Toxic effects of X-linked adrenoleukodystrophy-associated, very long chain fatty acids on glial cells and neurons from rat hippocampus in culture

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Received December 21, 2007; Revised February 18, 2008; Accepted March 2, 2008

Saturated very long chain fatty acids (VLCFAs; ≥C22:0) accumulate in X-linked adrenoleukodystrophy (X-ALD, OMIM 300100), a severe hereditary neurodegenerative disease, due to peroxisomal impairment. Previous studies analysed the development of X-ALD in humans and gene knockout animal models. However, the toxic effect of VLCFA leading to severe symptoms with progressive and multifocal demyelination, adrenal insufficiency and inflammation still remains unclear. To understand the toxic effects of VLCFA in the brain, here we exposed neural cells to VLCFA and analysed the cellular consequences. We found that oligodendrocytes and astrocytes challenged with docosanoic- (C22:0), tetracosanoic- (C24:0) and hexacosanoic acids (C24:0) die within 24 h. VLCFA-induced depolarization of mitochondria in situ and increased intracellular Ca²⁺ level in all three brain cell types provides indications about the mechanism of toxicity of VLCFA. Interestingly, VLCFAs affect to the largest degree the myelin-producing oligodendrocytes. In isolated mitochondria, VLCFAs exert a detrimental effect by affecting the inner mitochondrial membrane and promoting the permeability transition. In conclusion, we suggest that there is a potent toxic activity of VLCFA due to dramatic cell physiological effects with mitochondrial dysfunction and Ca²⁺ deregulation. This provides the first evidence for mitochondrial-based cell death mechanisms in neurodegenerative disease with peroxisomal defects and subsequent VLCFA accumulation.

INTRODUCTION

In some severe neurodegenerative diseases, such as the X-linked adrenoleukodystrophy (X-ALD, OMIM 300100), the most common inherited peroxisomal disorder, saturated very long chain fatty acids (VLCFAs) with 22 or more carbon atoms accumulate at abnormally high concentrations. VLCFA can be found predominantly in brain white matter, adrenal cortex and also in testes and plasma of patients suffering from X-ALD (1–3). Besides the adult form, the adrenomyeloneuropathy with a milder phenotype, the childhood cerebral form shows a rapidly progressing demyelination of central nervous system and leads to total disability during the first decade (reviewed in 2). Clinically, the cerebral or juvenile ALD is characterized by multifocal inflammatory demyelination of the central nervous system, adrenal insufficiency and a rapid fatal outcome (reviewed in 4). X-ALD is caused by mutations in the ABCD1 gene mapped to Xq28, which encodes for the ATP-binding cassette (ABC) transporter adrenoleukodystrophy protein (ALDP). This protein is located in the peroxisomal membrane (reviewed in 5). The exact function of ALDP is currently still unclear. However, it is known that the impairment of the function of ALDP leads to a disturbed degradation of VLCFA via peroxisomal β-oxidation, and therefore these fatty acids are accumulated (6–9). Degradation of VLCFA and branched-chain fatty acids is initiated exclusively in peroxisomes. CoA-derivatives of branched-chain fatty acids undergo three cycles of β-oxidation. Then the products are shuttled to mitochondria for complete oxidation (10–12). Such sequential degradation pathways are unknown for VLCFA. In contrast, short-, medium- and long-chain saturated fatty acids are degraded predominantly by mitochondria (10,11). It has been shown that the expression of ALDP in adult mouse and in human is restricted to astrocytes, microglial cells and oligodendrocytes, but is not found in neurons (13).
Although the genetic basis of most peroxisomal disorders has been investigated in detail (14–16), the molecular mechanism of the cellular abnormalities, including the severe neurodegeneration, is still unknown. The influence of VLCFA accumulation on different brain cell types in the pathogenesis of X-ALD is an unresolved issue. It is possible that the incorporation of VLCFA might destabilize cell membranes (17) or disturb the microenvironment of cells, which leads to dysfunction and death of vulnerable brain cells (reviewed in 18).

Glia cells seem to be involved in the progress of X-ALD. However, it is not known whether oligodendrocyte cell death, and therefore demyelination, is a primary or a secondary event in X-ALD. Microglia is most likely activated to release proinflammatory cytokines in cooperation with astrocytes, which can trigger oligodendrocyte cell death as a secondary step (reviewed in 19). In active lesions of X-ALD brain, macrophages and most prominently astrocytes express large amounts of TNF-α (20). It has been shown that TNF-α is toxic for oligodendrocytes and can trigger the death of these cells, in addition to the death of neurons (21). Furthermore, the survival of neurons depends on functional intactness of the other cell types in the brain, namely astrocytes and glial cells. The death of glial cells causes the death of neurons in many acute and chronic disorders (reviewed in 22). Therefore, acute death of brain cells plays probably a significant part in the progression of X-ALD. A main trigger of cell death is a dramatic increase of intracellular Ca²⁺ level and mitochondrial dysfunction. Permeability transition (PT) of the inner mitochondrial membrane (IMM) leads to a breakdown of the mitochondrial membrane potential (ΔΨ) and therefore to a failure of mitochondrial functions (23,24).

