Plasmalogens participate in very-long-chain fatty acid-induced pathology

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Peroxisomes are organelles responsible for multiple metabolic pathways including, the biosynthesis of plasmalogens, a class of phospholipids, and the β-oxidation of very-long-chain fatty acids (VLCFA). Lack of peroxisomes or dysfunction in any of their normal functions is the cellular basis for human peroxisomal disorders. Here we used mouse models to understand and define the biochemical and cellular determinants that mediate the pathophysiological consequences caused by peroxisomal dysfunctions. We investigated the role and effects of cellular plasmalogens and VLCFA accumulation in liver, testis and nervous tissue using Pex7 and Abcd1 knockout (KO) mice. In addition, we also generated a Pex7:Abcd1 double KO mouse to investigate how different peroxisomal dysfunctions modulate cellular function and pathology. We found that plasmalogens function as fundamental structural phospholipids and protect cells from damage caused by VLCFA accumulation. In testis, plasmalogens protect spermatocytes from VLCFA-induced degeneration and apoptosis. In nervous tissue, we found that gliosis, inflammatory demyelination and axonopathy caused by accumulation of VLCFA are modulated by plasmalogens. Our findings demonstrate the importance of normal peroxisomal functioning and allow the understanding of the pathological causality of peroxisomal dysfunctions. Nervous tissue deficient in plasmalogens is more prone to damage, illustrating the importance of plasmalogens in peroxisomal disorders including Zellweger syndrome and X-linked adrenoleukodystrophy.

Keywords: peroxisome; plasmalogen; very-long-chain fatty acids; demyelination; neuropathy

Abbreviations: C26:0 = hexacosanoic acid; CMAP = compound muscle action potentials; DKO = double knockout; KO = knockout; MNCV = motor nerve conductance velocity; VLCFA = very-long-chain-fatty acids; WT = wild type; X-ALD = X-linked adrenoleukodystrophy

Introduction

The importance of peroxisomes for the normal functioning of cells, tissues and even organisms is underscored by the causative effects of peroxisomal dysfunction. The recognition of various human disorders with impaired peroxisomal functioning has helped the identification of genes involved in peroxisome formation (Weller et al., 2003) and the identification and characterization of the extensive repertoire of functions performed by peroxisomes (Wanders and Waterham, 2006). These human peroxisomal disorders vary in the age of onset, clinical presentation, tissues affected and pathology. Knowing that peroxisomes participate in at least eight different metabolic pathways (Wanders and Waterham, 2006) it has become a challenging task to unravel the physiological link between a given peroxisomal dysfunction and its pathophysiological consequence.

Biological processes that are affected in peroxisomal disorders include endochondral ossification, neuronal migration and myelination (Faust et al., 2005). In order to understand the pathophysiological mechanisms behind the metabolic defects caused by peroxisome deficiencies we, and others, have generated mouse models for several human peroxisomal disorders...
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(Baes and Van Veldhoven, 2003; Brites et al., 2004a). The Pex7 KO mouse serves as a model for Rhizomelic Chondrodysplasia Punctata (RCDP) (Purdue et al., 1999; Brites et al., 2003). The genetic inactivation of Pex7 in the Pex7 KO mouse impairs the import into peroxisomes of proteins carrying a peroxisomal targeting signal type 2 (PTS2), and leads to defects in plasmalogens. Phytic acid α-oxidation and VLCFA β-oxidation (Brites et al., 2003). Throughout life, Pex7 KO mice have impaired biosynthesis of plasmalogens, a plasmalogen phospholipid characterized and distinctive from other glycerophospholipids by the presence of an α,β-unsaturated ether bond (also called vinyl ether bond) at the sn-1 position of the glycerol backbone (Brites et al., 2004b). Plasmalogens comprise all plasmalogen phospholipids containing ethanolamine or choline in the head group of the glycerol backbone. In mammals, the distribution and composition of plasmalogens vary among different tissues: nervous tissues, kidney and testes have relatively high levels of plasmalogens whereas liver has very low amounts of plasmalogens. Nervous tissues, kidney and testes are also characterized by high levels of plasmalogen ethanolamine whereas heart and skeletal muscle have high levels of plasmalogen choline (Brites et al., 2004b). In Pex7 KO mice the impaired plasmalogens biosynthesis affects all forms of plasmalogen phospholipids (Brites et al., 2003).

