abe, M. Honsho, H. Nakanishi, R. Taguchi, Y. Fujiki, Very-long-chain polyunsaturated fatty acids accumulate in phosphatidylcholine of fibroblasts from patients with Zellweger syndrome and acyl-CoA oxidase1 deficiency, Biochim. Biophys. Acta 1841 (2014) 610-619. https://doi.org/10.1016/j.bbalip.2014.01.001.

[7] T. Kobayashi, N. Shinnoh, A. Kondo, T. Yamada, Adrenoleukodystrophy protein-deficient mice represent abnormality of very long chain fatty acid metabolism, Biochem. Biophys. Res. Commun. 232 (1997) 631-636. https://doi.org/10.1006/bbrc.1997.6340.

[8] S. Takashima, K. Toyoshi, T. Itoh, N. Kajiwara, A. Honda, A. Ohba, S. Takemoto, S. Yoshida, N. Shimozawa, Detection of unusual very-long-chain fatty acid and ether lipid derivatives in the fibroblasts and plasma of patients with peroxisomal diseases using liquid chromatography-mass spectrometry, Mol. Genet. Metab. 120 (2017) 255-268. https://doi.org/10.1016/j.ymgme.2016.12.013.

[9] A.B. Moser, N. Kreiter, L. Bezman, S. Lu, G.V. Raymond, S. Naidu, H.W. Moser, Plasma very long chain fatty acids in 3,000 peroxisome disease patients and 29,000 controls, Ann. Neurol. 45 (1999) 100-110. https://doi.org/10.1002/1531-8249(199901)45:1<100::aid-art16>3.0.co;2-u.

[10] W.C. Hubbard, A.B. Moser, A.C. Liu, R.O. Jones, S.J. Steinberg, F. Lorey, S.R. Panny, R.F. Vogt, D. Macaya, C.T. Turgeon, S. Tortorelli, G.V. Raymond, Newborn screening for X-linked adrenoleukodystrophy (X-ALD): validation of a combined liquid chromatography-tandem mass spectrometric (LC-MS/MS) method, Mol. Genet. Metab. 97 (2009) 212-220. https://doi.org/10.1016/j.ymgme.2009.03.010.

[11] K. Herzog, M.L. Pras-Raves, M.A. Vervaart, A.C. Luyf, A.C. van Kampen, R.J. Wanders, H.R. Waterham, F.M. Vaz, Lipidomic analysis of fibroblasts from Zellweger spectrum disorder patients identifies disease-specific phospholipid ratios, J. Lipid Res. 57 (2016) 1447-1454. https://doi.org/10.1194/jlr.M067470.

[12] C. Wiesinger, M. Kunze, G. Regelsberger, S. Forss-Petter, J. Berger, Impaired very long-chain acyl-CoA β-oxidation in human X-linked adrenoleukodystrophy fibroblasts is a direct consequence of ABCD1 transporter dysfunction, J. Biol. Chem. 288 (2013) 19269-19279. https://doi.org/10.1074/jbc.M112.445445

[13] M. Morita, N. Shimozawa, Y. Kashiwayama, Y. Suzuki, T. Imanaka, ABC subfamily D proteins and very long chain fatty acid metabolism as novel targets in adrenoleukodystrophy, Curr. Drug Targets 12 (2011) 694-706. https://doi.org/10.2174/138945011795378577.

[14] S. Kemp, J. Berger, P. Aubourg, X-linked adrenoleukodystrophy: Clinical, metabolic, genetic and pathophysiological aspects, Biochim. Biophys. Acta 1822 (2012) 1465-1474. https://doi.org/10.1016/j.bbadis.2012.03.012.

[15] M. Engelen, S. Kemp, M. de Visser, B.M. van Geel, R.J. Wanders, P. Aubourg, B.T. Poll-The, X-linked adrenoleukodystrophy (X-ALD): clinical presentation and guidelines for diagnosis, follow-up and management, Orphanet J. Rare Dis. 13 (2012) 7:51. https://doi.org/10.1186/1750-1172-7-51.

[16] I. Ferrer, J.P. Aubourg, A. Pujol, General aspects and neuropathology of X-linked adrenoleukodystrophy, Brain Pathol. 20 (2010) 817-830. https://doi.org/10.1111/j.1750-3639.2010.00390.x.

[17] A.S. Paintlia, A.G. Gilg, M. Khan, A.K. Singh, E. Barbosa, I. Singh, Correlation of very long chain fatty acid accumulation and inflammatory disease progression in childhood X-ALD: implications for potential therapies, Neurobiol. Dis. 14 (2003) 425-439. https://doi.org/10.1016/j.nbd.2003.08.013.