We previously investigated the toxicity of the saturated, branched fatty acid phytanic acid, a marker of Refsum disease, and discovered that phytanic acid toxicity is mediated by multiple mitochondrial dysfunctions (25–28). It is currently completely unknown how brain cells react to VLCFA accumulation. Here, we present the first report which studies in detail the physiological consequences of VLCFA application in different neural cell types, namely oligodendrocytes, astrocytes and neurons. We used a mixed cell culture system from rat hippocampus as well as isolated mitochondria. In the present study, cell physiological consequences caused by exposure to docosanoic (C22:0, behenic acid)-, tetracosanoic (C24:0, lignoceric acid)- and hexacosanoic acids (C26:0, cerotic acid) were analysed. We made a multiparametric analysis to investigate (i) the induction of cell death, (ii) changes of intracellular Ca²⁺ level, (iii) production of reactive oxygen species (ROS) and (iv) alterations in mitochondrial functions. The detailed analysis of the role of VLCFA in the neural cell culture system provides new insights into the mechanisms underlying diseases such as X-ALD.

**RESULTS**

**VLCFAs induce cell death of oligodendrocytes and astrocytes**

VLCFAs are elevated in patients suffering from peroxisomal disorders, such as X-ALD. Here, we attempted to elucidate the physiological events of the pathogenesis associated with VLCFA accumulation. Therefore, we analysed the consequences of VLCFA application to neural cells. Figure 1A shows that in oligodendrocytes from rat brain the cell death, measured as a release of lactate dehydrogenase (LDH) activity, was increased after a 24 h incubation with C24:0 and C26:0 (40 μM; 55% of total LDH activity), 6-fold above control (vehicle treatment with α-cyclodextrin). Incubation with C22:0 (40 μM) for 24 h resulted in a 3-fold increase in oligodendrocyte cell death, which was not significantly different from the effect of C24:0 and C26:0. Palmitic acid (Palm, C16:0), however, which we used as a reference control fatty acid, did not enhance the death of oligodendrocytes (Fig. 1A) or astrocytes (Fig. 1B) at a concentration of 40 μM. Even at a higher concentration of 100 μM, Palm was without any effect (data not shown).

Demyelination and astrocyte activation are observed in X-ALD (reviewed in 5). This suggests that glial cells are largely affected by VLCFA accumulation. For that reason, we also exposed astrocytes to VLCFA, to test whether the cytotoxic effect is cell-type-specific. After 24 h of incubation with C22:0, C24:0 and C26:0, astrocyte cell death increased around 5-fold above the control value (Fig. 1B, 40% of total LDH activity). There was no significant difference between the three fatty acids. The cell death after incubation for 24 h was slightly lower in astrocytes than in oligodendrocytes, which suggests a higher vulnerability of oligodendrocytes to VLCFA application. The cell death of astrocytes after VLCFA application increased with increasing exposure time. Astrocyte cell death increased from 5-fold (40% of total LDH activity) to 8-fold (70% of total LDH activity) above the control within an incubation period of 3 days (Fig. 1B and C). In summary, VLCFAs exert a strong cytotoxic activity in oligodendrocytes and astrocytes derived from rat brain.

**Intracellular Ca²⁺ response due to acute application of VLCFA**

To study the mechanism of cell toxic effects of VLCFA, we analysed whether application of C22:0, C24:0 and C26:0 causes changes of intracellular Ca²⁺. For comparison, we applied Palm which we had already used in our previous studies (25). At first, we analysed the intracellular Ca²⁺ concentration in oligodendrocytes, which showed a strong increase in cell death after VLCFA application. Figure 2A displays the representative traces of the kinetics of C22:0-induced intracellular Ca²⁺ increase monitored with Fura-2. For comparison, we give the stable control trace obtained with vehicle solution containing α-cyclodextrin (1 mg/ml). Within the 7 min period of application indicated by the black bar below the traces, C22:0 (40 μM) evoked a dramatic increase of the intracellular Ca²⁺ concentration with a fast appearance. The irregular oscillations were not synchronous between neighbouring cells. Lower concentrations (20 and 10 μM) of C22:0 evoked a smaller Ca²⁺ increase. We found similar reactions after application of C24:0 and C26:0 (traces not shown). Owing to the fact that the VLCFA-induced Ca²⁺ responses were irregular, we analysed the mean maximal Ca²⁺ level obtained during the application time. This quantification is shown in Figure 2B. The maximal Ca²⁺ level achieved
Because the cells did not react synchronously to the VLCFA application, we analysed not only the maximal Ca\(^{2+}\) level but also the area under the curves of the Ca\(^{2+}\) responses reached during the application period. Figure 4A represents the quantitative analysis of the maximal Ca\(^{2+}\) peak in the three cell types. Also in astrocytes and neurons, there was a significant increase in the Ca\(^{2+}\) concentration with a 3–4-fold rise caused by 40 \(\mu\)M of C22:0, C24:0 and C26:0, but oligodendrocytes displayed the strongest increase in Ca\(^{2+}\) level (11–14-fold). It should be emphasized that in these cases all cells were studied in parallel under identical conditions. There was no significant difference in the maximal Ca\(^{2+}\) concentrations achieved by C22:0, C24:0 and C26:0.