Plasmalogens have been implicated in several biological processes where they can affect membrane fluidity, mediate signal transduction and protect against oxidative stress (Wanders and Waterham, 2006). Nevertheless, the in vivo role(s) of plasmalogens remains elusive and the usage of mouse models defective in plasmalogens may provide a better understanding of their function. Phenotypically, the Pex7 KO mouse display, amongst others, defects in embryonic neuronal migration and endochondral ossification (Brites et al., 2003). The impairment in neuronal migration found in Pex7 KO mice is relatively mild when compared with the Pex5 KO mice (Baes et al., 1997), a mouse that lacks functional peroxisomes and consequently, all peroxisomal functions. The observation that deficient β-oxidation alone does not affect neuronal migration in mice (Baes et al., 2002), suggests that multiple peroxisomal impairments possibly including defects in plasmalogens biosynthesis and β-oxidation, may be required to disrupt and modulate the process of neuronal migration.

Normal peroxisomal metabolism is also required for myelination of the nervous system, given that dysmyelination and demyelination are frequently observed in patients with deficiencies in peroxisomal functions (Faust et al., 2005), including X-linked Adrenoleukodystrophy (X-ALD). X-ALD is a complex peroxisomal disorder caused by mutations in the ABCD1 gene and biochemically characterized by the abnormal accumulation of VLCFA (Moser et al., 1984; Berger and Gartner, 2006). Despite its broad range of clinical presentations and outcomes, X-ALD can be divided into a severe variant presenting with a rapidly developing cerebral inflammatory demyelination, and a milder variant characterized by a slower-developing non-inflammatory axonopathy, also designated Adrenomyeloneuropathy (AMN) (Powers et al., 1992, 2000; Moser, 1997). Several independent mouse models of X-ALD, in which the Abcd1 gene has been disrupted (Abcd1 KO mice), have been generated (Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997), but despite the accumulation of VLCFA in target organs, the mice do not develop the characteristic clinical hallmarks of X-ALD. Although some controversy exists about the function of ALDP, the protein encoded by the ABCD1 gene, and the nature of the β-oxidation defect in X-ALD (McGuinness et al., 2003; Moser et al., 2007), the hypothesis that VLCFA accumulation, at least in mice, is caused by defective transport of these fatty acids across the peroxisomal membrane is still supported by the fact that ALDP belongs to the ATP-binding cassette (ABC) transporter superfamily (Stefkova et al., 2004; Wanders et al., 2007) and by the observation of reduced rates of VLCFA β-oxidation in fibroblasts (Kobayashi et al., 1997; Lu et al., 1997; Pujo et al., 2002) and hepatocytes (Kobayashi et al., 1997) isolated from Abcd1 KO mice.

To investigate the development of pathophysiological alterations caused by peroxisomal dysfunctions we have generated the Pex7:Abcd1 double KO (DKO) mice. These DKO mice allowed us to investigate the role and the effects of plasmalogens and VLCFA under in vivo conditions during postnatal development. Our findings indicate that plasmalogens modulate the pathology caused by VLCFA accumulation with detrimental consequences for spermatocyte development, myelination and axonal survival.

Materials and methods

Animal experiments

Mice were genotyped from genomic DNA isolated from toe clippings using the TissueDirect Multiplex PCR system (GenScript Corporation). Primers for the wild type (WT) Pex7 allele were 5’-TCCCAATCTCGAGACACGCGTGTA-3’ and 5’-ATGCACAGTTCACCTCGTCTGCGAAAACCG-3’; and for the mutant Pex7 allele primers were 5’-CTACGCTGACAGCTCAACGTCTTAAGACCCG-3’ and 5’-GTACCGCTGCTACAGGCTTACAC-3’. The mutant Pex7 allele primer were amplifying a fragment of 450 bp and primers for the mutant Pex7 allele primers were 5’-CTACGCTGACAGCTCAACGTCTTAAGACCCG-3’ and 5’-GTACCGCTGCTACAGGCTTACAC-3’. Amplifying a fragment of 300 bp. Genotyping of the Abcd1 alleles was performed with primers 5’-CACACGCCCTCTCCTCCTTAAAGC-3’, 5’-CTCGTGTGCTTACGCCAATCGG-3’ and 5’-CTTGCATGCCTCCCTTTCTTGACG-3’, amplifying a fragment of 217 bp for the WT allele and a fragment of 117 bp for the mutant Abcd1 allele.

Pex7 heterozygous mice (Pex7+/-; genotype Ay+/-) in a Swiss Webster background were crossed with Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory). From the F1 we crossed Abcd1 KO mice (Abcd1+/-; genotype aa+/+) in a C57BL/6J:129S1 mixed background (The Jackson Laboratory).
analyses. Experiments and mouse manipulations were approved by the University of Amsterdam Animals Experiments Committee.

