© 2024. This manuscript version is made available under the CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by-nc-nd/4.0/
The published version is available via https://doi.org/10.1016/j.bbalip.2024.159452.

Protective effect of oleic acid against very long-chain fatty acid-induced apoptosis in peroxisome-deficient CHO cells

Hanif Ali, Mone Yamanishi, Keigo Sunagawa, Mizuki Kumon, Rumana Yesmin Hasi, Mutsumi Aihara, Ryushi Kawakami, Tamotsu Tanaka*

Graduate School of Technology, Industrial and Social Sciences, Tokushima University, Tokushima, 770-8513, Japan

*To whom correspondence should be addressed
e-mail: tanaka.tamotsu@tokushima-u.ac.jp

Abstract

Very long-chain fatty acids (VLCFAs) are degraded exclusively in peroxisomes, as evidenced by the accumulation of VLCFAs in patients with certain peroxisomal disorders. Although accumulation of VLCFAs is considered to be associated with health issues, including neuronal degeneration, the mechanisms underlying VLCFAs-induced tissue degeneration remain unclear. Here, we report the toxic effect of VLCFA and protective effect of C18:1 FA in peroxisome-deficient CHO cells. We examined the cytotoxicity of saturated and monounsaturated VLCFAs with chain-length at C20-C26, and found that longer and saturated VLCFA showed potent cytotoxicity at lower accumulation levels. Furthermore, the extent of VLCFA-induced toxicity was found to be associated with a decrease in cellular C18:1 FA levels. Notably, supplementation with C18:1 FA effectively rescued the cells from VLCFA-induced apoptosis without reducing the cellular VLCFAs levels, implying that peroxisome-deficient cells can survive in the presence of accumulated VLCFA, as long as the cells keep sufficient levels of cellular C18:1 FA. These results suggest a therapeutic potential of C18:1 FA in peroxisome disease and may provide new insights into the pharmacological effect of Lorenzo's oil, a 4:1 mixture of C18:1 and C22:1 FA.

Keywords:

Peroxisome disease, Very long-chain fatty acids, Oleic acid, Apoptosis

Abbreviations: ABCD1, ATP binding cassette subfamily D member 1; ALD, X-linked adrenoleukodystrophy; ALDP, adrenoleukodystrophy protein; BSA, FA-free bovine serum albumin; CHO, Chinese hamster ovary; DAPC, 1,2-diarachidoyl-sn-glycero-3-phosphocholine; DAPI, 4',6'-Diamidino-2-phenylindole; ER, Endoplasmic reticulum; EtOH, ethanol; FAs, fatty acids; FAMEs, fatty acid methylesters; FBS, fetal bovine serum; GC, gas chromatography; IP, isopropanol; 7-KC, 7-ketocholesterol; LO, Lorenzo's oil; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MS/MS, tandem mass spectrometry; MU-VLCFAs, monounsaturated very long-chain fatty acids; Na, NaOH; PBD, peroxisome biogenesis disorders; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; PS, phosphatidylserine; SCD1, stearoyl-CoA desaturase-1; SFAs, saturated fatty acids; SM, sphingomyelin; S-VLCFAs, saturated very long-chain fatty acids; TDYA, 10,12-tricosadiynoic acid; TFA, total fatty acids; TAG, triacylglycerol; VLCFAs, very long-chain fatty acids; ZS, Zellweger syndrome.

1. Introduction

Peroxisomes, which are highly multifunctional organelles present in virtually all eukaryotic cells, play an essential role in the synthesis of lipids as well as in their degradation [1–6]. In mammals, peroxisomes oxidize long-chain hydrocarbons, including very long–chain fatty acids (VLCFAs; C ≥ 20), long-chain dicarboxylic acids, polymethylene-interrupted unsaturated FAs, and certain xenobiotics [1–6]. Proper peroxisomal function is indispensable for normal human development as well as for the optimal functioning of vital organs, including neurons, adrenal glands, liver, and kidneys. The requirement of peroxisome functions has become evident from a fact that genetic defects in peroxisomal function lead to a variety of health issues known as peroxisomal disorders [7–10].

Peroxisomal disorders can be classified into two categories: peroxisome biogenesis disorders (PBD); and single peroxisomal enzyme deficiencies. Zellweger syndrome (ZS) is a PBD caused by impaired peroxisomal functions leading to the accumulation of VLCFAs presenting as distinctive biochemical characteristics. The most common phenotypes of ZS are neuronal migration defects, dysmyelination and neural heterotopia [11–14]; there is currently no available treatment for recovery. The peroxisomal enzymes, acyl-CoA oxidase 1 and D-bifunctional protein, are required for peroxisomal β-oxidation [11–14]. Deficiency in these enzymes results in excessive accumulation of VLCFAs, leading to severe neurodegeneration [11–13, 15].

X-linked adrenoleukodystrophy (ALD), is the most common peroxisomal disorder, occurring in approximately 1 in 14,700 births. ALD is caused by mutations in *ABCD1* gene which encodes the peroxisomal transmembrane protein, ALDP [16–22]. The primary role of ALDP is the transportation of VLCFA as CoA-esters into the peroxisomal matrix, where they undergo chain-shortening via β-oxidation [16–22]. The loss of ALDP function leads to the accumulation of VLCFAs, mainly C24:0, C26:0 and C26:1, in the plasma and tissues [23–38]. Accumulation of these VLCFAs in blood is a definitive diagnostic marker of this disease. Our recent investigation also revealed the accumulation of ceramide with these VLCFAs in the blood of ALD patients [39]. ALD is characterized by progressive demyelination of both central and peripheral nervous systems, accompanied by adrenal insufficiency and inflammatory processes in the demyelinated areas [40–44].

Excessive VLCFAs (C24:0 and C26:0 FAs) have been shown to induce toxicity in various neural cell types, leading to plasma membrane changes and oxidative stress, as well as lysosomal and mitochondrial dysfunction [45–54]. These observations suggest that VLCFAs accumulation may act as a causal factor in neurodegeneration. However, this assumption may be too simplistic, because some ALD patients with elevated plasma VLCFAs are known to exhibit mild or no neurological

symptoms. We also observed that a healthy female carrier with mutated *ABCD1* gene showed elevated VLCFA-ceramide [39]. Currently, the link between VLCFA accumulation and the progression of demyelination/neurodegeneration remains unclear.

To gain a better understanding of the association between VLCFA accumulation and peroxisome disease symptoms, it is crucial to explore the effects exerted by VLCFAs on various cellular functions, including the myelinating of cells. However, metabolic regulation of endogenous or exogenous VLCFAs, as well as cellular events induced by abnormally accumulated VLCFAs remain unclear. One of difficulties of biological experiments on VLCFAs is extremely low aqueous solubility of VLCFAs. Recently, we developed a method for dispersing VLCFAs as an albumin complex in aqueous medium [55,56]. Using this technique, we analyzed the uptake [55] and cytotoxicity of VLCFAs [56] in various cells including peroxisome-deficient cells [55,56].

In this study, we found that saturated and longer VLCFAs, such as C26:0 FA, show potent toxicity in peroxisome-deficient cells at low levels of accumulation. We also found that VLCFA-induced cell death was associated with cellular C18:1 FA reduction. Notably, supplementation of C18:1 effectively rescued the cells from VLCFA-induced apoptosis. These findings indicated the therapeutic potential of C18:1 FA in peroxisome disease.

2. Materials & Methods

2.1. Materials

FA-free bovine serum albumin (BSA), 10,12-tricosadiynoic acid (TDYA), arachidic acid (C20:0 FA), paullinic acid (C20:1 FA), nervonic acid (C24:1 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 purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Palmitoleic acid (C16:1 FA) and oleic acid (C18:1 FA) were purchased from Nacalai Tesque (Kyoto, Japan). 4',6'-Diamidino-2-phenylindole (DAPI) solution was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,2-Diheptadecanoyl-*sn*-glycero-3-phosphocholine (17:0/17:0 PC) and 1,2-Diarachidoyl-*sn*-glycero-3-phosphocholine (20:0/20:0 PC, 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). Hexacosenoic acid (C26:1 FA) was purchased from Larodan (Michigan, United States). Proteinase K, RNase, 100bp DNA ladder, and BlueJuice™ Gel Loading Buffer were purchased from Invitrogen (Carlsbad, CA, USA). TLC plates coated with silica gel (Art 5721; Merck, Darmstadt, Germany) were used. All other reagents used were of reagent grade.

2.2. Cell culture

Chinese hamster ovary (CHO-K1) cells were sourced from RIKEN Cell Bank (Tsukuba, Japan). CHO-zp102 cells were generated via the deletion of *Pex5*, the gene encoding a receptor for peroxisome-targeting signal-1, as described previously [57,58]. All cell lines used in the experiments were within passages 3 and 10. The experiments were performed after the cells reached confluence.

2.3. Preparation of FA/albumin complex for gel filtration column chromatography

We developed a method to solubilize VLCFAs in aqueous medium by forming an FA/albumin complex in the presence of a small amount of isopropanol (IP; FA/IP/BSA) [55,56]. In brief, 300 nmol of FA (C18:1, C24:0, C24:1 and C26:0 FA) dissolved in 50 μL IP was warmed to 37 °C, while 6.6 mg of BSA was dissolved in 950 μL of phosphate-buffered saline (PBS) at 37 °C. The FA/IP solution was mixed with the BSA solution in a water bath, and sonicated at 150 W for 1–5 min at 37 °C. The resulting solution containing the FA/albumin complex was further incubated in a water bath at 37 °C for 60 min. The molar ratio of FA/BSA was 3:1 and the maximum concentration of IP in the PBS was 2.5% (v/v). We also prepared FA/BSA complex via a conventional method, using FA

sodium salt (FA/Na/BSA). In brief, 300 nmol of FA (C18:1, C24:0, C24:1 and C26:0 FA) in 50 μL IP was transferred to a glass tube. After evaporation under N_2 flow, 100 μL of 100 mM NaOH was added, following which the preparation was heated at 65 °C in a water bath for 5 min, while 6.6 mg of BSA was dissolved in 900 μL of PBS at 37 °C. These solutions were thoroughly mixed via sonication as described above. The molar ratio of FA/NaOH/BSA was 3:6:1. The FA samples prepared using the FA/IP/BSA and FA/Na/BSA methods were fractionated via gel filtration chromatography. The FAs were eluted with 0.2 M NaCl in 10 mM Tris-HCl buffer (pH 7.5). Subsequently, FA was extracted via the Bligh and Dyer method [59] under acidic conditions and subjected to methanolysis to determine the amounts of FA using gas chromatography (GC), as described below. The protein amounts in each fraction were determined by the Bradford method with the bovine serum albumin (BSA) as the standard.

To determine the solubility of C24:0 FA, as the albumin complex, various amounts of C24:0 FA (600, 1000, 1500 and 2000 nmol) were dissolved either in 100 μL IP or ethanol (EtOH) at 37 °C. These solutions were then mixed with 1.9 mL of PBS containing BSA at 37 °C. These preparations were thoroughly mixed via sonication as described above. The C24:0 FA samples were subsequently filtered through a 0.22 μm filter, following which the amounts of FA in the filtrates were determined via GC. The molar ratio of FA/BSA was 3:1 and the maximum concentration of both IP and EtOH in the PBS was 5 % (v/v).

2.4. Cytotoxicity assay

The CHO cells were seeded in 35-mm dishes at 2×10^5 cells/dish at 37 °C in a CO_2 incubator. Following a 24 h incubation period, various amounts of FA were introduced in the form of the FA/BSA complex, which was prepared using the FA/IP/BSA method [55,56], for the time periods indicated. In another set of experiments, C18:1 FA were co-incubated with various VLCFAs for the time periods indicated. Control cells were treated only with the vehicle (BSA + IP). Following the incubation, cell viability was assessed using a trypan blue dye exclusion assay. Results were expressed as a percentage of the vehicle-treated control cells. In most experiments, the final concentration of IP in the medium was 1% or lower. Up to 2% IP concentrations in the medium did not affect cell viability, as previously described [55].