To get an evidence for the impact of this Ca\(^{2+}\) rise, we analysed the glutamate-induced Ca\(^{2+}\) response. Glutamate exerts a strong Ca\(^{2+}\)-induced excitotoxicity (29–31). We found a dramatic increase in intracellular Ca\(^{2+}\) with glutamate (500 \(\mu\)M): in oligodendrocytes 14.2-fold, in astrocytes 6.3-fold and in neurons 6.0-fold above the control value (data not shown). VLCFA induced an intracellular Ca\(^{2+}\) rise comparable to the glutamate-induced Ca\(^{2+}\) increase. In addition, the VLCFA-Ca\(^{2+}\) data were evaluated as area under the curve to analyse the complex Ca\(^{2+}\) responses (Fig. 4B). Also these data clearly show that oligodendrocytes are more sensitive to VLCFA than astrocytes and neurons. The control fatty acid Palm did not evoke a deregulation of intracellular Ca\(^{2+}\) homoeostasis in all three cell types.

VLCFA-induced instantaneous ROS production and depolarization of mitochondria in neural cells in situ

Recent reports demonstrate that long-chain saturated and unsaturated fatty acids, such as Palm, phytanic acid or arachidonic acid, enhance the ROS production in various cell types (25,32,33). Therefore, we also studied the generation of cellular ROS in the mixed hippocampal cell culture due to short-term (15 min) application of VLCFA. However, we could not detect any significant change in ROS formation in oligodendrocytes, astrocytes and neurons when they were treated with VLCFA (data not shown). This is surprising, since VLCFA induced an increase of the intracellular Ca\(^{2+}\) level which might also stimulate cellular ROS generation (31). As a control, we tested the increase in the ROS generation when mitochondria were impaired by the addition of antimycin A, an inhibitor of complex III of the respiratory chain. The antimycin A-induced ROS generation was 7–, 6- and 13-fold above the control value in oligodendrocytes, astrocytes and neurons, respectively (data not shown).

To further understand the cellular mechanisms of toxicity of VLCFA, we examined a possible depolarization of mitochondria in situ. Using mixed hippocampal cell cultures, the cells were first loaded with the membrane potential probe Rh123, and thereafter treated for 10 min with VLCFA (40 \(\mu\)M). Then, a complete depolarization of the mitochondria was achieved by the protonophoric uncoupler carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP). FCCP was applied together with the F1F0-ATPase inhibitor oligomycin (Oli), which prevents partial repolarization of the IMM due to hydrolysis of glycolytic ATP.

**Figure 1.** Cell death induction in oligodendrocytes (A) and astrocytes (B and C) by VLCFA application. Cells were incubated with docosanoic- (C22:0), tetracosanoic- (C24:0) and hexacosanoic acids (C26:0) and Palm (C16:0) for time period and concentrations indicated. The supernatant was collected and LDH activity was analysed as percent of total activity. The amount of total LDH activity is LDH activity of lysed cells plus that of supernatant. The horizontal black line represents the LDH activity measured with vehicle α-cyclodextrin; 1 mg/ml alone. Data shown are means from four individual cell preparations and analysis was performed twice for each condition.

\(P < 0.05\) compared with control.

during the application of VLCFA (C22:0, C24:0 and C26:0) increased with raising the concentration from 10 to 40 \(\mu\)M. Even 10 \(\mu\)M of VLCFA caused a significant Ca\(^{2+}\) increase in oligodendrocytes. The Ca\(^{2+}\) levels after application of 40 \(\mu\)M of VLCFA were 6–8-fold higher than the control level.

To determine whether there are possibly cell-type-specific effects, we next applied the VLCFA to a mixed cell culture derived from rat brain hippocampus, containing oligodendrocytes, astrocytes and neurons. The cell types were identified after each experiment with specific antibodies. Figure 3 shows the representative traces of intracellular Ca\(^{2+}\) rise in oligodendrocytes, astrocytes and neurons. VLCFAs (40 \(\mu\)M) were applied for 7 min, as indicated by the black bar below the traces. C22:0, C24:0 and C26:0 evoked an increase in Ca\(^{2+}\) level in each cell type. Also for astrocytes and neurons, the traces showed irregular Ca\(^{2+}\) concentration increases, but with lower amplitude than in oligodendrocytes.
As described in Materials and Methods, the release of Rh123 during the final depolarization with FCCP and Oli was quantified to obtain a value for the mitochondrial membrane potential ($\Delta \psi$). With exposure to the vehicle $\alpha$-cyclodextrin (1 mg/ml), we detected a large amount of FCCP–Oli-releasable Rh123 dye, indicating a high control $\Delta \psi$ value in each cell type (presented as 100% in Fig. 5, horizontal line). Incubation with VLCFA (40 $\mu$M) reduced the FCCP–Oli-releasable Rh123 fluorescence in all three cell types, which demonstrates a depolarization. The decrease in $\Delta \psi$ was strongest with C22:0 (40% relative to control). C24:0 and C26:0 decreased the Rh123 release by ~20% below the control value. However, there was no cell-type-specific difference in the effect of VLCFA to reduce the mitochondrial potential. Palm did not influence $\Delta \psi$ (data not shown).