Cell culture
Skin-derived fibroblasts were grown from skin biopsies taken from 6 months old mice and cultured in Dulbecco’s modified Eagle’s medium (DMEM), containing 4.5 g/l glucose and L-glutamine, 20% FBS, 100U penicillin/ml, 100 μg/ml streptomycin, 150 μg/ml fungizone and, supplemented with 25 μmol Hepes. All studies were performed between passages 16 and 22.

Biochemical analyses
Tissues and fibroblasts were homogenized in phosphate buffer solution (PBS) by sonication. The homogenates were cleared by centrifugation at 900 g for 5 min and protein was measured using the DC Protein Assay kit (Bio-Rad) using BSA as standard. For the measurement of VLCFA and phytanic acid lysate corresponding to 300 μg of protein was used and for the measurement of plasmalogens lysate corresponding to 200 μg of protein was used. All biochemical assays, including β-oxidation in cultured fibroblasts (Wanders et al., 1995), measurement of VLCFA and phytanic acid levels using a coupled gas chromatography-mass spectrometry method (Vreken et al., 1998) and measurement of plasmalogen levels using gas chromatography (Dacremont and Vincent, 1995) were performed as described.

Histological analyses
Pieces of harvested tissues were fixed by immersion in buffered formalin at 4 °C for 48 h, processed for paraffin embedding and sectioned on a Leica RM2255 microtome, according to routine procedures. Paraffin sections, 5 μm thick, were deparaffinized in Histoclear II (National Diagnostics), rehydrated using decreasing concentrations of ethanol and used for routine histological or immunohistochemical analyses. Routine histological stainings included H&E, nuclear fast red and luxol fast blue stainings. For immunohistochemical analyses we used the Vectorstain Elite peroxidase ABC kit (Vector Laboratories) according to the manufacturer’s protocol and 3,3'-diaminobenzidine tetra-chloride (DAB; Sigma) as a substrate. Processed sections were counterstained with hematoxylin QS (Vector Laboratories), dehydrated in graded ethanol solutions, cleared in Histoclear II and mounted in DPX (Fluka). For immunohistochemical analyses the following primary antibodies and procedures were used: mouse anti-nestin (1:100; Millipore), rabbit anti-GFAP (1:500; DAKOcytomation) after microwave in Tris:EDTA solution pH 9.0; rabbit anti-cleaved caspase 3 (1:100; Cell Signaling Technology) after microwave in citrate solution pH 6.0; rat anti-F4/80 (1:20; Serotec) after microwave in citrate solution pH 6.0; and, goat anti-MBP (1:100; SantaCruz). All secondary antibodies (Vector Laboratories) were biotinylated, and used at 1:100 dilution. In sciatic nerves, MBP detection was performed as described above but after the secondary antibody, slides were incubated with 1:100 dilution of streptavidin-FITC (DACKyoto) and observed under UV light from a deuterium lamp using a 450–490 nm excitation filter and a 510–565 nm emission filter. Slides were examined on a Zeiss Axioshot microscope and photographed using a Leica DFC320 camera.

Western blot analysis
Lysates of cerebrum and cerebellum from 10 months old WT and KO mice (n=3 per genotype) were prepared by sonication in PBS containing 0.2% Triton X100 and protease inhibitor cocktail (Roche). Protein samples (20 μg) were separated on 12.5% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked with 3% skim dried milk (Fluka) in PBS containing 0.01% Tween20 (w/v) and probed with antibodies against MBP (see above) and mouse anti-2',3’-cyclic nucleotide 3’ phosphodiesterase (CNPase, Neomarkers) both at 1:250 dilution in blocking buffer. Mouse anti-β-actin (1:5000, Sigma) was used as a protein loading control. Membranes were developed with BCIP/NBT (Promega) after incubation with alkaline phosphatase-labeled secondary antibodies (Biosource). Blots were scanned and quantification was performed using the AIDA software.

Electrophysiology
Mice were anesthetized with 100 mg/kg ketamin and 10 mg/kg xylazine and placed on a warm pad at a temperature of ~30–34 °C. Recordings of compound muscle action potentials (CMAP) were obtained on PowerLab 4/25T (AD instruments) using Chart5 software. Recording needle electrodes were placed subcutaneously in the foot pad and supramaximal stimulation of sciatic nerves was performed distally at the level of the ankle and proximally at the sciatic notch. Conduction velocities were calculated as: (proximal distance–distal distance)/(proximal latency–distal latency), with latencies corresponding to the time lapse between the stimulus and the onset of the CMAP and expressed in meters per second.