Treatment with TDYA, a peroxisomal oxidation inhibitor, was conducted as previously described [56]. In brief, 24 h-serum starved CHO-K1 cells were incubated with 30 μM TDYA prior to the addition of FA. Following incubation for 6 h, FA was added for 48 h in the presence or absence of

TDYA. Following incubation, the cells were fixed for visualization via fluorescence microscopy, as described below.

2.5. FA uptake assay

The FA uptake assay was conducted as described previously [55,56]. In brief, CHO cells were seeded at 1×10^6 cells per dish in 100-mm petri dishes with 10 mL of FBS-containing medium for 24 h at 37 °C in a CO_2 incubator. Cells were subjected to serum starvation for 24 h, following which VLCFAs were introduced as FA/BSA complexes prepared using the FA/IP/BSA method [55,56]. In another set of experiments, C18:1 FA at indicated concentrations was co-incubated with VLCFAs. Following incubation, cells were washed with FBS-free medium containing 1% BSA cells and harvested. Cellular lipids were extracted according to the method described by Bligh and Dyer [59] 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). The amounts of FA obtained from cellular lipids were calculated based on the relative peak areas obtained via GC analysis. GC was conducted using a capillary column (DB-225, 0.25 μm film thickness, 30 m length, 0.25 mm ID; Agilent Technologies, Santa Clara, CA, USA). Oven temperature was maintained at 100 °C for 0.5 min and then raised to 195 °C at a rate of 25 °C/min, to 205 °C at a rate of 3 °C/min, and to 240 °C at 8 °C/min, and then maintained constant for 10 min. Oven temperature was reduced to 100 °C prior to injection of the next sample.

Amounts of C24:0 FA incorporated into the various lipid classes were determined via GC. Cellular lipids were separated by TLC developed with petroleum ether:diethyl ether:acetic acid = 80:20:1 (v/v/v). For the isolation of sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE), chloroform:methanol:28% ammonia = 60:35:8 (v/v/v) was utilized. The isolated lipids were then subjected to methanolysis and analyzed using GC as described above.

2.6. Nuclear staining via DAPI (4',6'-Diamidino-2-phenylindole)

VLCFA-treated CHO cells were fixed with 4% paraformaldehyde in PBS for 20 min at 37 °C, permeabilized with 0.1% Triton X-100 in PBS for 5 min at 37 °C, and washed using PBS. Next, the cells were incubated with DAPI (10 μg/mL) at 37 °C for 30 min, washed with PBS, and immediately observed via fluorescence microscopy as described previously [56]. Condensed or fragmented nuclei were indicative of apoptotic cells, while intact nuclei were indicative of viable cells. The numbers of total and apoptotic cells in each microscopic field were counted and the percentage of living cells was

calculated.

2.7. DNA fragmentation assay

A DNA fragmentation assay was conducted as described previously [56]. In brief, subsequent to VLCFA treatments, the conditioned medium and adherent cells were collected and centrifuged at 2,100 rpm for 5 min at 4 °C. The resultant pellet was treated with 0.2% SDS and 0.2 mg/mL proteinase K, and the lysate so obtained was treated with 5 M NaCl and EtOH to precipitate DNA. The DNA precipitate was treated with RNase, and loaded on 1.5% agarose gel, separated via electrophoresis, and analyzed for fragmentation under UV light.

2.8. Lipidomics analysis

The extracted cellular lipids were dissolved in 100 µL of chloroform/methanol/water (1:2:0.2, V/V/V) and subjected to lipidomics analysis. Chromatographic separation was performed using an Acquity UPLC Peptide BEH-C18 column (50 x 2.1 mm; 1.7 µm; Waters, Ireland) on an Ultimate 3000 UHPLC (Dionex) with Q Exactive Orbitrap mass spectrometer (Thermo, CA). Flow rate was set at 0.3 mL/min and column temperature was maintained at 45 °C. A gradient elution with acetonitrile, methanol and water (1:1:3, v/v/v) containing 5 mM ammonium acetate and 10 nM EDTA (mobile phase A), and 100 % isopropanol with 5mM ammonium acetate and 10 nM EDTA (mobile phase B) was used. The volume of sample injected into the column was 5 µL. The gradient profile was as follows; 0-1 min 0% (B), 5 min 40% (B), 7.5-12 min 64% (B), 12.5 min 82.5% (B) 19 min 85% (B), 20 min 95% (B), 20.1-25 min 0% (B). The temperature of the sample was maintained at 4 °C. Lipid were detected via mass spectrometry using a quadrupole/time-of-flight mass spectrometer TripleTOF 6600 (SCIEX, Framingham, MA, USA) in the high-resolution mode for MS1 (~35,000 FWHM) and high sensitivity mode for MS2 (~20,000 FWHM) with data-dependent MS/MS acquisition. Parameters included MS1 and MS2 mass ranges of m/z 70-1250, MS1 and MS2 accumulation time was 250 msec, and 100 msec, respectively. Lipidomics data were processed using metabolomics software, MS-DIAL [60].

2.9. Statistical analysis

One-way ANOVA followed by the post-hoc Tukey test was used to compare more than two groups, while the Student's t-test was performed for comparison between 2 groups.

3. Results

3.1. Characterization of the FA/IP/BSA method for solubilization of VLCFAs

We recently reported that introduction of VLCFAs as an IP solution into an aqueous BSA solution enables the formation of a FA/albumin complex [55,56]. In this study, we characterized the FA/BSA complex prepared via our method. When 600, 1000, 1500, and 2000 nmol of C24:0 FA in IP solution (100 μL) was introduced into aqueous BSA solution (1.9 mL), the amount of C24:0 FA passed through the 0.22 μm filter was 93.5, 95.5, 81.3, and 57.5 % of introduced FA, respectively (Fig. 1A). This indicated that the maximum concentration of C24:0 FA solubilized as the albumin complex by our method is around 500 μM. We also found that the maximum concentration of solubilized C24:0 FA was higher with IP as an introduction solvent than that with EtOH (Fig. 1A). It should be noted that VLCFAs were not dissolved when BSA was absent in the solution (data not shown). This result indicated that VLCFAs were not solubilized as FA micelles.

Formation of the FA/BSA complex was confirmed via gel filtration column chromatography. We found that both C24:0 FA and BSA were present in the same eluted fraction when the FA/BSA sample prepared by the FA/IP/BSA method was fractionated. On the other hand, C24:0 FA was not present in the BSA fraction of samples prepared using the FA/Na/BSA method (Fig. 1B). A similar experiment was conducted with C24:1 FA. We found that C24:1 FA and BSA were eluted in the same fraction in both samples prepared by FA/Na/BSA and FA/IP/BSA (Fig. 1C). We determined recoveries of C24:0, C26:0, C24:1 and C18:1 FA based on the amount of FA present in BSA-eluted fraction of gel filtration column chromatography, and found that the recoveries of these FA prepared by FA/IP/BSA method were 73.5, 63.5, 60.2 and 62.2 % of starting FA, respectively. By contrast, neither C24:0 FA nor C26:0 FA was recovered when samples were prepared using the conventional FA/Na/BSA method (Fig. 1D). These results indicated that FA/IP/BSA has advantageous on the preparation of saturated VLCFA/BSA complex, whereas, both FA/Na/BSA and FA/IP/BSA methods is applicable to unsaturated VLCFAs, as described previously [56].

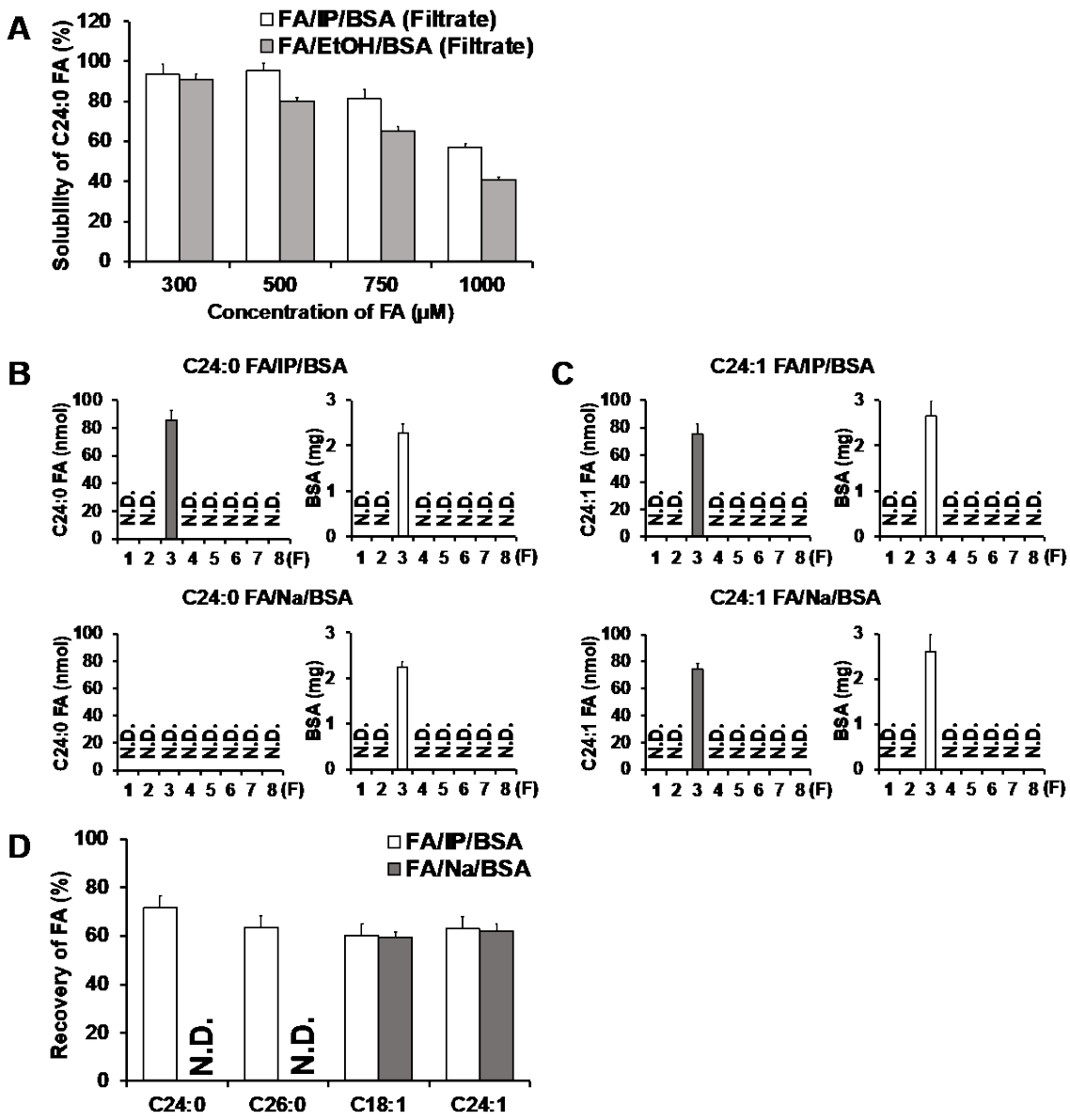


Fig. 1. Confirmation of the formation of the VLCFA/BSA complex by membrane filtration and gel filtration. Various amounts of C24:0 FA dissolved in either isopropanol (IP) or ethanol (EtOH) were converted to their BSA complex by the FA/IP/BSA method or the FA/EtOH/BSA method, as described in 2.3. After filtration with 0.22 μm filter, amount of FA in the filtrate was determined by GC (A). The FA/BSA complex (300 μM) prepared via the FA/IP/BSA method or the FA/Na/BSA method was subjected to a gel filtration column chromatography for fractionation. Each fraction was subjected to protein assay (BSA quantification) and GC analysis (fatty acid quantification) as described in section 2.5 (B, C). The amount of FA present in BSA fraction was used for calculation of the recovery (D). Values represent the mean ± S.D. of three independent experiments. N.D. denotes not detected.