### Interaction of VLCFA with isolated mitochondria

The interaction of VLCFA with the inner membrane of brain mitochondria was substantiated by examining their effect on oxygen uptake. We measured the influence of VLCFA on
the respiration of mitochondria in the resting state (Fig. 6A) and in ADP-activated state 3 (Fig. 6B). C22:0 and C24:0, applied at concentrations of 120 nmol/mg of protein (25 μM), significantly stimulated the respiration of resting mitochondria, whereas C26:0 was inactive (Fig. 6A). The stimulation of respiration in the resting state indicates that VLCFAs increase the permeation of H\(^{+}\) across the IMM. In addition, C22:0 and C24:0 significantly inhibit the phosphorylating respiration by >20% (Fig. 6B). Thus, C22:0 and C24:0, but not C26:0, induce both an uncoupling of mitochondria (Fig. 6A) and an inhibition of the electron flow in the respiratory chain (Fig. 6B).

Moreover, we also applied a very sensitive method to demonstrate the detrimental effect of VLCFA on the IMM. When mitochondria oxidize succinate, they exhibit high reverse electron transport (RET)-supported ROS production. This process is very sensitively inhibited by depolarization of the IMM or reduction of the electron transport from complex II to complex I (34,35). We used this as a tool for analysing the influence of VLCFA. VLCFA by themselves did not induce ROS production (data not shown). Figure 7A exemplifies the succinate-supported ROS production by rat brain mitochondria (RBM). To estimate ROS production, we determined the formation of the fluorescent resorufin from Amplex Red which is due to oxidation by released H\(_2\)O\(_2\). Addition of the protonophoric uncoupler FCCP (0.5 μM) nearly completely abolished the H\(_2\)O\(_2\) release into the medium. In Figure 7A, the basal RET-linked ROS production by RBM is given by slope 1, and its decline due to depolarization by FCCP application is shown by slope 2.

The detrimental activity of VLCFA on IMM, as deduced from the inhibition of the RET-linked ROS production, is presented in Figure 7B (slope 2 as % of slope 1). The effect of VLCFA at 50 and 100 nmol/mg protein (equivalent to 10 and 20 μM) on the ROS generation was tested. The vehicle α-cyclodextrin had no significant effect. C22:0 decreased the ROS production at 100 nmol/mg protein by
FCCP reduced the ROS production by \(~\sim 90\%\) (Fig. 7B).

The opening of the PT pore (PTP) in the IMM is enhanced by depolarization (reviewed in 36). Therefore, we also elucidated the possible consequence of VLCFA-linked mitochondrial depolarization on the sensitivity of isolated mitochondria to undergo PT. For that purpose, the sudden release of accumulated Ca\(^{2+}\) from isolated mitochondria due to the change in \(\Delta\psi\) was measured.

Figure 7C and D shows experiments carried out with isolated rat liver mitochondria. These mitochondria were used as a model system here, because it is well known that brain mitochondria display much lower PTP-related experimental signals. Mitochondria were incubated with 0.05 \(\mu\)M of calcium green (CaG5N) as extra-mitochondrial fluorescent probe of Ca\(^{2+}\). As can be seen in Figure 7C, a Ca\(^{2+}\) pulse first increases the CaG5N fluorescence, which returns thereafter to baseline, as the mitochondria sequester the Ca\(^{2+}\) added. The following exposure to C22:0, C24:0 and C26:0 (10 and 20 nmol/mg protein, equivalent to 10 and 20 \(\mu\)M) with \(\alpha\)-cyclodextrin as vehicle caused a subsequent increase in CaG5N fluorescence, which indicates PTP opening due to the complete release of the accumulated Ca\(^{2+}\). The shorter fatty acid C22:0 leads to a more rapid (4 min) release of Ca\(^{2+}\) than C24:0 (8 min) and C26:0 (10 min). As described before (28), the time period required for reaching 50\% of the maximal Ca\(^{2+}\) release (\(t_{50\%}\) PT) was used as an index of vulnerability (Fig. 7D). Thus, VLCFA sensitization for an induction of PT in isolated mitochondria was revealed, where the effect of C26:0 was milder than that of C22:0 and C24:0.

