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Astrocytes and mitochondria from adrenoleukodystrophy protein (ABCD1)-deficient mice reveal that the adrenoleukodystrophy-associated very long-chain fatty acids target several cellular energy-dependent functions

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ABSTRACT

X-linked adrenoleukodystrophy (X-ALD) is a severe neurodegenerative disorder resulting from defective ABCD1 transport protein. ABCD1 mediates peroxisomal uptake of free very-long-chain fatty acids (VLCFA) as well as their CoA-esters. Consequently, VLCFA accumulate in patients' plasma and tissues, which is considered as pathogenic X-ALD triggering factor. Clinical symptoms are mostly manifested in neural tissues and adrenal gland. Here, we investigate astrocytes from wild-type control and a genetic X-ALD mouse model (*Abcd1*-knockout), exposed to supraphysiological VLCFA (C22:0, C24:0 and C26:0) concentrations. They exhibit multiple impairments of energy metabolism. Furthermore, brain mitochondria from *Abcd1*$^{-/-}$ mice and wild-type control respond similarly to VLCFA with increased ROS generation, impaired oxidative ATP synthesis and diminished Ca^{2+} uptake capacity, suggesting that a defective ABCD1 exerts no adaptive pressure on mitochondria. In contrast, astrocytes from *Abcd1*$^{-/-}$ mice respond more sensitively to VLCFA than wild-type control astrocytes. Moreover, long-term application of VLCFA induces high ROS generation, and strong in situ depolarization of mitochondria, and, in *Abcd1*$^{-/-}$ astrocytes, severely diminishes the capability to revert oxidized pyridine nucleotides to NAD(P)H. In addition, observed differences in responses of mitochondria and astrocytes to the hydrocarbon chain length of VLCFA suggest that detrimental VLCFA activities in astrocytes involve defective cellular functions other than mitochondria. In summary, we clearly demonstrate that VLCFA increase the vulnerability of *Abcd1*$^{-/-}$ astrocytes.

1. Introduction

With a minimal incidence of 1:20,000 births, X-linked adrenoleukodystrophy (X-ALD, OMIM 300100) is one of the most common peroxisomal disorders. Striking biochemical feature of X-ALD is the enrichment of very long chain fatty acids (VLCFA), such as C24:0, C26:0, and possibly also C26:1, in plasma and tissue of patients suffering from X-ALD [1,2]. Characteristic for X-ALD is mutations in the *Abcd1* gene, encoding for the peroxisomal ATP-binding cassette (ABC)-transporter adrenoleukodystrophy protein (ALDP/ABCD1) [3]. Defective *Abcd1* gene decreases the uptake of VLCFA as well as the CoA-esters of VLCFA into peroxisomes and thus, impairs the degradation of VLCFA by the peroxisomal β-oxidation pathway [4,5].

Elevated VLCFA levels appear to be correlated to the clinical outcome, which is characterized in the most severe cases by lethal progressive and multifocal demyelination, adrenal insufficiency, and inflammation [6]. The clinical phenotypic expression of X-ALD has a very broad variability, ranging from the fatal childhood cerebral form (cALD) to the more slowly progressing adult form, the adrenomyeloneuropathy (AMN) [7]. No correlation between mutations in the *ABCD1* gene and clinical phenotypes could be observed in several studies, suggesting that further genetic or environmental factors might contribute to the disease state [8–11].

Abbreviations: ABCD1, peroxisomal ATP-binding cassette transporter 1; ALDP, adrenoleukodystrophy protein; AMN, adrenomyeloneuropathy; cALD, cerebral ALD; CRC, Ca^{2+} retention capacity; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle's medium; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; FCS, fetal calf serum; FET, forward electron transport; HBSS, Hanks balanced salt solution; IMM, inner mitochondrial membrane; MBM, mouse brain mitochondria; MDA, malondialdehyde; Oli, oligomycin; PI, propidium iodide; PTP, permeability transition pore; RET, reverse electron transport; Rh123, Rhodamin 123; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; VLCFA, very long chain fatty acids; X-ALD, X-linked adrenoleukodystrophy; $\Delta\psi_m$, mitochondrial membrane potential; WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium

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The mouse model for X-ALD, the *Abcd1*-knockout mouse, which was developed independently in three laboratories [12–14] displays biochemical abnormalities, like reduced VLCFA β-oxidation and accumulation of VLCFA, similar to the situation seen in patients. However, *Abcd1*-knockout mice develop a late onset neurological phenotype that resembles the AMN phenotype and not cALD [15].