3.2. Cytotoxicity of extracellular VLCFAs in peroxisome-deficient CHO cells

Previously, we observed that incubation with C20:0, C20:1, C22:0, C22:1 and C24:1 FA, at 10-30 μM for 48 h was toxic to peroxisome-deficient CHO cells. Whereas, incubation with C24:0 and C26:0 FA up to 30 μM was not toxic [56]. The uptake assay revealed that the uptake of C24:0 and C26:0 FA into the CHO cells was strongly suppressed as compared to that of cytotoxic C20:0, C20:1, C22:0, C22:1 and C24:1 FA, respectively. In this study, we found that incubation with higher concentrations (~ 150 μM) of C24:0 and C26:0 FA for a longer time (~ 72h) was toxic to peroxisome-deficient cells. As shown in Fig. 2A, 100 μM C24:0 and 150 μM C26:0 FA treatment decreased cell viability by about 35 and 40 %, respectively, in peroxisome-deficient cells after 72 h. We also found that 30 μM C26:1 FA decreased cell viability to around 10 % in peroxisome-deficient cells after 72 h. Whereas, C20:0, C20:1, C22:0, C22:1 and C24:1 FA below 30 μM were found to exert cytotoxicity. Notably, concentrations up to 100 μM C16:0, C16:1, C18:0, and C18:1 FA treatments showed no cytotoxic effects on peroxisome-deficient cells (Fig. 2A). In contrast to peroxisome-deficient cells, C24:0 and C26:0 FA treatment did not exert any cytotoxic effects on wild-type CHO cells even at higher concentrations and longer incubation times (Fig. 2B). Our results reconfirmed that VLCFAs, except for high concentrations of C20:0 FA, did not exert cytotoxic effects on wild-type cells [56]. The difference in susceptibility toward cytotoxic VLCFAs between peroxisome-deficient cells and wild-type cells is attributable largely to peroxisome activity that ensures the clearance of VLCFAs as described in our previous report [55,56].

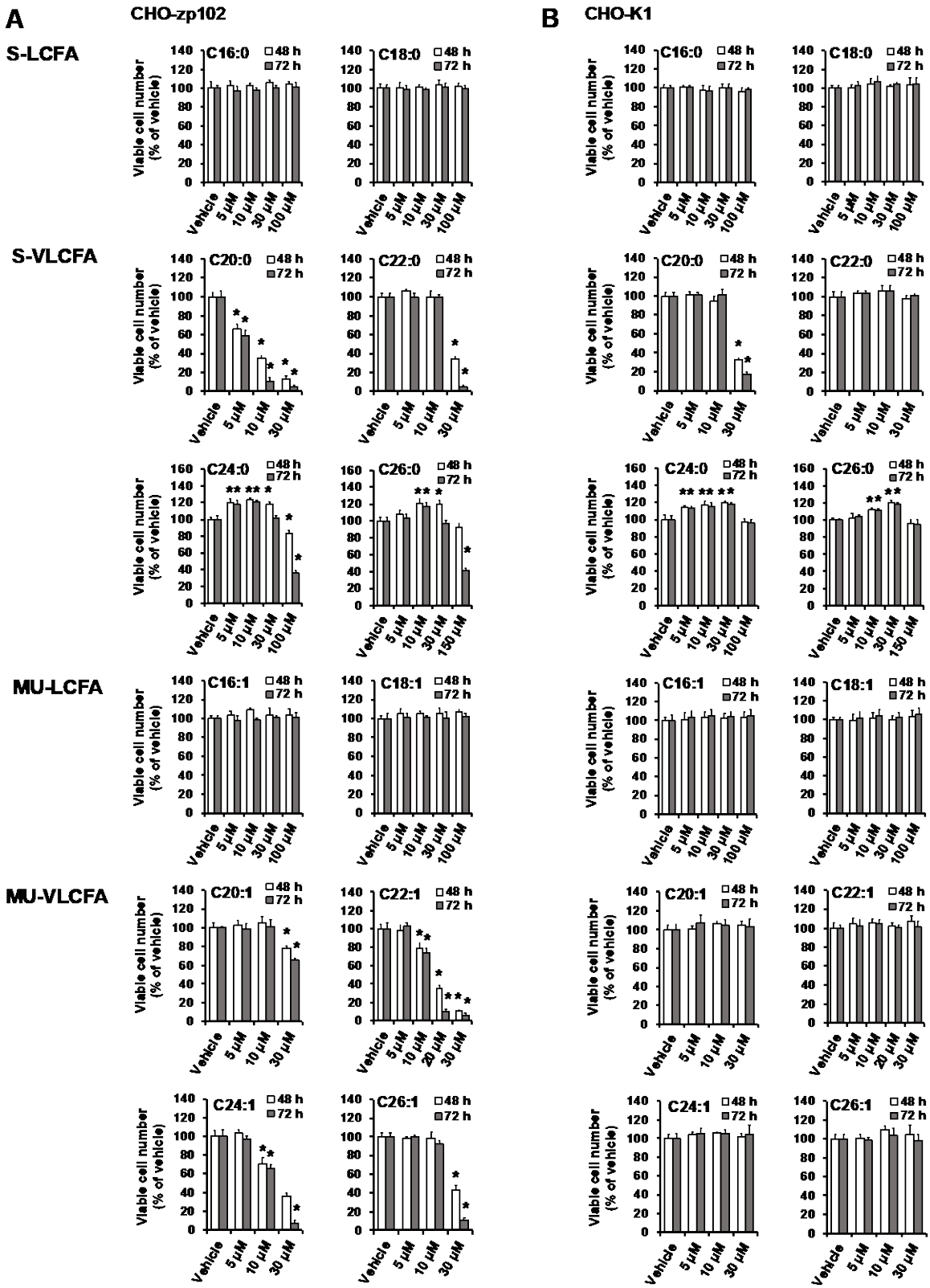

A
CHO-zp102
B
CHO-K1
S-LCFA
S-VLCFA
MU-LCFA
MU-VLCFA
C16:0
C18:0
C20:0
C22:0
C24:0
C26:0
C16:1
C18:1
C20:1
C22:1
C24:1
C26:1
48 h
72 h
Viable cell number (% of vehicle)
Vehicle
5 µM
10 µM
20 µM
30 µM
100 µM
150 µM

Fig. 2. Toxicity of VLCFAs in peroxisome-deficient CHO cells.

CHO-zp102 (peroxisome-deficient) and CHO-K1 (wild-type) cells were incubated in serum-free medium with indicated concentrations of FAs for 48 and 72 h (A, B). Cell viabilities were determined via a trypan blue exclusion test. The data shown are the percentages of the values of the vehicle-treated control cells. Values represent the mean ± S.D. of three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test; $*p < 0.05$ vs. vehicle.

3.3. FA composition of cellular lipids supplemented with various VLCFAs

We examined the FA composition of cellular lipids of peroxisome-deficient (CHO-zp102) cells and its wild-type (CHO-K1) cells incubated with various VLCFAs (Fig. 3). We found that amount of VLCFAs accumulated in cellular lipids was higher in shorter VLCFAs than longer VLCFAs in these cells. For example, peroxisome-deficient cells incubated with 30 μM of C20:0 FA resulted in the accumulation of 79 nmol/dish of C20:0 FA in cellular lipids (Fig. 3A). Whereas, incubation of the peroxisome-deficient cells with the same concentration of C26:0 FA, resulted in the accumulation of only 6 nmol/dish of C26:0 FA in cellular lipids (Fig. 3D). This was also the case for monounsaturated VLCFAs. Incubation with 30 μM C22:1 and C26:1 FA induced the accumulation of 120 nmol/dish and 20 nmol/dish of the monounsaturated VLCFAs, respectively, in the cellular lipids of peroxisome-deficient cells (Fig. 3E, G). Although a similar tendency was also observed in wild-type cells, the accumulation levels of VLCFAs were much less in wild-type cells compared with that of peroxisome-deficient cells. This was due to the fact that most parts of the VLCFAs taken up in wild-type cells were immediately metabolized with peroxisome-dependent fashion [55,56].

The potency of cytotoxic VLCFAs was compared in terms of their accumulation levels in cellular lipids (Table 1). An accumulation level of 44 nmol/dish of C20:0 FA, which was equivalent to 25 % of the total fatty acids (TFA) in cellular lipids, resulted in the death of 65 % of peroxisome-deficient cells. On the other hand, an accumulation of 16 nmol/dish of C26:0 FA, which was equivalent to 8 % of TFA in cellular lipids induced a similar magnitude of cytotoxicity, indicating that longer VLCFAs exert cytotoxicity in peroxisome-deficient cells at lower levels of accumulation. Similarly, the accumulation level of C26:1 FA (20 nmol/dish; 8 % of TFA) that induced cell death in 60-65 % of peroxisome-deficient cells was lower than that for C22:1 FA (70 nmol/dish, 28 % of TFA). This comparison also indicated the tendency that saturated VLCFAs has potent cytotoxicity as compared to the corresponding monounsaturated one. These results indicated that, even in the low level,

accumulation of saturated and longer VLCFA causes profound damage to the peroxisome-deficient cells.