**DISCUSSION**

The aim of the present study was to analyse the consequences of VLCFA accumulation on physiological properties of neural cells. Here, we demonstrate for the first time that VLCFAs (C22:0, C24:0 and C26:0) are toxic to rat brain cells. Saturated VLCFAs (\(\geq\)C22), mainly hexacosanoic acid (C26:0) and tetracosanoic acid (C24:0) accumulate at abnormal high concentrations in patients suffering from diseases with peroxisomal deficiency, such as X-ALD, Zellwegers syndrome and infantile Refsum disease. The peroxisomal disease X-ALD has an incidence of 1:15 000 in France (37) and 1:20 000 in USA (38). C24:0 and C26:0 increase dramatically in the plasma of X-ALD patients. In contrast, a mild increase in C22:0 concentration was observed (39). The plasma level of
C26:0 in X-ALD patients was found to be $1.52 \pm 0.56 \mu g/ml$ (reviewed in 40). For analysis of short-term cell physiological consequences of VLCFA, we used in our experiments concentrations between 10 and 40 mM ($\approx 10$ and 20 $\mu M$). Data were calculated as percent (slope 2 of slope 1) for each experiment. The vehicle $\alpha$-cyclodextrin (control) was used at 0.25 and 0.5 mg/ml, respectively. FCCP was used as a positive control. (C and D) VLCFA-triggered PT in isolated mitochondria. Measurement of sudden $Ca^{2+}$ release by isolated rat liver mitochondria after addition of VLCFA. Mitochondria (1 mg of protein) were suspended in 1 ml incubation medium supplemented with glutamate/malate (5 mM/5 mM) and 0.05 $\mu M$ CaG5N. (C) Fluorescence traces of CaG5N showing the uptake of $Ca^{2+}$ from the medium after the pulse of 50 nmol CaCl$_2$. Afterwards, VLCFAs (C22:0, C24:0, C26:0) were added (10 nmol/mg of protein). VLCFAs initiate a subsequent sudden release of accumulated $Ca^{2+}$ from mitochondria. This indicates the onset of PTP opening. (D) The time to reach 50% of the maximal $Ca^{2+}$ release ($t_{50\%}$ PT) was calculated from traces exemplified in (C) and was depicted as a function of the concentration of VLCFA used (10 and 20 nmol/mg = 10 and 20 $\mu M$). Data were obtained from three individual mitochondrial preparations. *$P < 0.05$ compared with control.

X-ALD is the only genetic disease known to be caused by a peroxisomal ABC transporter gene defect, namely ABCD1. This neurodegenerative disorder is characterized by progressive central nervous system demyelination and VLCFA accumulation (2,19). The loss of ALDP, an ABC half-transporter of the peroxisomal membrane, is responsible for the abnormality in peroxisomal $\beta$-oxidation of VLCFA. Three additional peroxisomal ABC half-transporters have been identified: the ALD-related protein, the more distantly related PMP70 protein and the PMP70-related (P70R) protein (5,42).

In mammals, in the brain particularly myelin is rich in VLCFA (43). Indeed, there is a strong correlation between plasma VLCFA concentration and severity of phenotype in peroxisomal disorders (1). The accumulation of VLCFA at high concentrations in the brain is likely to be associated with myelin loss, oligodendrocyte death and activation of...
The death of oligodendrocytes could be a primary or secondary event (reviewed in 5). It has not yet been clarified whether the accumulation of VLCFA in X-ALD itself causes demyelination or triggers cell death. Microglia dysfunction in X-ALD is also very important. Other studies suggest that VLCFA might destabilize membranes (reviewed in 4), are incorporated into myelin (44) or can perturb the microenvironment of cells, resulting in dysfunction and death of vulnerable cells (18). VLCFAs are incorporated into membranes of neural cells. This enrichment of membranes with VLCFA appears to be the earliest biochemical abnormality in X-ALD brain tissue (45). Incorporated VLCFAs desorb from membranes by orders of magnitude slower than ‘classical’ free fatty acids (17). Consequently, the distorting effects on the arrangement of membrane constituent are more permanent than that of short-chain free fatty acids. Our results demonstrate that the VLCFAs cause cell death of the myelin-producing oligodendrocytes and astrocytes (Fig. 1).

Fatty acids play a decisive role in many steps of inflammation, chemotaxis, cell adhesion and cell activation. The toxicity of fatty acids to cells depends on the length and saturation (41). In our previous work, we found that phytanic acid a saturated branched-chain fatty acid caused rapid cell death of cultured astrocytes. In contrast, Palm over the same time period and at the same concentration did not affect the survival of astrocytes (25). Moreover, we reported that phytanic acid impairs the Ca\(^{2+}\) homeostasis, depolarizes mitochondria and induces the generation of ROS (25). Additionally, phytanic acid sensitizes isolated mitochondria for PT (28). All these findings strongly suggest that phytanic acid initiates astrocyte cell death by activating the mitochondrial route of apoptosis (26).