[18] C.R. Vargas, M. Wajner, L.R. Sirtori, L. Goulart, M. Chiochetta, D. Coelho, A. Latini, S. Llesuy, A. Bello-Klein, R. Giugliani, M. Deon, C.F. Mello, Evidence that oxidative stress is increased in patients with X-linked adrenoleukodystrophy, Biochim. Biophys. Acta. 1688 (2004) 26-32. https://doi.org/10.1016/j.bbadis.2003.10.004.

[19] S. Hein, P. Schönfeld, S. Kahlert, G. Reiser, Toxic effects of X-linked adrenoleukodystrophy-associated, very long chain fatty acids on glial cells and neurons from rat hippocampus in culture, Hum. Mol. Genet. 17 (2008) 1750–1761. https://doi.org/10.1093/hmg/ddn066.

[20] M. Doria, T. Nury, D. Delmas, T. Moreau, G. Lizard, A. Vejux, Protective function of autophagy during VLCFA-induced cytotoxicity in a neurodegenerative cell model, Free Radic. Biol. Med. 137 (2019) 46-58. https://doi.org/10.1016/j.freeradbiomed.2019.04.016.

[21] T. Nury, M. Doria, G. Lizard, A. Vejux, Docosahexaenoic acid attenuates mitochondrial alterations and oxidative stress leading to cell death induced by very long-chain fatty acids in a mouse oligodendrocyte model, Int. J. Mol. Sci. 21 (2020) 641. https://doi.org/10.3390/ijms21020641.

[22] M. Baarine, K. Ragot, A. Athias, T. Nury, Z. Kattan, E.C. Genin, P. Andreoletti, F. Ménétrier, J.M. Riedinger, M. Bardou, G. Lizard, Incidence of Abcd1 level on the induction of cell death and organelle dysfunctions triggered by very long chain fatty acids and TNF-α on oligodendrocytes and astrocytes, Neurotoxicology 33 (2012) 212-228. https://doi.org/10.1016/j.neuro.2011.10.007.

[23] J. López-Erauskin, J. Galino, M. Ruiz, J.M. Cuezva, I. Fabregat, D. Cacabelos, J. Boada, J. Martínez, I. Ferrer, R. Pamplona, F. Villarroya, M. Portero-Otín, S. Fourcade, A. Pujol, Impaired mitochondrial oxidative phosphorylation in the peroxisomal disease X-linked adrenoleukodystrophy, Hum. Mol. Genet. 22 (2013) 3296-3305. https://doi.org/10.1093/hmg/ddt186.

[24] M.C. van de Beek, R. Ofman, I. Dijkstra, F. Wijburg, M. Engelen, R. Wanders, S. Kemp, Lipid-induced endoplasmic reticulum stress in X-linked adrenoleukodystrophy, Biochim.

Biophys. Acta Mol. Basis Dis. 1863 (2017) 2255-2265. https://doi.org/10.1016/j.bbadis.2017.06.003.

[25] H. Ali, K. Morito, R.Y. Hasi, M. Aihara, J. Hayashi, R. Kawakami, K. Kanemaru, K. Tsuchiya, K. Sango, T. Tanaka, Characterization of uptake and metabolism of very long-chain fatty acids in peroxisome-deficient CHO cells, Biochim. Biophys. Acta. 1867 (2022) 159088. https://doi.org/10.1016/j.bbalip.2021.159088.

[26] J. Zeng, S. Deng, Y. Wang, P. Li, L. Tang, Y. Pang, Specific inhibition of acyl-CoA oxidase-1 by an acetylenic acid improves hepatic lipid and reactive oxygen species (ROS) metabolism in rats fed a high fat diet, J. Biol. Chem. 292 (2017) 3800-3809. https://doi.org/10.1074/jbc.M116.763532.

[27] C. Chu, L.F. Mao, H. Schulz, Estimation of peroxisomal beta-oxidation in rat heart by a direct assay of acyl-CoA oxidase, Biochem. J. 302 (1994) 23-29. https://doi.org/10.1042/bj3020023.

[28] M. Morita, S. Shinbo, A. Asahi, T. Imanaka, Very long chain fatty acid β-oxidation in astrocytes: contribution of the ABCD1-dependent and -independent pathways, Biol. Pharm. Bull. 35 (2012) 1972-1979. https://doi.org/10.1248/bpb.b12-00411.