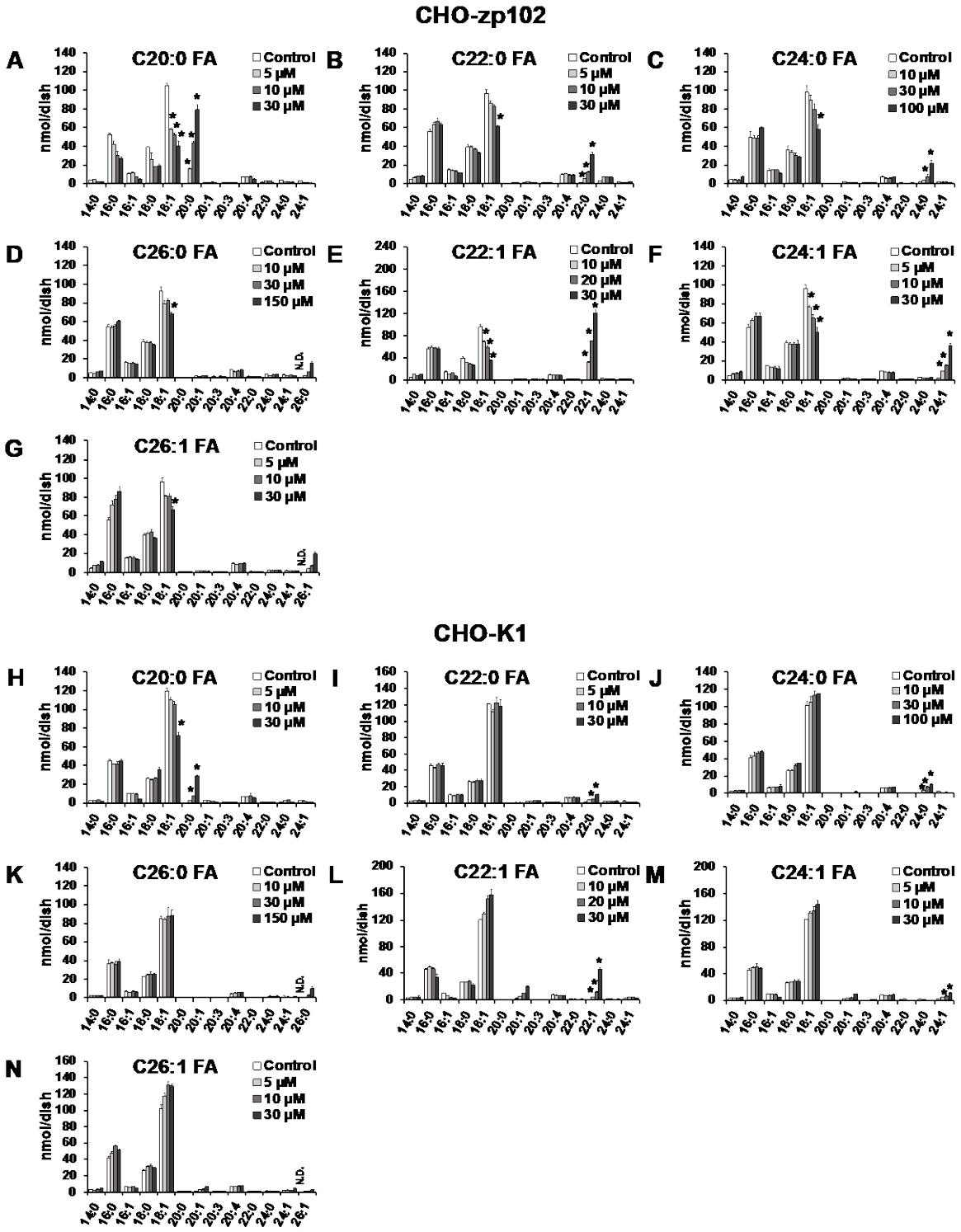


Fig. 3. Fatty acid composition of CHO-K1 and CHO-zp102 cells incubated with various VLCFAs.
CHO-zp102 (peroxisome-deficient) and CHO-K1 (wild-type) cells were incubated with indicated concentrations of VLCFAs in serum-free medium for 24 h (A–N). The cells were collected after 24 h and subjected to lipid extraction. The fatty acid methyl esters were analyzed via GC, as mentioned in section 2.5. Values represent the mean ± S.D. of three independent experiments. *S*tatistically significance was set at $*p < 0.05$ (Student's t-test); N.D.: not detected.

Table 1. Toxicity of VLCFAs as a function of the accumulation of cellular lipids.
Values shown here were obtained via the experiments shown in Figs. 2 and 3. The concentration of FA in the medium were as follows; C20:0 FA (10 μM), C22:0 FA (30 μM), C24:0 FA (100 μM), C26:0 FA (150 μM), C22:1 FA (20 μM), C24:1 FA (30 μM), C26:1 FA (30 μM). Values represent the mean ± S.D. of three independent experiments.

	CHO-zp102 (peroxisome-deficient cells)			**CHO-K1 (wild-type cells)**		
	Accumulation		Viable cell number	Accumulation		Viable cell number
	nmol/dish	% of TFA	% of vehicle	nmol/dish	% of TFA	% of vehicle
Saturated						
C20:0 FA	44 ± 2	25 ± 0.3	35 ± 3	28 ± 1	15 ± 0.1	30 ± 2
C22:0 FA	32 ± 2	14 ± 0.1	35 ± 2	10 ± 2	5 ± 0.1	Not toxic
C24:0 FA	22 ± 3	11 ± 0.3	35 ± 3	10 ± 2	4 ± 0.6	Not toxic
C26:0 FA	16 ± 1	8 ± 0.2	40 ± 3	10 ± 1	5 ± 0.5	Not toxic
Monounsaturated						
C22:1 FA	70 ± 2	28 ± 0.6	35 ± 3	12 ± 1	5 ± 0.1	Not toxic
C24:1 FA	35 ± 3	15 ± 0.2	35 ± 3	10 ± 2	4 ± 0.2	Not toxic
C26:1 FA	20 ± 3	8 ± 0.7	40 ± 4	4 ± 1	1 ± 0.1	Not toxic

3.4. Association of cellular C18:1 FA with VLCFA-induced cell death

As shown in Fig. 2, significant reductions in the cell viability occurred when peroxisome-deficient cells were incubated with ≥ 5 μM C20:0, 30 μM C22:0, 100 μM C24:0 and 150 μM C26:0 FA. In these experiments, remarkable decreases in the level of cellular C18:1 FA were observed (Fig. 3). Such decreases were also observed in the experiments with toxic level of monounsaturated VLCFAs

(C22:1, C24:1 and C26:1 FA) in peroxisome-deficient cells. The VLCFA-induced toxicity was observed only in the experiment with 30 μM C20:0 FA in wild-type cells. In this experiment, the decrease in the level of cellular C18:1 FA was remarkable (Fig. 2, 3). It should be mentioned that cellular lipid analysis was performed at 24 h of incubation with VLCFAs, the time of which is before onset of cell death (~ 48h).

Previously, we showed that cytotoxicity of C20:0 FA was enhanced in the presence of peroxisomal oxidation inhibitor, TDYA, in wild-type CHO cells [56]. Here, we found that treatment with TDYA enhanced C20:0 FA cytotoxicity along with accelerating the decrease in cellular C18:1 FA (Fig. 4A, B). Data pertaining to the decrease in cellular C18:1 FA levels (Fig. 4A) and the extent of C20:0 FA-induced cell death (Fig. 4B) were plotted (Fig. 4C). The results indicated that these two cellular events were correlated. The threshold reduction level of cellular C18:1 FA at which apparent cytotoxicity observed is around 20-30 nmol reduction from the control level. This threshold reduction level of C18:1 FA seemed to be applicable to other VLCFAs with cytotoxic concentration (Fig. 2, 3). These results indicated the association between cellular C18:1 FA and VLCFA-induced cell death.

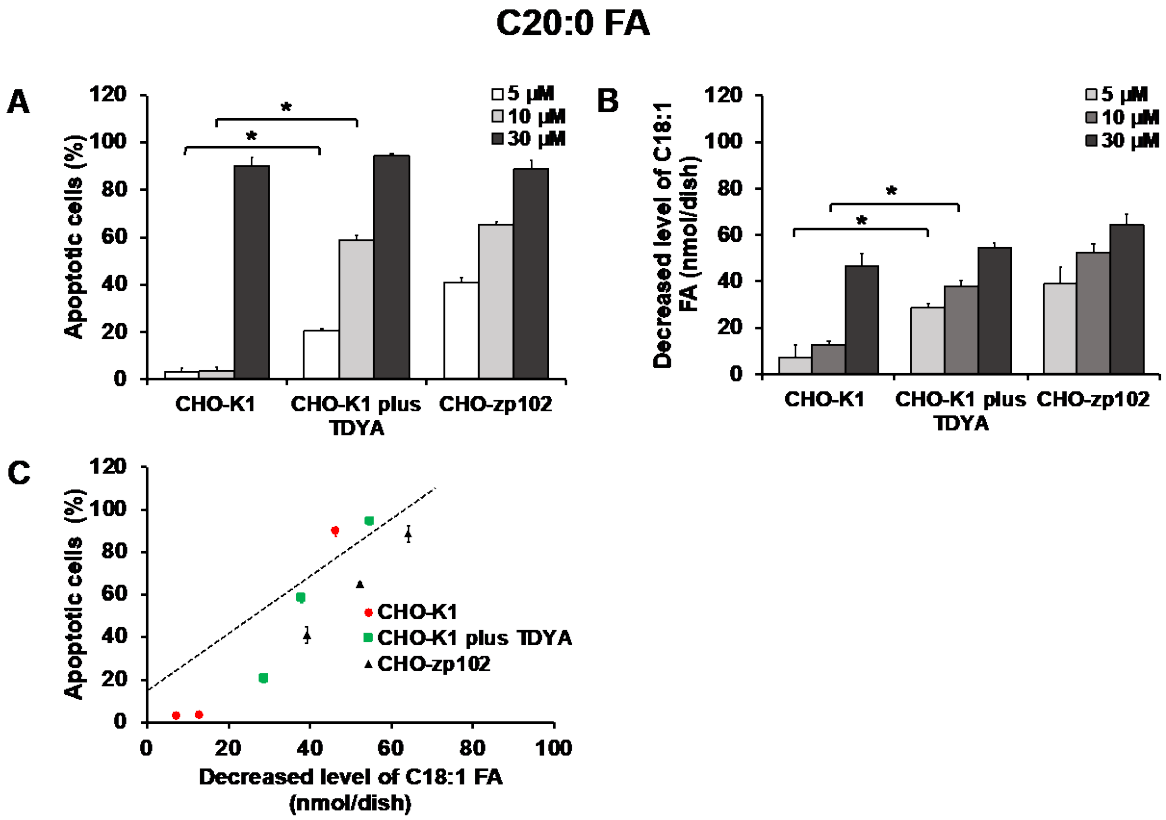


Fig. 4. The correlation between decreased levels of C18:1 FA and cellular toxicity.

CHO-K1 (wild-type) and CHO-zp102 (peroxisome-deficient) cells were incubated with indicated concentrations of C20:0 FA in serum-free medium for 48 h in the absence or presence of 30 μM TDYA, followed by DAPI staining aimed at counting cells showing apoptotic nuclei. Cellular lipids were extracted from the cells followed by methyl esterification to determine FA composition by GC. The percentage of apoptotic cells (A) and decreased level of C18:1 FA as compared to non-treated control cells (B), and their correlation (C) are shown. Values represent means ± S.D. of three independent experiments. *S*tatistically significance was set at $*p < 0.05$ (Student's t-test).

3.5. Protective effect of C18:1 FA against VLCFA-induced apoptosis in peroxisome-deficient CHO cells.

Supplementation with C18:1 FA rescued peroxisome-deficient cells from cell death induced by all VLCFAs tested (Fig. 5A–F). For example, treatment with 10 μM C20:0 FA for 48 h reduced the viability of peroxisome-deficient cells to 33.4 % of that of the non-treated control cells. The cell viability recovered to 67.1, 79.8, and 89.4 % following co-treatment with C18:1 FA at 5, 10, and 30 μM, respectively (Fig. 5A). Supplementation with C18:1 FA also restored the reduced cellular C18:1 FA levels in a dose-dependent fashion (Fig. 5A). It should be noted that accumulation level of C20:0 FA remained unchanged regardless of C18:1 FA supplementation, indicating that the protective effect of C18:1 FA was not due to the inhibition of C20:0 FA uptake. Another example is C26:1 FA. Treatment with 30 μM C26:1 FA reduced cell viability to 40 % of that of the control cells. The C26:1 FA-induced cytotoxicity was almost completely reversed by the co-administration of 30 μM C18:1 FA with restoring reduced cellular C18:1 FA levels without affecting the accumulation level of C26:1 FA (Fig. 5F).

Previously, we showed that VLCFA-induced cell death in peroxisome-deficient cells was primarily due to apoptosis, as represented by DNA fragmentation [56]. In this study, we found that supplementation with C18:1 FA inhibited morphological changes in the nuclei (Fig. 5G) as well as DNA fragmentation (Fig. 5H), induced by C24:0 FA. These results imply that peroxisome-deficient cells can survive, as long as the cells keep sufficient levels of cellular C18:1 FA, even if the cells accumulate cytotoxic level of VLCFA.