VLCFA at a concentration of 40 \(\mu\)M clearly induced cell death of oligodendrocytes and astrocytes in a time period of 1–3 days. Palm in comparison did not influence the survival of these cells. It has been reported that Ca\(^{2+}\) overload and mitochondrial dysfunction trigger cascade leading to cell death (reviewed in 46). An overload of intracellular Ca\(^{2+}\) can cause PT of the IMM, which initiates cell death (reviewed in 47). Alterations in mitochondrial ultrastructure and changes in the expression and activity of mitochondrial respiratory chain complexes have been reported in human and in mouse models of Zellweger syndrome (48,49). Others have suggested the involvement of mitochondrial dysfunction in X-ALD (50,51). They found no primary mitochondrial defects in knockout animals. However, their results do not exclude that secondary mitochondrial dysfunction occurs in affected tissues, such as the degenerating central nervous system. Furthermore, the knockout mouse model (ABCD1 \(-/-\)) did not show the clinical symptoms of X-ALD patients. Knock-out mice develop an adult adrenomyeloneuropathy-like phenotype (52).

To clarify the mechanism, which leads to the observed cell death after VLCFA incubation, we analysed cell physiological consequences. We observed a dramatic increase in intracellular Ca\(^{2+}\) level in oligodendrocytes (Fig. 2) and an increase, albeit somewhat lower, in astrocytes and neurons after VLCFA (C22:0, C24:0 and C26:0) application (Figs 3 and 4). This dramatic effect on intracellular Ca\(^{2+}\) homeostasis was similar to the Ca\(^{2+}\) rise observed with the excitotoxic stimulus glutamate. The myelin-forming oligodendrocytes seem to be more susceptible to exposure to VLCFA. The mechanism, which evokes the increase in intracellular Ca\(^{2+}\) level, is still unclear. However, the characteristic of the traces of the Ca\(^{2+}\) response (Figs 2 and 3) are unlike receptor-mediated increase, as shown before in astrocytes (53,54). The cells display irregular asynchronous Ca\(^{2+}\) oscillations. Interestingly, among these neural cells, the oligodendrocytes showed the strongest increase in intracellular Ca\(^{2+}\) (Fig. 4). This suggests a high vulnerability of these myelinating cells. The cell death of oligodendrocytes detected here is consistent with the observation of myelin and oligodendrocyte loss in X-ALD (reviewed in 5). Nevertheless, the increased death of cells treated with VLCFA over more than 1 day is not a primary result of increased ROS formation. On the other hand, it has been shown that free radicals may play an important role in diseases such as X-ALD (55,56).

We found that short-term exposure to VLCFA did not induce ROS generation in the mixed cell culture. However, our findings here do not exclude an involvement of ROS in X-ALD pathogenesis. Currently, we do not yet know whether long-term exposure of brain cells to VLCFA might trigger an increase in oxidative stress and damage. Previous studies using isolated mitochondria demonstrated that long-chain fatty acids can stimulate ROS production by inhibition of the forward electron transport in the respiratory chain (57–59).

The functional disturbance of mitochondria is critical for cell survival, as recently reported for phytanic acid and its toxic activities (25,26,57,60). Here, we examined the effects of VLCFA on isolated mitochondria. We found that VLCFA decreased the mitochondrial potential (Fig. 7B; indicated as a decrease of RET-dependent ROS production). This result clearly proves that VLCFAs have deleterious effects on the IMM. Similarly, VLCFAs lowered the mitochondrial potential in cultured oligodendrocytes, astrocytes and neurons in situ (Fig. 5). We suggest that the depolarization of the IMM by increasing the permeability to protons (Fig. 6A) might be due to incorporation of VLCFA into the inner membrane, which disturbs the well-ordered phospholipids arrangement. Our experiments show that VLCFAs sensitize mitochondria for PT (Fig. 7C and D).

Our data indicate that the different VLCFAs exert toxic effects through different pathways. The shortest fatty acid C22:0, which is relatively mildly increased in plasma of X-ALD patients, exerted a strong effect on mitochondrial physiology. In contrast, C24:0 and C26:0 had mild or no effect on mitochondria. Thus, C22:0 and in part C24:0 might cause cell death by mitochondrial-based mechanisms. The longest fatty acid (C26:0), which is very important in peroxisomal defects, exerts toxic effects mainly through dysfunction of the Ca\(^{2+}\) homeostasis. Because of the fact that mainly C24:0 and C26:0 accumulate in plasma of patients with X-ALD peroxisomal defects, we suggest that these VLCFAs cause cell death by a combination of disturbance of Ca\(^{2+}\) homeostasis and mitochondrial dysfunction.

In conclusion, the present work demonstrates for the first time that VLCFAs exert toxic activity on brain cells. However, it should be noted that our experiments were performed on cells with normal ALDP function. It is likely that in ALDP-deficient cells, VLCFA could
cause more severe dysfunctions, which could potentiate the observed toxicity.