Data analysis
Data are expressed as means ± SD of measurements. Statistical comparisons were performed using Wilcoxon Mann-Whitney test, and significance was defined as P<0.05.

Results
Generation and phenotypic characterization of Pex7:Abcd1 double KO
To study the pathophysiological consequences of multiple peroxisomal deficiencies we generated a mutant mouse with a combined defect in both plasmalogen biosynthesis and VLCFA β-oxidation. The Pex7:Abcd1 double KO (hereafter referred to as DKO) was obtained after crossing Pex7 heterozygous males with homozygous Abcd1 KO females. At birth, Pex7 KO and DKO mice were smaller than WT and Abcd1 KO mice, displayed hypotonia and ~50% of Pex7 KO and DKO pups died within 2 days of birth. Weight measurements throughout postnatal development consistently demonstrated the impaired development of Pex7 KO and DKO mice when compared to WT and Abcd1 KO mice (Fig. 1A and B). The body weights of DKO mice at P15 (Fig. 1A) and at 3 months of age (data not shown) were more reduced than those of Pex7 KO mice but after 6 months of age Pex7 and DKO mice had similar body weights (Fig. 1B). A factor contributing to the decreased body weight was the extremely reduced amount of white adipose tissue found upon dissection of Pex7 KO and DKO mice (data not shown). Abnormal movement and posture were characteristics found in DKO mice that were not evident in Pex7 KO mice. Pex7 KO and DKO mice that survived past the weaning age had a life-span between 9 and 14 months of age. During this
period, DKO mice started to develop tremors and hindlimb ataxia. These phenotypic differences between Pex7 KO and DKO mice suggested a synergy between the two different biochemical abnormalities and their pathological consequences.

Plasmalogen levels in brain, kidney and liver did not differ between Pex7 KO and DKO mice and were extremely reduced (<5%) when compared to WT or Abcd1 KO mice (Fig. 1C). Levels of phytanic acid were very low and did not differ between the different mice (Fig. 1D) fed the standard diet. These results illustrate that the defect in \( \alpha \)-oxidation has no consequences under standard feeding conditions. Only when mice were fed a diet supplemented with phytol (the precursor of phytanic acid) was a drastic accumulation of phytanic acid observed (Fig. 1D and Brites et al., 2003).

Synergistic effect on VLCFA accumulation in tissues from DKO mice

To evaluate the effects of the combined deficiency of the Pex7 and Abcd1 mutant alleles we measured \( \beta \)-oxidation in skin-derived fibroblasts from WT and mutant mice (Fig. 2A). Whereas mitochondrial \( \beta \)-oxidation of stearic acid was normal in all mutant cell lines, we found a 40% reduction in the \( \beta \)-oxidation rate of the VLCFA hexacosanoic acid (C26:0) in the cell lines from Abcd1, Pex7 and DKO mice (Fig. 2A). Interestingly, despite having similar rates of hexacosanoic acid \( \beta \)-oxidation, we found that the levels of VLCFA in the skin-derived fibroblasts from DKO mice were significantly increased when compared to fibroblasts from Abcd1 and
Fig. 2 Impaired peroxisomal β-oxidation and accumulation of VLCFA in fibroblasts and tissues. (A) Mitochondrial β-oxidation of stearic acid (C16:0) and peroxisomal β-oxidation of hexacosanoic acid (C26:0) in skin-derived fibroblasts from WT (n = 6), Abcd1 KO (n = 5), Pex7 KO (n = 5) and DKO (n = 6). All KO mice showed specific impairments in peroxisomal β-oxidation. Error bars indicate standard deviation. *P < 0.05 compared to WT mice. (B) Levels of VLCFA in fibroblasts from the different mice (n ≥ 3 for each genotype). (C) Levels of VLCFA in different tissues. Measurements in testis were performed in P20 mice (n = 3 for all genotypes). Measurements in all other organs were performed in 6-month old WT (n = 5), Abcd1 KO (n = 5), Pex7 KO (n = 4) and DKO (n = 5) mice, showing a tissue-dependent accumulation of VLCFA in Pex7 KO mice in contrast to the generalized accumulation of VLCFA in DKO mice. The different fatty acids have the same color code as in (B). Error bars indicate standard deviation. *P < 0.05 compared to WT mice; #P < 0.05 compared to Abcd1 KO mice; †P < 0.05 compared to Pex7 KO mice.
Pathological alterations in non-nervous tissue

We next sought to evaluate the pathological changes in known target tissues and in tissues where we found a differential accumulation of VLCFA. As shown above, normal levels of VLCFA were detected in livers of Pex7 KO mice, but in livers of DKO mice increased levels of VLCFA were observed which were even higher that in Abcd1 KO mice. Histological analysis of livers from WT and mutant mice did not reveal any major changes but we did find increased fibrosis and infiltration surrounding the portal area of livers from Abcd1 and DKO mice (data not shown).