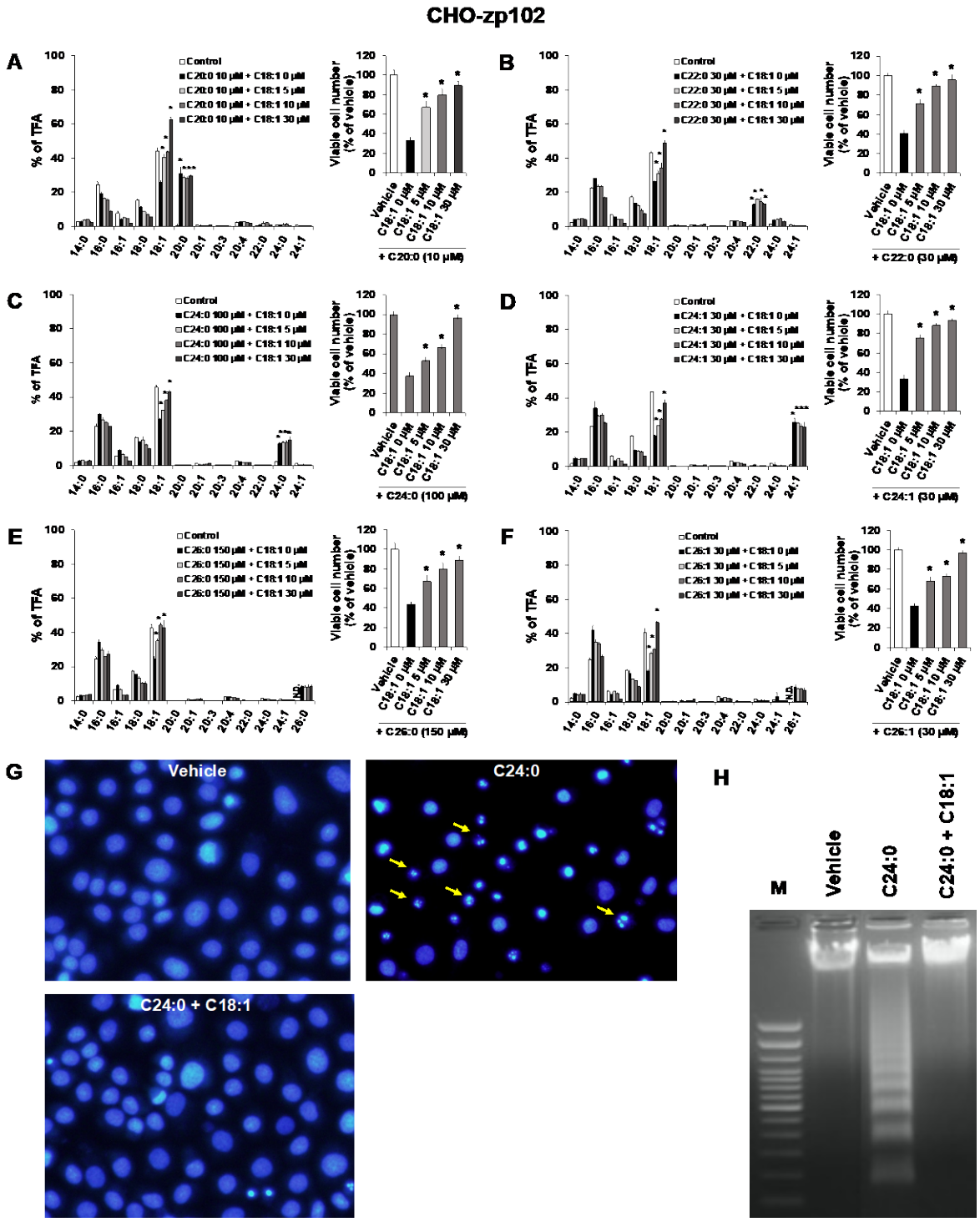


Fig. 5. Effects of C18:1 FA on FA composition and VLCFA-induced apoptosis in peroxisome-deficient CHO cells.

CHO-zp102 (peroxisome-deficient) cells were cultured in serum-free medium with various concentration

of VLCFAs for 48 h (C20:0, C22:0, C24:1, and C26:1) or 72 h (C24:0 and C26:0) in the presence or absence of C18:1 FA (A–F). Cell viability and FA levels were analyzed as described in section 2.4, and 2.5, respectively. Values represent the mean ± S.D. of three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test; $*p < 0.05$ vs. vehicle. N.D.: not detected. Fluorescence microscopy of DAPI-stained CHO-zp102 cells treated with 100 μM C24:0 FA with or without 30 μM C18:1 FA for 72 h (G). The data are representatives of three independent experiments. Electrophoresis of DNA of CHO-zp102 cells treated with 100 μM C24:0 FA for 72 h with or without 30 μM C18:1 FA (H). DNA fragmentation was analyzed using 1.5% agarose gel electrophoresis. M indicates the 100-bp DNA ladder size marker.

3.6. Lipidomics analyses of peroxisome-deficient CHO cells loaded with C24:0 FA

We conducted a lipidomic analysis of peroxisome-deficient cells to gain a comprehensive understanding of lipid alteration triggered by C24:0 FA treatment. The number of lipid molecules annotated by our UHPLC-QE orbitrap/MS/MS method was around 400. Principal component analysis (PCA) was used to explore notable differences between sample groups. The PCA score plot revealed a clear separation between the control group and the C24:0 FA-treated group at different concentrations, indicating that distinct changes had occurred between these groups (Fig. 6A). The PCA-loading plot was performed with a metabolomics software, MS-DIAL to determine the specific lipid species responsible for such changes. The PCA-loading plot revealed that primary molecules contributing to the differences between these groups were largely PC and LPC species, such as PC 36:2 and LPC 24:0, as shown by red circles in Fig. 6B. In addition, PE 36:2 and SM 42:2 looked to contributed to the differences observed between groups (Fig. 6B).

Changes in the composition of the molecular species of each lipid class was analyzed. Increases in the levels of PC species, such as PC 40:0, PC 42:1, PC 42:2, PC 44:4, PC 46:5, PC 46:6, PC 48:0, were observed in C24:0 FA-treated cells (Fig. 7). These are considered to be PC species containing C24:0 FA. By contrast, remarkable decreases in the levels of PC 34:2, PC 36:1 and PC 36:2 were observed in cells treated with C24:0 FA. Fragment analyses of respective mass spectra indicated that PC 34:2, PC 36:1 and PC 36:2 mainly consisted of 16:1/18:1, 18:0/18:1 and 18:1/18:1 PC, respectively. Among them, a decrease in the level of 18:1/18:1 PC (PC 36:2) was notable. The molecular species, 18:1/18:1 PE, was also significantly reduced in response to C24:0 FA uptake (Supplementary Fig. 1). Lipidomics analysis performed in peroxisome-deficient cells co-treated with C24:0 FA (100 μM) and C18:1 FA (30 μM) revealed that reduction in the level of 36:2, but not 34:2

or 36:1, was completely recovered both PC and PE. These results indicated the importance of phospholipids with 18:1/18:1 for cell survival against membrane modification caused by toxic levels of VLCFA (Fig. 7, Supplementary Fig. 1).

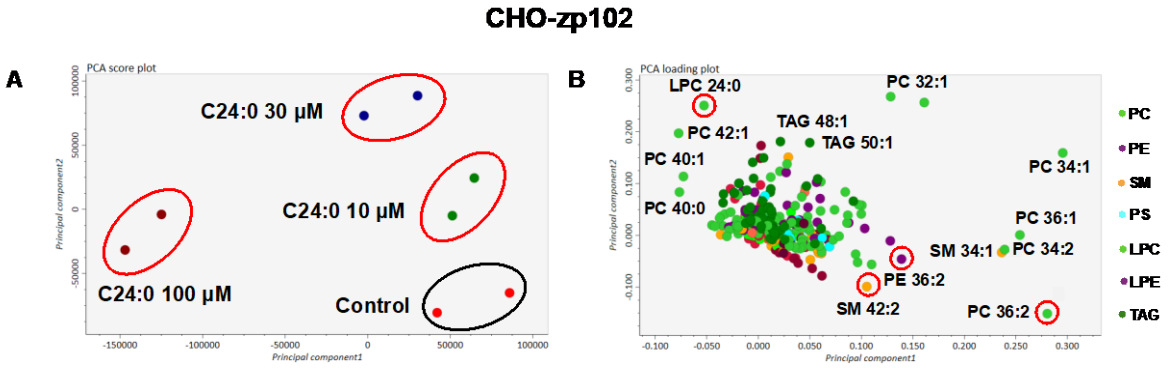


Fig. 6. Lipidomics of peroxisome-deficient CHO cells incubated with C24:0 FA.

CHO-zp102 (peroxisome-deficient) cells were cultured in serum-free medium with (10, 30 and 100 μM) or without (control) C24:0 FA for 24 h. The cellular lipids were extracted and subjected to LC-MS/MS as described in 2.8. Principal component analysis (PCA) was performed to characterize lipid alterations using metabolomics software, MS-DIAL. PCA score plot showing clear separation between the control group and C24:0 FA (10, 30 and 100 μM) treated groups (A). PCA loading plot showing the variables contributing most to the separation (B). Each data point represents an individual lipid ion. The major significant lipid ions are indicated on the plot. PC: phosphatidylcholine, PE: phosphatidylethanolamine, SM: sphingomyelin, PS: phosphatidylserine, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, TAG: triacylglycerol.

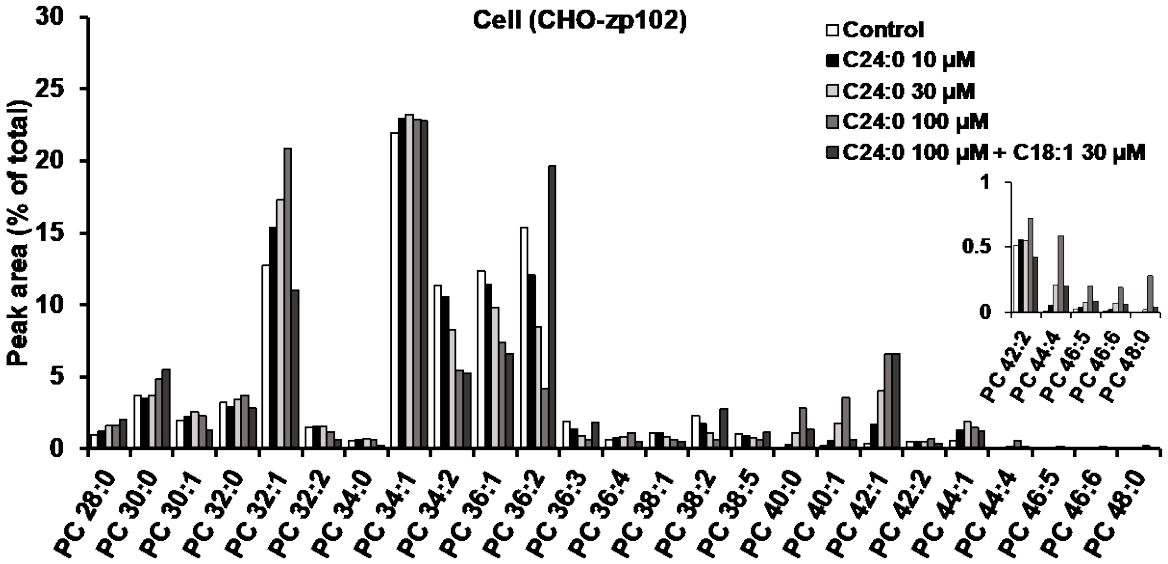
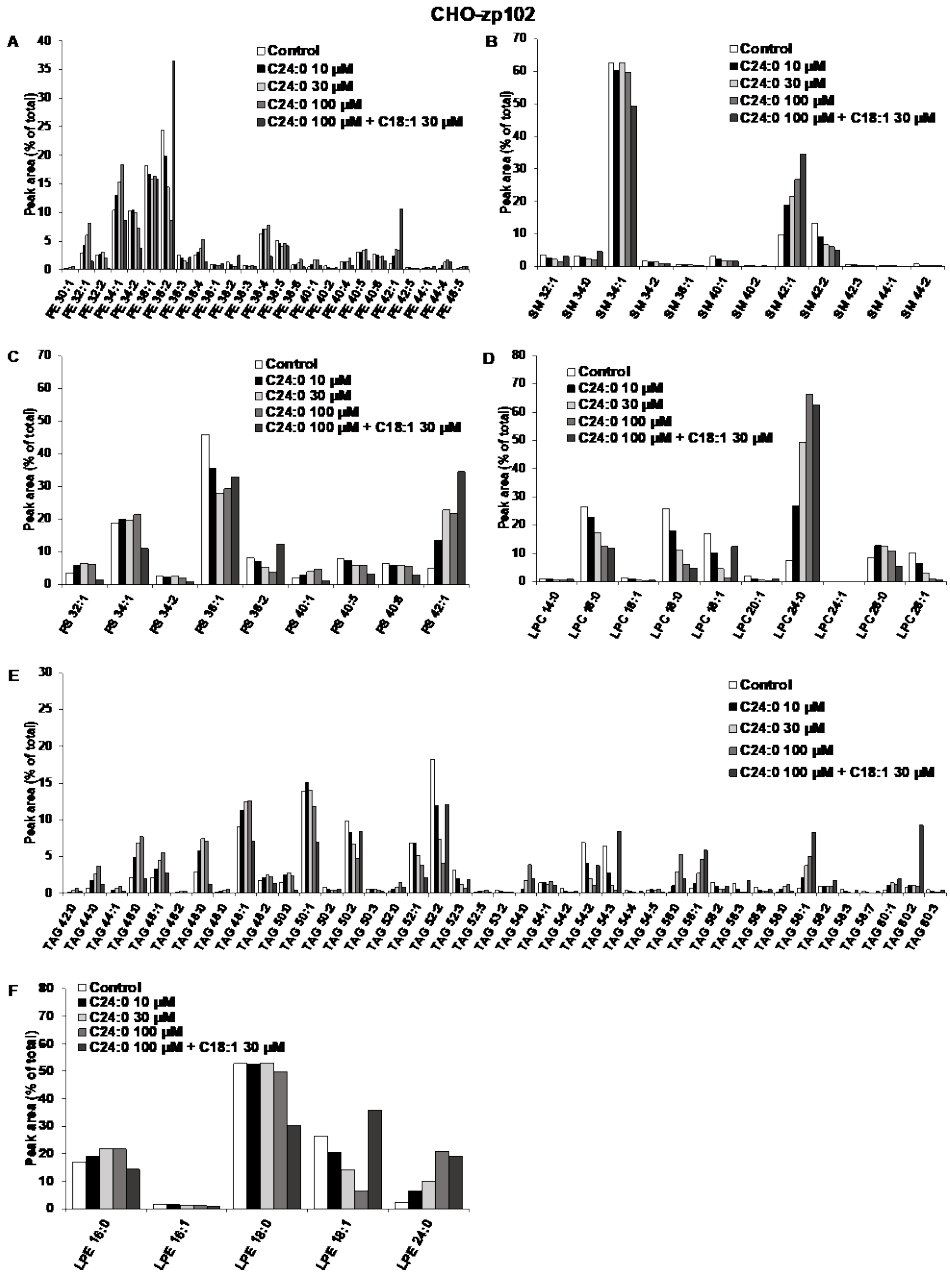