**MATERIALS AND METHODS**

**Materials**

The culture medium TNB-100, DMEM (Dulbecco’s modified Eagle’s medium), stabilized glutamine (N-acetyl-L-alanyl-L-glutamine), penicillin/streptomycin and fetal calf serum were from Biochrom AG (Berlin, Germany), Ultroser G serum replacement and B27 supplement were from Gibco (Eggenstein, Germany); the acetoxyethyl ester of Fura-2 (Fura-2 AM), dihydroidothidium (DHE), Rhodamin 123 (Rh123), secondary Alexa antibodies, Hoechst33342 and Pluronic from Molecular Probes (Eugene, OR, USA). Primary antibody rabbit anti-Synaptotagmin 1 was from Synaptic System (Göttingen, Germany) and chicken anti GFAP from Chemicon (Billerica, USA). Docosanoic-(behenic acid), tetracasoic-(lignoceric acid) and hexacosanoic acid (ceroticin acid) were from Larodan (Malmö, Sweden) and palmitic acid (Palm) was obtained from ICN. Cytotoxicity detection kit was from Roche Diagnostics GmbH (Mannheim, Germany). Amplex Red and Calcium Green-5N were supplied by Invitrogen (Eugene, OR). All other chemicals were from Sigma.

**Hippocampal cell culture**

Mixed hippocampal cell culture containing oligodendrocytes, astrocytes and neurons were prepared from post-natal days 1–2 Wistar rat pups as already described (31). All experiments conformed to guidelines from Sachsen Anhalt, Germany, on the ethical use of animals, and all efforts were made to minimize the number of animals used. The preparation medium contained 1 mM pyruvate, 10 mM glucose, 6 μg/ml DNase I type IV, 1 mg/ml bovine serum albumin, 2 mM stabilized glutamine and 1% (v/v) antibiotics mixture (penicillin/streptomycin) in a Ca\(^{2+}\)-free HEPES-buffered salt solution (10 mM HEPES, pH 7.2). For the digestion of the hippocampi, the preparation medium was supplemented with 4 mg/ml trypsin type XI. Cells were plated on glass cover slips coated with poly-L-lysine (PLL) yielding a density of 100 cells/mm\(^2\). Cell culture medium TNB-100 was supplemented with 10 mM HEPES, 5% (v/v) heat-inactivated horse serum, 2% (v/v) Ultroser G and 1% (v/v) antibiotics mixture. The cell cultures were used for experiments within 16–20 days in culture. Cells were fed in a 2-day interval and 24 h before the experiment.

**Cell identification**

After each experiment, oligodendrocytes, astrocytes and neurons were identified in the mixed culture via immunostaining as described earlier (31). Therefore, we used antibodies against GFAP (astrocyte marker) and synaptotagmin (neuronal pre-synaptic marker). Additionally, we used antibody against 2',3'-cyclic nucleotide 3'-phosphodiesterase (oligodendrocyte marker) to discriminate mature oligodendrocytes in the mixed hippocampal cell culture. For staining the different cell types, cells were fixed after kinetic measurements with 4% freshly prepared paraformaldehyde solution (4% sucrose and 120 mM phosphate buffer, pH 7.4).

**Oligodendrocyte and astrocyte cell culture**

Purified primary oligodendrocytes and astrocytes were prepared as a mixed glial cell culture from newborn rats according to a previously published method (61,62). After 7 days in culture, oligodendrocytes were separated from the astrocytic layer as described (63). Cells were cultured in DMEM containing 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 10% CO\(_2\) at 37°C. After shaking the mixed glial cell culture for 16 h in an orbital shaker (Innova 4230, incubator shaker; 200 rpm, 37°C) supernatant with oligodendrocytes was filtered through a nylon mesh (40 μm). Oligodendrocytes were cultured for 24 h in a cell flask (25 cm\(^2\)) before removing the cells from resting astrocytes by vigorously shaking by hand. Oligodendrocytes were then seeded on PLL-coated plates at a density of 4 × 10\(^4\) cells per well (48-well plate) or cover slip (22 mm\(^2\)). Purified astrocytes were seeded after shaking at a density of 2 × 10\(^3\) cells per well (48-well plate) or 2 × 10\(^5\) cells per dish (21 cm\(^2\)). The medium was changed every second day. For experiments, cells were used between days 13 and 15 in culture, which means 4–6 days after separation from mixed glial cell culture.