Like in other peroxisomal diseases, the molecular mechanism of the X-ALD pathogenesis is still elusive. There are two different research paradigms to uncover the X-ALD pathogenesis. Impairment of axonal function by demyelination is a hallmark of several neurodegenerative diseases caused by mutated peroxisomal proteins, most prominently in X-ALD or the Refsum syndrome. According to one view, oligodendrocytes with impaired peroxisomes fail to support white matter maintenance. This is probably due to the lack of peroxisomal mutations to provide oligodendrocytes with essential neuroprotective functions in order to protect against axon degeneration and neuroinflammation [16]. This conclusion is based on reports showing that peroxisomes may function to inactivate ROS in cells, such as oligodendrocytes [17]. Nevertheless, peroxisomes may also be considered as a main source of the generation of cellular reactive oxygen species (ROS) [18]. Moreover, a clear correlation between enhanced tissue levels of VLCFA and inflammatory disease progression in childhood X-ALD has been reported [19].

In addition, it has been claimed that oxidative stress is the major factor in the pathogenesis of X-ALD [20–24]; and in mitochondrial mPTP function [25]. Moreover, oxidative stress in turn generates inflammatory responses of cytokine and chemokine mediators, thereby promoting generalized peroxisomal dysfunctions, and, therefore, cell loss during the inflammatory demyelinating disease [21]. Enhanced oxidative stress was found by different groups using the X-ALD mouse model [24,26]. Moreover, C22:0, C24:0 and C26:0 were shown to induce neuronal damage by causing morphological and functional changes in mitochondria of a human neuroblastoma cell line [27]. Furthermore, fibroblasts exposed to C26:0 exhibit enhanced generation of ROS, which is coupled with the decreased mitochondrial membrane potential ($\Delta\psi_m$) [24]. Oxidative stress in X-ALD has been documented as enhanced lipid peroxidation, reduced plasmalogen level, and decreased antioxidant defense resulting in increased GSSG/GSH ratio [20,26,28–32].

Therefore, an alternative strategy to reveal the role of VLCFA in the pathogenesis of X-ALD is to analyze VLCFA-associated events triggering the onset of X-ALD pathogenesis (see for recent reviews [33,34]).

ABCD1 gene expression in the brain of adult mouse and human is mostly restricted to astrocytes, microglia cells, and oligodendrocytes [35]. Dysfunction of ABCD1 in glial cells seems to have a special role in the pathogenesis of X-ALD, since both oligodendrocytes and astrocytes are important for myelination. Astrocytes influence the myelination via secretion of factors or formation of cell–cell contacts to oligodendrocytes [36]. Furthermore, astrocytes are known to synthesize lipids and supply these to other cells for myelination and synaptogenesis [37,38]. Previously, we could already demonstrate that excess of VLCFA is toxic for neural cells, especially for glial cells [39,40]. In these studies we revealed that VLCFA cause detrimental changes in hippocampal neurons, astrocytes and oligodendrocytes by disturbing the intracellular Ca^{2+} homeostasis and mitochondrial functions, which finally lead to death of cells. The strongest impairment was seen in the myelin-producing oligodendrocytes. However, until now no studies have been done concerning the toxicity of VLCFA in neural cells of the *Abcd1*-knockout mouse model.