Fig. 7. Molecular species composition of PC in peroxisome-deficient CHO cells treated with C24:0 FA- or C24:0 + C18:1 FA.

CHO-zp102 (peroxisome-deficient) cells were cultured in serum-free medium with indicated concentrations of C24:0 for 24 h in the presence or absence of 30 μM C18:1 FA. Lipidomics were performed with LC-MS/MS as described in 2.8. The compositional alteration of PC is shown as a % of the total. Values represent the average of data from two separate culture dishes. Similar results were obtained from two independent experiments performed on separate days.



Supplementary Fig. 1. Molecular species composition of PE, SM, PS, LPC, TAG and LPE in peroxisome-deficient CHO cells treated with C24:0 FA- or C24:0 + C18:1 FA.

CHO-zp102 (peroxisome-deficient) cells were cultured in serum-free medium with indicated concentrations of C24:0 for 24 h in the presence or absence of 30 μM C18:1 FA. Lipidomics were performed with LC-MS/MS as described in 2.8. Compositional alterations in these lipid classes are shown as a % of the total. Values represent the average of data from two separate culture dishes. Similar results were obtained via two independent experiments performed on separate days (A–F). PE: phosphatidylethanolamine, SM: sphingomyelin, PS: phosphatidylserine, LPC: lysophosphatidylcholine, TAG: triacylglycerol, LPE: lysophosphatidylethanolamine.

4. Discussion

Previously, we developed a method for solubilizing VLCFAs by forming a VLCFA/BSA complex and examined the uptake and toxicity of VLCFAs with C20-C26 in peroxisome-deficient CHO cells [55,56]. Our results indicated that shorter VLCFAs exerted higher cytotoxicity at lower concentrations compared to longer ones [56]. In fact, C20:0 FA showed toxicity starting from 5 μM, whereas, C24:0 FA did not exert any toxicity even at 30 μM. However, comparison of cytotoxicity of VLCFAs based on their concentration in a medium would not reflect their intracellular toxicity. Because, longer VLCFAs were hardly incorporated into the peroxisome-deficient cells under such conditions [55,56]. In fact, the amount of C26:0 FA incorporated in to the peroxisome-deficient CHO cells was around 6 nmol/dish, whereas, 79 nmol/dish of C20:0 FA was incorporated into the cells when the cells were incubated with these VLCFAs at 30 μM (Fig. 3). Considering the characteristic accumulation of C24:0, C26:0 and C26:1 FA in the body of patients of peroxisome disease [23–38], effects of accumulation of the longer VLCFAs in the cells are important for pathological elucidation of the diseases. In this study, we found that incubation of C24:0 and C26:0 FA at higher concentrations for longer times led to substantial accumulations of these VLCFAs. Using this altered culture conditions with our solubilization technique, we evaluated the association between the toxicity of VLCFAs and their accumulation levels. We found that accumulation of saturated and longer VLCFAs causes profound damage to the peroxisome-deficient cells, compared to the damage caused by unsaturated or shorter ones. For example, C26:0 FA killed peroxisome-deficient cells when its accumulation reached 8 % of TFA. By contrast, C20:0 FA, killed cells when its accumulation reached 25 % of TFA (Table 1). These observations are consistent with those of previous studies indicating that longer SFAs exerted more potent cytotoxicity [61,62].

Several mechanisms have been proposed as those underlying the cytotoxicity of VLCFAs. These include oxidative stress and endoplasmic reticulum (ER) stress, as well as dysfunctional lysosomes and mitochondria [45–54]. Considering that longer and saturated VLCFAs exerts potent cytotoxicity as compared to shorter and unsaturated one, stresses causing from membrane distortion and loss of membrane flexibility, which has been known as ER stress [63–65], seems to trigger the apoptosis of the peroxisome-deficient cells. The survival effect of C18:1 FA against VLCFA-induced apoptosis is also explainable as relieve of such ER stress as described below.

Here, we found that the cytotoxicity of VLCFAs was associated with decreased levels of cellular C18:1 FA. This relationship was observed in all experiments that investigated different VLCFAs accumulations in peroxisome-deficient CHO cells as well as in TDYA-treated wild-type CHO cells.

We also found that supplementation with C18:1 FA rescue the cells from VLCFA-induced apoptosis. Importantly, cellular level of VLCFA was not so changed by the coaddition of C18:1 FA. This indicated that C18:1 FA was able to prevent VLCFA-induced damage to peroxisome-deficient cells without affecting VLCFA levels. We found that C18:1 FA did not rescue peroxisome-deficient cells from UV- or staurosporine-induced apoptosis (data not shown), indicating that C18:1 FA does not affect the execution process of apoptosis. These findings suggested that intracellular C18:1 FA may ameliorate the ER stress by buffering membrane stress induced by the accumulation of VLCFAs. A possible scheme is depicted in Fig. 8, where 18:1/18:1 phospholipid species stabilizes the cellular membrane distorted by VLCFAs. This notion was led from the lipidomics results showing dramatical decrease of C36:2 (mainly 18:1/18:1 species) of PC and PE in response to addition of C24:0 FA. The fact that supplementation with C18:1 FA restored C36:2 species of PC and PE as well as cell viability was taken into consideration in this hypothesis. This membrane smoothing action of phospholipids with 18:1/18:1 explains the ability of C18:1 FA to rescue cells without changing cellular VLCFAs levels. Smooth and flexible membrane will contribute to the membrane functions, such as vesicle traffic and the solvent role for membrane enzymes.

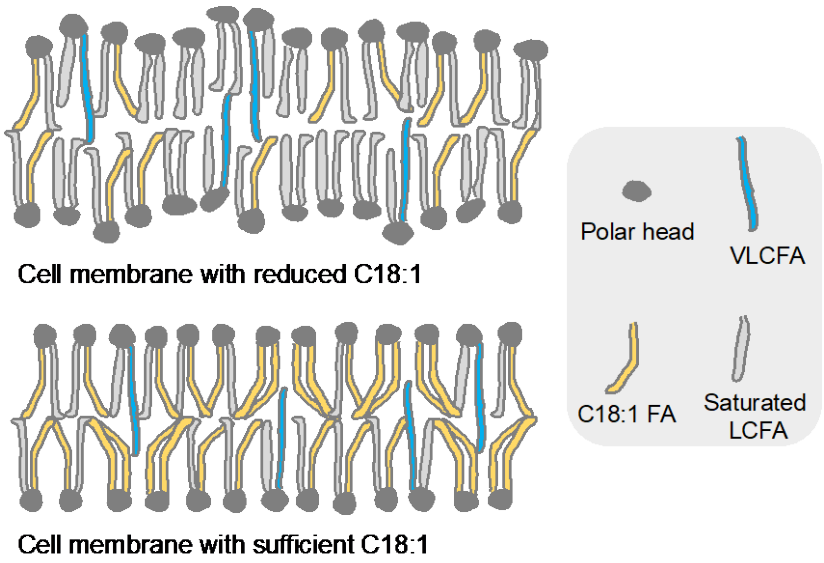
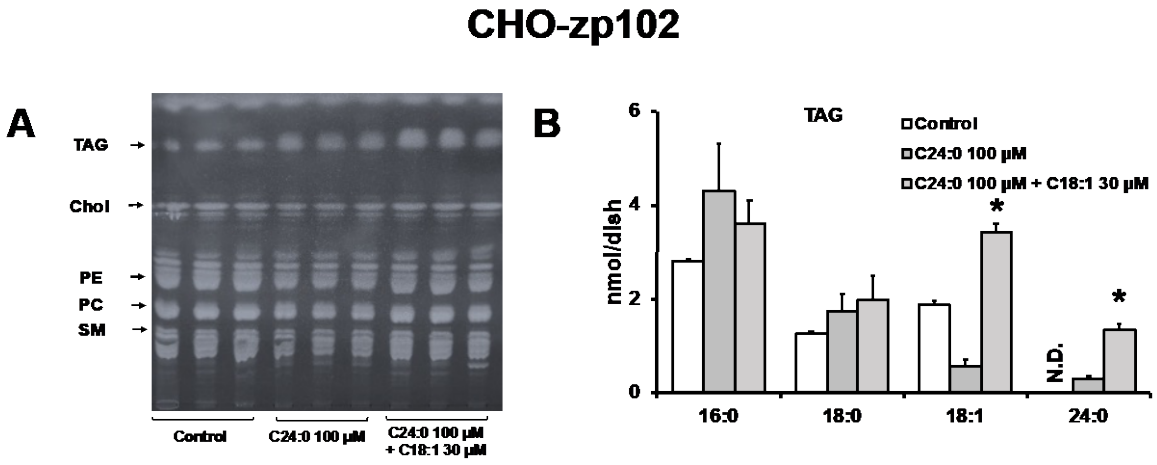


Fig. 8. Schematic representation of buffering action of 1,2-dimonounsaturated phospholipids (typically 18:1/18:1) against membrane distortion stress induced by VLCFA-containing phospholipids.