**Measurements of intracellular Ca\(^{2+}\) level, mitochondrial membrane potential and generation of ROS in living cells**

All experiments with living cells were measured with an imaging system (TILL Photonics, Planegg, Germany) attached to a Zeiss Axioscope microscope. For the experiments, cells were bathed in Hanks balanced salt solution (HBSS, concentrations in mM: KCl, 5.44; KH\(_2\)PO\(_4\), 0.44; Na\(_2\)HPO\(_4\), 0.34; MgCl\(_2\), 0.49; MgSO\(_4\), 0.41; NaCl, 132; HEPES, 10; glucose, 5.56; NaHCO\(_3\), 4.17; CaCl\(_2\), 1.26), which was completely exchanged within 0.5 min by a continuous superfusion system. Substances were applied by addition to the superfusate. All experiments were performed at 36°C. For measurement of intracellular Ca\(^{2+}\) concentration, cells were loaded with the acetoxyethyl ester of the Ca\(^{2+}\)-sensitive fluorophore Fura-2 AM (2 μM, 0.02% Pluronic, 30 min, 15 min post-incubation with HBSS). The dye remains intracellular after cleavage by non-specific esterase activity. Fluorescence signals were acquired at 510 nm emission during alternative excitation at 340 nm (Fura-2 bound to free Ca\(^{2+}\)) and 380 nm (unbound Fura-2 molecule). For analysis, the ratio of fluorescence intensities was obtained at 340 and 380 nm excitation; the region of interest was defined over intracellular space.

Mitochondrial membrane potential (ΔΨ) in living cells was detected with the cationic dye Rh123 (10 μM) as reported before (64). Cells were loaded with the dye for 30 min. The dye was excited at 520 nm and fluorescence was detected at >590 nm. The ΔΨ was calculated as an increase in Rh123 fluorescence over the mitochondria-free nucleus evoked by the uncoupler FCCP (4 μM) in combination with oligomycin (Oli, 10 μM), an inhibitor of F\(_{1}\)F\(_{0}\) ATPase. FCCP/Oli-mediated depolarization under control conditions was taken as 100%.
If an agonist depolarized the mitochondria, the release of Rh123 was calculated as percent to release under control condition.

The measurement of ROS generation is done by the oxidation of DHE to ethidium cation and subsequent binding to nuclear DNA, which is detectable at 520 nm excitation and >590 nm emission. To avoid artificial generation of ROS by higher DHE loads, 1 \mu M was added directly with the substances to the cells without preincubation (65). As a vehicle for VLCFA application, we used \alpha-cyclodextrin (66,67), which enables the study of VLCFA directly on living cells.

Preparation of isolated mitochondria

Mitochondria from rat brain and liver were prepared as described by (27,68). Protein contents in the stock suspensions were determined by the Biuret method using bovine serum albumin as standard. For measurement, mitochondria were suspended in incubation buffer (110 mM mannitol, 60 mM KCl, 60 mM Tris, 10 mM KH2PO4, 2 \mu M EGTA, pH 7.4) supplemented with pyruvate plus malate (5 mM/5 mM) or glutamate plus malate (5 mM/5 mM) or succinate (10 mM) as respiratory substrates. Functional intactness was characterized by determining the respiratory control ratio, which was \geq 5 for all preparations.

Fluorimetric measurements with isolated mitochondria

The fluorimetric measurements were done with a Perkin-Elmer Luminescence spectrometer LS 50B. Measurement of mitochondrial Ca2+ uptake and release was done using Calcium Green-5N (CaG5N). Briefly, mitochondria (1 mg of protein) were suspended in 1 ml of incubation buffer supplemented with pyruvate plus malate (5 mM/5 mM) or glutamate plus malate (5 mM/5 mM) or succinate (10 mM) as respiratory substrates. Functional intactness was characterized by determining the respiratory control ratio, which was \geq 5 for all preparations.

Oxygen uptake

Oxygen uptake by mitochondria was measured using an oxygraph (Oroboros Oxygraph®; Bioenergetics and Biomedical Instruments, Innsbruck, Austria). Briefly, an aliquot of the mitochondrial stock suspension (1 mg of protein) was added to 2 ml of incubation buffer supplemented with pyruvate plus malate (5 mM/5 mM).

Assay of cell death

Cells were prepared as already described and seeded in PLL-coated wells of a 48-well plate in a density of 2 \times 10^3/well (astrocytes) or \times 4 \times 10^3/well (oligodendrocytes). Cells were grown for 3 days. Afterwards, medium was changed from DMEM with FCS to TNB-100 (described earlier) without FCS and substances were added. After a defined time period, the supernatant was collected and the activity of LDH was measured (absorption: 492 nm). For quantification of total amount of LDH, the LDH activity was measured in the supernatant and in the lysate of the remaining cells. All data were calculated to background absorption of the buffer used.

Data analysis

Statistical analysis was performed using Prism 3.0 software (GraphPad Software, San Diego, CA, USA). Significance was examined by the analysis of variance and Kruskal–Wallis post-test. Results were classified as non-significant (ns; \( P > 0.05 \)) or significant (\( *P < 0.05 \)). Mean values are calculated \( \pm \) s.e.m.

ACKNOWLEDGEMENT

We thank P. Grüneberg, Dr A. Schneider and H. Goldammer for expert technical assistance.

Conflict of Interest statement: There are no conflicts of interest.

FUNDING

The study was supported by ELA foundation.

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