The degradation of VLCFA is orchestrated by the peroxisomal and mitochondrial β-oxidation pathways (see for review [41]). This combined degradation by both organelles is disturbed in X-ALD. From this fact the important question arises: Does the biogenesis of mitochondria respond to the defective ABCD1 protein, leading to mitochondrial functions in the ABCD1-deficient phenotype that are different from that of the normal phenotype? Indeed, there are the following controversial reports on alterations of mitochondrial functions in adrenoleukodystrophy. On the one side, structural abnormalities of mitochondria in cells of X-ALD mice have been proposed to indicate impaired mitochondrial functions [42,43], whereas on the other side it was reported that normality in size, structure and localization of mitochondria in muscle can be detected in an ABCD1-deficient mouse model for X-ALD [44]. Rates of oxygen uptake of phosphorylating isolated skeletal muscle mitochondria from ABCD1-deficient and wild-type mice also did not differ [44]. In contrast, from experiments with permeabilized spinal cord slices of the ABCD1-deficient mouse model revealed that the phosphorylating respiration is decreased by 20 to 25%, when the respiration was stimulated by excess of ADP and succinate [45]. This decrease in the phosphorylating respiration might be attributed to an oxidatively impaired F_OF_1-ATP synthase activity [45] and/or to the noxious activity of an enhanced level of VLCFA in the spinal cord slice tissue. The latter view is indicated by the observation that the oligomycin-sensitive respiration of fibroblast cultures prepared from control ABCD1-deficient mice (metabolizing galactose) was similar, but decreased in the presence of 50 μM of C26:0 [45]. Moreover, reduced contents of mitochondria were found in neural tissues from the spinal cords of the same mouse model [46].

Despite the fact that X-ALD is severely manifested in neural tissue, most studies in the field of X-ALD basic research were done using *Abcd1*$^{-/-}$ fibroblasts of patients or mice. Therefore, the present study was designed to illuminate the influence of VLCFA accumulation on glial cells of wild-type control mice and those from an X-ALD mouse model (*Abcd1*$^{-/-}$). Here, we investigated the influence of VLCFA on the cellular properties of ROS production, various mitochondrial parameters, cellular Ca^{2+} handling, and the reduction of a tetrazolium electron acceptor (WST-1) to the water-soluble formazan dye, and, finally, induction of cell death in astrocytes.

Here, we report that astrocytes from wild-type control and ABCD1-deficient mice, which were exposed to supraphysiological concentrations of the VLCFA (C22:0, C24:0 and C26:0) exhibit multiple impairments of the astrocytic energy metabolism. In contrast, the functional parameters of mitochondria from brain tissue of *Abcd1*$^{-/-}$ mice do not differ from those of the wild-type control. This finding suggests that a defective peroxisomal ABCD1 exerts no adaptive pressure on the mitochondria. Moreover, mitochondria and astrocytes respond to VLCFA with different lengths of the hydrocarbon chain in a different manner, indicating that mitochondria are not the only target of detrimental VLCFA activities in astrocytes.

In summary, astrocytes from *Abcd1*$^{-/-}$ mice respond more sensitively to VLCFA than those from wild-type control. Thus, long-term application of VLCFA causes higher ROS generation and a stronger in situ depolarization of mitochondria. In addition, VLCFA diminished severely in *Abcd1*$^{-/-}$ astrocytes the capability to reduce the tetrazolium electron acceptor (WST-1) to the formazan dye. Taken together, these findings may help to establish new concepts for finding treatments for X-ALD.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and fetal calf serum (FCS) were obtained from Biochrom AG (Berlin, Germany). The fluorescent dyes Fura-2AM, dihydroethidium (DHE) and Rhodamin 123 (Rh123) as well as Pluronic and the dead cell apoptosis kit with annexin V FITC and propidium iodide (PI) were from Molecular Probes Invitrogen (Karlsruhe, Germany). Water-soluble tetrazolium salt WST-1 was from Roche (Mannheim, Germany). Lactate oxidase assay was from Labor & Technik (Berlin, Germany). Behenic acid (C22:0), lignoceric acid (C24:0), and cerotic acid (C26:0) were obtained from Larodan Fine Chemicals AB (Malmö, Sweden). All other chemicals were from Sigma Aldrich (Taufkirchen, Germany).

2.2. Cell culture and treatment

All experiments with animals conformed to the guidelines of Sachsen-Anhalt (Germany) on the ethical use of animals, and all efforts

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