Another mechanism underlying the ameliorative effect of C18:1 FA is the removal of VLCFA from membrane phospholipids via esterification into TAG, a molecule which stores energy. This mechanism has been proposed as an explanation of ameliorative effect of C18:1 FA on ER stress

induced by C16:0 FA [66–69]. We found that addition C18:1 FA promotes TAG accumulation via the conversion of excess C24:0 FA to TAG stores (Supplementary Fig. 2). Our lipidomics analysis also revealed the acylation of C24:0 FA from PC to TAG. The enhanced formation of TAG will lead to the formation of lipid droplets, which protect the cells against lipotoxicity by sequestration of toxic FA from ER [70].



Supplementary Fig. 2. Change in the amount of TAG in peroxisome-deficient CHO cells incubated with C24:0 FA in the presence or absence of C18:1 FA.

CHO-zp102 (peroxisome-deficient) cells were cultured in serum-free medium with indicated concentrations of C24:0 FA for 24 h in the presence or absence of 30 μM C18:1 FA. Cellular lipids were separated via TLC (A). Isolated TAG was subjected to methyl esterification for quantification of FA by GC (B). Values represent the means ± S.D. of three independent experiments. PC: phosphatidylcholine, PE: Phosphatidylethanolamine, SM: sphingomyelin, Chol: cholesterol, TAG: triacylglycerol. Significance was analyzed by two-way ANOVA followed by Student's t-test (B). *$p < 0.05$ indicates a significant difference between indicated groups. N.D.: not detected.

The mechanisms underlying the decrease in cellular C18:1 FA levels with increasing VLCFA accumulation remain unclear. A possible explanation is that fatty acid-chain elongation and desaturation systems present in the ER are hampered due to membrane distortion caused by accumulated VLCFAs. Further study on the VLCFA toxicity will clarify the mechanism underlying the decrease in C18:1 FA as well as the rescue effects exerted by C18:1 FA.

It has been shown that 7-ketocholesterol (7-KC), a toxic compound produced by the oxidation of cholesterol, was increased in the plasma of ALD patients [71]. Importantly, C18:1 FA has been shown to prevent the death of nerve cells induced by the 7-KC [72,73]. It is important to determine the level

of 7-KC in VLCFA-treated peroxisome-deficient CHO cells to clarify the involvement of 7-KC in the VLCFA-induced apoptosis. These multiple approaches for clarification of the mechanisms underlying VLCFA-induced damage will contribute better understanding of the pathology of peroxisome diseases.

ALD is the most frequent peroxisome disorder that shows elevation of VLCFAs. Although abnormal accumulation of VLCFAs is believed to be associated with the symptoms of ALD, the association between the accumulation of VLCFAs and the clinical severity of ALD remains obscure [16–38]. We have also observed healthy female carriers with elevated VLCFA-ceramide [39]. In this study, we found that peroxisome-deficient cells can survive in the presence of toxic level of VLCFAs, as long as the cells keep sufficient levels of cellular C18:1 FA. Considering this, it may be possible to hypothesize that the elevated VLCFAs with reduced C18:1 FA is at risk for severe symptoms. In this regard, decreased levels of C18:1 FA have been reported in altered lesions of patients with ALD [74,75]. In addition, Ras et al., have been reported that SCD1 expression is associated with the recovery of deteriorated motor behaviors in ALD zebra fish model [76]. Currently, elevated VLCFAs in blood is used as a marker in the diagnosis of peroxisome disease. Clinical surveys focusing on both VLCFA and C18:1 FA may help elucidate the pathology of the disease.

Therapies that are currently available for ALD include hormone replacement therapy, hematopoietic stem cell transplantation, and a dietary oil known as Lorenzo's oil (LO). LO, which is a mixture of C18:1 and C22:1 FA in a 4:1 ratio, reportedly normalizes blood C26:0 levels of ALD patients, possibly by competing for the microsomal elongation system [77,78], and believed to prevent neuronal degenerations. However, LO appears to be ineffective against ongoing neurological degeneration, with varying degrees of effectiveness among patients [79–82]. In this study, we found that C18:1 FA protect the peroxisome-deficient cells from VLCFA-induced damage, whereas C22:1 FA, being a peroxisomal substrate, promotes cell death [56]. Our findings provide new insights into the mechanisms of pharmacological effect of LO, and raises questions regarding the constituent FAs in LO.

In summary, we established a VLCFA-accumulated disease model using peroxisome-deficient CHO cells. Using this disease model, we demonstrate that C18:1 FA exerts a protective effect against the VLCFA-induced apoptosis in peroxisome-deficient cells. Although our model is useful for analyzing the metabolism and toxicity of VLCFAs, FA composition in human tissues may vary quite considerably. In addition, tissues that are characteristically found to be damaged in peroxisomal disorders are nerve tissues consisting of myelinated neurons [16–38]. Further studies are required to

elucidate the effects of VLCFAs and C18:1 FA using disease models of neuronal cells, oligodendrocytes and Schwann cells for understanding of the peroxisome diseases.

Acknowledgments/grant support

This study was partly supported by the Chugai Foundation for Innovative Drug Discovery Science (to H.A. and T.T.) and JSPS KAKENHI (Grant Number 22K06114 to T.T.). We appreciate Dr. Kohei Nishino for providing technical assistance in the analysis of lipidomics data.

References

[1] M. Schrader, H.D. Fahimi, The peroxisome: still a mysterious organelle, Histochem. Cell Biol. 129 (2008) 421–440, https://doi.org/10.1007/s00418-008-0396-9.

[2] R.J. Wanders, H.R. Waterham, Biochemistry of mammalian peroxisomes revisited, Annu. Rev. Biochem. 75 (2006) 295–332, https://doi.org/10.1146/annurev.biochem.74.082803.133329.

[3] M. Schrader, H.D. Fahimi, Mammalian peroxisomes and reactive oxygen species, Histochem. Cell Biol. 122 (2004) 383–393. https://doi.org/10.1007/s00418-004-0673-1.

[4] S.D. Nguyen, M. Baes, P.P. Van Veldhoven, Degradation of very long chain dicarboxylic polyunsaturated fatty acids in mouse hepatocytes, a peroxisomal process, Biochim. Biophys. Acta 1781 (2008) 400–405, https://doi.org/10.1016/j.bbalip.2008.06.004.

[5] 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.

[6] 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.

[7] H.R. Waterham, M.S. Ebberink, Genetics and molecular basis of human peroxisome biogenesis disorders, Biochim. Biophys. Acta 1822 (2012) 1430–1441, https://doi.org/10.1016/j.bbadis.2012.04.006.

[8] 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.

[9] 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.

[10] J. Berger, F. Dorninger, S. Forss-Petter, M. Kunze, Peroxisomes in brain development and function, Biochim. Biophys. Acta 1863 (2016) 934–955, https://doi.org/10.1016/j.bbamcr.2015.12.005.

[11] S.J. Steinberg, G. Dodt, G.V. Raymond, N.E. Braverman, A.B. Moser, H.W. Moser, Peroxisome biogenesis disorders, Biochim. Biophys. Acta 1763 (2006) 1733–1748, https://doi.org/10.1016/j.bbamcr.2006.09.010.

[12] M. Depreter, M. Espeel, F. Roels, Human peroxisomal disorders, Microsc. Res. Tech. 61 (2003) 203–223, https://doi.org/10.1002/jemt.10330.

[13] 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.

[14] 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 oxidase 1 deficiency, Biochim. Biophys. Acta 1841 (2014) 610–619, https://doi.org/10.1016/j.bbalip.2014.01.001.

[15] H. Rosewich, H.R. Waterham, R.J. Wanders, S. Ferdinandusse, M. Henneke, D. Hunneman, J. Gärtner, Pitfall in metabolic screening in a patient with fatal peroxisomal beta-oxidation defect, Neuropediatrics 37 (2006) 95–98, https://doi.org/10.1055/s-2006-923943.

[16] 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.

[17] J. Mosser, A.M. Douar, C.O. Sarde, P. Kioschis, R. Feil, H. Moser, A.M. Poustka, J.L. Mandel, P. Aubourg, Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters, Nature 361(1993) 726–730, https://doi.org/10.1038/361726a0.

[18] S. Kemp, F.L. Theodoulou, R.J. Wanders, Mammalian peroxisomal ABC transporters: from endogenous substrates to pathology and clinical significance, Br. J. Pharmacol. 164 (2011) 1753–1766, https://doi.org/10.1111/j.1476-5381.2011.01435.x.

[19] 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.

[20] 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.

[21] J. Berger, S. Forss-Petter, F.S. Eichler, Pathophysiology of X-linked adrenoleukodystrophy, Biochimie. 98 (2014) 135–142, https://doi.org/10.1016/j.biochi.2013.11.023.

[22] A.B. Moser, R.O. Jones, W.C. Hubbard, S. Tortorelli, J.J. Orsini, M. Caggana, B.H. Vogel, G.V. Raymond, Newborn screening for X-linked adrenoleukodystrophy, Int. J. Neonatal. Screen. 2 (2016) 15, https://doi.org/10.3390/ijns2040015.

[23] S. Kemp, R. Wanders, Biochemical aspects of X-linked adrenoleukodystrophy, Brain Pathol. 20 (2010) 831–837, https://doi.org/10.1111/j.1750-3639.2010.00391.x.

[24] 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.

[25] M.C. McGuinness, J.F. Lu, H.P. Zhang, G.X. Dong, A.K. Heinzer, P.A. Watkins, J. Powers, K.D. Smith, Role of ALDP (ABCD1) and mitochondria in X-linked adrenoleukodystrophy, Mol. Cell Biol. 23 (2003) 744–753, https://doi.org/10.1128/MCB.23.2.744-753.2003.

[26] H.W. Moser, K.D. Smith, P.A. Watkins, J. Powers, A.B. Moser, X-linked adrenoleukodystrophy, The Metabolic and Molecular Bases of Inherited Disease, McGraw Hill, New York, 2001, pp. 3257–3301.

[27] H.W. Moser, A.B. Moser, K.K. Frayer, W. Chen, J.D. Schulman, B.P. O'Neill, Y. Kishimoto, Adrenoleukodystrophy: increased plasma content of saturated very long chain fatty acids, Neurology 31 (1981) 1241–1249, https://doi.org/10.1212/wnl.31.10.1241.

[28] I. Singh, A.E. Moser, H.W. Moser, Y. Kishimoto, Adrenoleukodystrophy: impaired oxidation of very long chain fatty acids in white blood cells, cultured skin fibroblasts, and amniocytes, Pediatr. Res. 18 (1984) 286–290, https://doi.org/10.1203/00006450-198403000-00016.

[29] D.K. Lee, N.P. Long, J. Jung, T.J. Kim, E. Na, Y.P. Kang, S.W. Kwon, J. Jang, Integrative lipidomic and transcriptomic analysis of X-linked adrenoleukodystrophy reveals distinct lipidome signatures between adrenomyeloneuropathy and childhood cerebral adrenoleukodystrophy, Biochem. Biophys. Res. Commun. 508 (2019) 563–569, https://doi.org/10.1016/j.bbrc.2018.11.123.

[30] 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.

[31] 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.

[32] 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.

[33] 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.

[34] 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.

[35] 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.

[36] 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.

[37] M. Igarashi, H.H. Schaumburg, J. Powers, Y. Kishmoto, E. Kolodny, K. Suzuki, Fatty acid abnormality in adrenoleukodystrophy, J. Neurochem. 26 (1976) 851–860, https://doi.org/10.1111/j.1471-4159.1976.tb04462.x.

[38] F. Valianpour, J.J. Selhorst, L.E. van Lint, A.H. van Gennip, R.J. Wanders, S. Kemp, Analysis of very long-chain fatty acids using electrospray ionization mass spectrometry, Mol. Genet. Metab. 79 (2003) 189–196, https://doi.org/10.1016/s1096-7192(03)00098-2.