[29] H. Singh, N. Derwas, A. Poulos, Beta-oxidation of very-long-chain fatty acids and their coenzyme A derivatives by human skin fibroblasts, Arch. Biochem. Biophys. 254 (1987) 526-533. https://doi.org/10.1016/0003-9861(87)90133-0.

[30] H. Singh, N. Derwas, A. Poulos, Very long chain fatty acid beta-oxidation by subcellular fractions of normal and Zellweger syndrome skin fibroblasts, Arch. Biochem. Biophys. 257 (1987) 302-314. https://doi.org/10.1016/0003-9861(87)90570-4.

[31] T. Plötz, A.S. von Hanstein, B. Krümmel, A. Laporte, I. Mehmeti, S. Lenzen, Structure-toxicity relationships of saturated and unsaturated free fatty acids for elucidating the lipotoxic effects in human EndoC-βH1 beta-cells, Biochim. Biophys. Acta Mol. Basis. Dis. 1865 (2019) 165525. https://doi.org/10.1016/j.bbadis.2019.08.001.

[32] S. Grösch, S. Schiffmann, G. Geisslinger, Chain length-specific properties of ceramides, Prog. Lipid Res. 51 (2012) 50-62. https://doi.org/10.1016/j.plipres.2011.11.001.

[33] Y. Ohno, S. Suto, M. Yamanaka, Y. Mizutani, S. Mitsutake, Y. Igarashi, T. Sassa, A. Kihara, ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis, Proc. Natl. Acad. Sci. USA 107 (2010) 18439-18444. https://doi.org/10.1073/pnas.1005572107.

[34] M. Isokawa, T. Sassa, S. Hattori, T. Miyakawa, A. Kihara, Reduced chain length in myelin sphingolipids and poorer motor coordination in mice deficient in the fatty acid elongase Elovl1, FASEB Bioadv. 1 (2019) 747-759. https://doi.org/10.1096/fba.2019-00067.

# Conclusion

# Conclusion

In this study, I developed a method to disperse VLCFAs in an aqueous medium by using a small amount of isopropanol with BSA under warmed conditions (37 ºC). I found that our method (FA/IP/BSA) can facilitate the solubility of saturated FA with chain length of C16 to C26 in aqueous medium. I also found that the conventional method (FA/Na/BSA) is only applicable to MU-VLCFAs and saturated-FA up to C16, but not to saturated FA above C18. Using this method, I examined metabolism and lipotoxicity of VLCFAs in peroxisomal-deficient cells and their wild-type cells.

- **Elongated VLCFAs (C24:0 FA and C26:0FA)**

I found that both uptake and peroxisomal β-oxidation of elongated VLCFAs (C24:0 FA and C26:0 FA) was actively operated in wild-type cells. As a result, accumulations of these VLCFAs in cellular lipids were not observed. In contrast, uptakes of the elongated VLCFAs (C24:0 FA and C26:0 FA) were considerably depressed levels in peroxisome-deficient cells. As a result, these VLCFAs did not so accumulate in the peroxisome-deficient cells.

- **Intermediate VLCFAs and MU-VLCFAs (C20:0 FA, C22:0, FA, C22:1 FA, C24:1 FA)**

Uptakes and metabolism of the intermediate VLCFAs, such as C20:0 FA, and MU-VLCFAs, such as C24:1 FA, in wild-type cells were regarded as “active uptake and active peroxisomal β-oxidation” type. This metabolic manner was similar to those observed in VLCFAs (C24:0 and C26:0). As a result, intermediate VLCFAs and MU-VLCFAs did not accumulate in wild-type cells. On the other hand, intermediate VLCFAs and MU-VLCFAs are extensively acylated to cellular lipids in peroxisome-deficient cells. This metabolic manner can be called “active uptake and acylation”. The accumulation of these types of VLCFAs seems to trigger the apoptotic switch in the peroxisome-deficient cells when they accumulated above threshold level. This is not limited to peroxisome-deficient cells. Wild-type cells also go apoptosis when peroxisomal β-oxidation was inhibited, or excess C20:0 FA was accumulated at a level above threshold.

From these results, it is concluded that peroxisomal β-oxidation plays an important role in the prevention of accumulation of apoptotic VLCFAs in the cells. Peroxisome disease patients, whose peroxisomal β-oxidation activity is impaired, are considered to be in dangerous state, especially when their cells are in a condition performing enhanced synthesis of VLCFAs. I hope the knowledge obtained in these studies will be helpful for better understanding of the pathology of peroxisome diseases.

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