[39] K. Morito, R. Shimizu, H. Ali, A. Shimada, T. Miyazaki, N. Takahashi, M.M. Rahman, K. Tsuji, N. Shimozawa, M. Nakao, S. Sano, M. Azuma, M. Nanjundan, K. Kogure, T. Tanaka, Molecular species profiles of plasma ceramides in different clinical types of X-linked adrenoleukodystrophy. J. Med. Invest. 70 (2023) 403-410, https://doi.org/10.2152/jmi.70.403.

[40] 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.

[41] 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.

[42] 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.

[43] 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.

[44] S. Kemp, I.C. Huffnagel, G.E. Linthorst, R.J. Wanders, M. Engelen, Adrenoleukodystrophy - neuroendocrine pathogenesis and redefinition of natural history, Nat. Rev. Endocrinol. 12 (2016) 606–615, https://doi.org/10.1038/nrendo.2016.90.

[45] M. Baarine, P. Andréoletti, A. Athias, T. Nury, A. Zarrouk, K. Ragot, A. Vejux, J.M. Riedinger, Z. Kattan, G. Bessede, D. Trompier, S. Savary, M. Cherkaoui-Malki, G. Lizard, Evidence of oxidative stress in very long chain fatty acid-treated oligodendrocytes and potentialization of ROS production using RNA interference-directed knockdown of ABCD1 and ACOX1 peroxisomal proteins, Neuroscience 213 (2012) 1–18, https://doi.org/10.1016/j.neuroscience.2012.03.058.

[46] 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.

[47] 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.

[48] 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.

[49] 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.

[50] 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.

[51] 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.

[52] J. Zhou, M.R. Terluk, P.J. Orchard, J.C. Cloyd, R.V. Kartha, N-Acetylcysteine reverses the mitochondrial dysfunction induced by very long-chain fatty acids in Murine Oligodendrocyte model of adrenoleukodystrophy, Biomedicines 9 (2021) 1826, https://doi.org/10.3390/biomedicines9121826.

[53] J. López-Erauskin, J. Galino, P. Bianchi, S. Fourcade, A.L. Andreu, I. Ferrer, C. Muñoz-Pinedo, A. Pujol, Oxidative stress modulates mitochondrial failure and cyclophilin D function in X-linked adrenoleukodystrophy, Brain 135 (2012) 3584–3598, https://doi.org/10.1093/brain/aws292.

[54] A. Zarrouk, A. Vejux, T. Nury, H.I. El Hajj, M. Haddad, M. Cherkaoui-Malki, J.M. Riedinger, M. Hammami, G. Lizard, Induction of mitochondrial changes associated with oxidative stress on very long chain fatty acids (C22:0, C24:0, or C26:0)-treated human neuronal cells (SK-NB-E), Oxid. Med. Cell Longev. 2012 (2012) 623257, https://doi.org/10.1155/2012/623257.

[55] 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.

[56] H. Ali, M. Kobayashi, K. Morito, R.Y. Hasi, M. Aihara, J. Hayashi, R. Kawakami, K. Tsuchiya, K. Sango, T. Tanaka, Peroxisomes attenuate cytotoxicity of very long-chain fatty acids, Biochim. Biophys. Acta 1868 (2023) 159259, https://doi.org/10.1016/j.bbalip.2021.159088.

[57] 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.

[58] 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.

[59] 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.

[60] H. Tsugawa, T. Cajka, T. Kind, Y. Ma, B. Higgins, K. Ikeda, M. Kanazawa, J. VanderGheynst, O. Fiehn, M. Arita, MS-DIAL: data- independent MS/MS deconvolution for comprehensive metabolome analysis, Nat. Methods 12 (2015) 523−526, https://doi.org/10.1038/nmeth.3393.

[61] 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.

[62] A.S. von Hanstein, D. Tsikas, S. Lenzen, A. Jörns, T. Plötz, Potentiation of lipotoxicity in human EndoC-βH1 β-cells by glucose is dependent on the structure of free fatty acids, Mol. Nutr. Food Res. 67 (2023) e2200582, https://doi.org/10.1002/mnfr.202200582.

[63] J.K. Ho, H. Moser, Y. Kishimoto, J.A. Hamilton, Interactions of a very long chain fatty acid with model membranes and serum albumin. Implications for the pathogenesis of adrenoleukodystrophy, J. Clin. Invest. 96 (1995) 1455-1463, https://doi.org/10.1172/JCI118182.

[64] N. Launay, M. Ruiz, L. Grau, F.J. Ortega, E.V. Ilieva, J.J. Martínez, E. Galea, I. Ferrer, E. Knecht, A. Pujol, S. Fourcade, Tauroursodeoxycholic bile acid arrests axonal degeneration by inhibiting the unfolded protein response in X-linked adrenoleukodystrophy, Acta. Neuropathol. 133 (2017) 283-301, https://doi.org/10.1007/s00401-016-1655-9.

[65] Y. Micoogullari, S.S. Basu, J. Ang, N. Weisshaar, N.D. Schmitt, W.M. Abdelmoula, B. Lopez, J.N. Agar, N. Agar, J. Hanna, Dysregulation of very-long-chain fatty acid metabolism causes membrane saturation and induction of the unfolded protein response, Mol. Biol. Cell. 31 (2020) 7-17, https://doi.org/10.1091/mbc.E19-07-0392.

[66] R. Volmer, K. van der Ploeg, D. Ron, Membrane lipid saturation activates endoplasmic reticulum unfolded protein response transducers through their transmembrane domains, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 4628–4633, https://doi.org/10.1073/pnas.1217611110.

[67] E. Karaskov, C. Scott, L. Zhang, T. Teodoro, M. Ravazzola, A. Volchuk, Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis, Endocrinology 147 (2006) 3398–3407, https://doi.org/10.1210/en.2005-1494.

[68] T. Coll, E. Eyre, R. Rodriguez-Calvo, X. Palomer, R.M. Sánchez, M. Merlos, J.C. Laguna, M. Vazquez-Carrera, Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells, J. Biol. Chem. 283 (2008) 11107-11116, https://doi.org/10.1074/jbc.M708700200.

[69] L.L. Listenberger, X. Han, S.E. Lewis, S. Cases, R.V.F. Jr, D.S. Ory, J.E. Schaffer, Triglyceride accumulation protects against fatty acid-induced lipotoxicity, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 3077-3082. https://doi.org/10.1073/pnas.0630588100.

[70] E. Jarc, T. Petan T, Lipid Droplets and the Management of Cellular Stress, Yale J. Biol. Med. 92 (2019) 435-452.

[71] T. Nury, A. Zarrouk, K. Ragot, M. Debbabi, J.M. Riedinger, A. Vejux, P. Aubourg, G. Lizard, 7-Ketocholesterol is increased in the plasma of X-ALD patients and induces peroxisomal modifications in microglial cells: Potential roles of 7-ketocholesterol in the pathophysiology of X-ALD, J. Steroid Biochem. Mol. Biol. 169 (2017) 123-136, https://doi.org/10.1016/j.jsbmb.2016.03.037.

[72] M. Debbabi, T. Nury, A. Zarrouk, N. Mekahli, M. Bezine, R. Sghaier, S. Grégoire, L. Martine, P. Durand, E. Camus, A. Vejux, A. Jabrane, L. Bretillon, M. Prost, T. Moreau, S.B. Ammou, M. Hammami, G. Lizard, Protective effects of α-tocopherol, γ-tocopherol and oleic Acid, three compounds of olive oils, and no effect of trolox, on 7-Ketocholesterol-induced mitochondrial and peroxisomal dysfunction in microglial BV-2 cells, Int. J. Mol. Sci. 17 (2016) 1973, https://doi.org/10.3390/ijms17121973.

[73] M. Debbabi, A. Zarrouk, M. Bezine, W. Meddeb, T. Nury, A. Badreddine, E.M. Karym, R. Sghaier, L. Bretillon, S. Guyot, M. Samadi, M. Cherkaoui-Malki, B. Nasser, M. Mejri, S. Ben-Hammou, M. Hammami, G. Lizard, Comparison of the effects of major fatty acids present in the Mediterranean diet (oleic acid, docosahexaenoic acid) and in hydrogenated oils (elaidic

acid) on 7-ketocholesterol-induced oxiapoptophagy in microglial BV-2 cells, Chem. Phys. Lipids 207 (2017) 151-170, https://doi.org/10.1016/j.chemphyslip.2017.04.002.

[74] O.A. Bizzozero, G. Zuñiga, M.B. Lees, Fatty acid composition of human myelin proteolipid protein in peroxisomal disorders, J. Neurochem. 56 (1991) 872-878, https://doi.org/10.1111/j.1471-4159.1991.tb02003.x.

[75] A.B. Moser, J. Borel, A. Odone, S. Naidu, D. Cornblath, D.B. Sanders, H.W. Moser, A new dietary therapy for adrenoleukodystrophy: biochemical and preliminary clinical results in 36 patients, Ann. Neurol. 21 (1987) 240-249, https://doi.org/10.1002/ana.410210305.

[76] Q. Raas, M.C. van de Beek, S. Forss-Petter, I.M. Dijkstra, A. Deschiffart, B.C. Freshner, T.J. Stevenson, Y.R. Jaspers, L. Nagtzaam, R.J. Wanders, M. van Weeghel, J.Y. Engelen-Lee, M. Engelen, F. Eichler, J. Berger, J.L. Bonkowsky, S. Kemp, Metabolic rerouting via SCD1 induction impacts X-linked adrenoleukodystrophy, J. Clin. Invest. 131 (2021) e142500, https://doi.org/10.1172/JCI142500.

[77] T. Sassa, T. Wakashima, Y. Ohno, A. Kihara, Lorenzo's oil inhibits ELOVL1 and lowers the level of sphingomyelin with a saturated very long-chain fatty acid, J. Lipid Res. 55 (2014) 524–530. https://doi.org/10.1002/ana.410210305.

[78] W.B. Rizzo, P.A. Watkins, M.W. Phillips, D. Cranin, B. Campbell, J. Avigan, Adrenoleukodystrophy: oleic acid lowers fibroblast saturated C22-26 fatty acids, Neurology 36 (1986) 357-361. https://doi.org/10.1212/wnl.36.3.357.

[79] H.W. Moser, G.V. Raymond, S.E. Lu, L.R. Muenz, A.B. Moser, J. Xu, R.O. Jones, D.J. Loes, E.R. Melhem, P. Dubey, L. Bezman, N.H. Brereton, A. Odone, Follow-up of 89 asymptomatic patients with adrenoleukodystrophy treated with Lorenzo's oil, Arch. Neurol. 62 (2005) 1073-1080. https://doi.org/10.1001/archneur.62.7.1073.

[80] M.A. Ahmed, R.V. Kartha, R.C. Brundage, J. Cloyd, C. Basu, B.P. Carlin, R.O. Jones, A.B. Moser, A. Fatemi, G.V. Raymond, A model-based approach to assess the exposure-response relationship of Lorenzo's oil in adrenoleukodystrophy, Br. J. Clin. Pharmacol. 81 (2016) 1058–1066, https://doi.org/10.1111/bcp.12897.

[81] B.M. van Geel, J. Assies, E.B. Haverkort, J.H. Koelman, B. Verbeeten, R.J. Wanders, P.G. Barth, Progression of abnormalities in adrenomyeloneuropathy and neurologically asymptomatic X-linked adrenoleukodystrophy despite treatment with "Lorenzo's oil, J. Neurol. Neurosurg. Psychiatry. 67 (1999) 290–299, https://doi.org/ 10.1136/jnnp.67.3.290.

[82] H.W. Moser, J. Borel, Dietary management of X-linked adrenoleukodystrophy, Annu. Rev. Nutr. 15 (1995) 379–397, https://doi.org/10.1146/annurev.nu.15.070195.002115.