Since male Pex7 KO mice are infertile (Brites et al., 2004a) we evaluated the testicular pathology in the different mouse mutants (Fig. 3). At P21, the first signs of pathology in testes of Pex7 KO mice included a slight disorganization of the seminiferous epithelium with respect to the localization of spermatocytes and round spermatids (Fig. 3C). At the same age the testes of Abcd1 KO mice lacking any obvious signs of pathological alterations (Fig. 3B), but the testes of DKO mice showed a severe pathology with very disorganized seminiferous epithelium lacking round spermatids and an increase in the number of abnormally multinucleated cells within the lumen of the seminiferous tubules (Fig. 3D and E), and these multinucleated cells were present in 53 ± 12% of DKO tubules. We next investigated if the loss of spermatocytes might be mediated by apoptosis. Testes of P21 mice were immunostained with an antibody against cleaved-caspase 3, a marker for apoptosis. Spermatocytes positive for cleaved-caspase 3 were evident in the seminiferous tubules of Pex7 (63 ± 3% of tubules contained cleaved-caspase 3 labeled cells) and DKO mice (76 ± 8% of tubules contained cleaved-caspase 3 labeled cells) (Fig. 3F and G). The multinucleated cells observed in DKO were not stained. The number of spermatocytes positive for cleaved-caspase 3 was increased in DKO mice when compared to Pex7 KO mice (Fig. 3H).

Effect of plasmalogens in VLCFA-induced demyelination

We next assessed the myelination status of the CNS of mutant mice by immunohistochemistry with an antibody against myelin basic protein (MBP; Fig. 5). At 3 months of age, Abcd1, Pex7 and DKO mice showed normal myelination in the corpus callosum, brainstem, spinal cord (data not shown) and cerebellum (Fig. 5B–D). Strikingly, 9–11 months old DKO showed thinning of the cerebellar white matter tracts with reduced staining for MBP, indicative of demyelination (Fig. 5H). The demyelination in DKO mice, as judged by the intensity of MBP staining in IHC, was detected throughout the CNS but was more apparent in smaller white matter tracts compared to large myelinated areas (e.g. corpus callosum; data not shown). No obvious demyelination or paraly was observed in Abcd1 KO mice (Fig. 5F). In Pex7 KO mice only a slight paraly was evident in the small white matter cerebellar tracts (Fig. 5G). Luxol fast blue stainings confirmed the demyelination in small white matter tracts of DKO mice and showed myelin paraly throughout the corpus callosum and the large cerebellar white matter tracts (Supplementary Fig. 1). Western blot
Fig. 3 Plasmalogens modulate the damaging effects of VLCFA accumulation in testis. (A–D) Histological analyses of testis from P21 WT (A) Abcd1 KO (B), Pex7 KO (C) and DKO (D) mice stained with nuclear fast red, showing degeneration of seminiferous tubules of DKO mice. Arrowheads in (D) point to multinucleated cells found in the lumen of seminiferous tubules. (E) Quantification of multinucleated cells (≥2 nuclei) per seminiferous tubule of mice from the different genotypes. n = 3 for all genotypes and scale bars are 50 μm. (F–G) Immunohistochemical detection of cleaved-caspase 3 in the seminiferous tubules of P21 Pex7 KO (F) and DKO (G) mice showing an increased number of apoptotic spermatocytes in DKO mice. Arrowheads in (G) point to multinucleated cells. (H) Quantification of cleaved-caspase 3 positive cells in seminiferous tubules of mice from the different genotypes. Slides were counterstained with hematoxylin. Scale bars are 50 μm. (I–L) Histological analyses of testis from 6-month old WT (I) Abcd1 KO (J), Pex7 KO (K) and DKO (L) mice stained with H&E, showing complete degeneration of the seminiferous tubules of Pex7 KO and DKO mice. Arrowheads in (L) denote the increased numbers of Leydig cells in the testis of DKO mice. Identification of Leydig cells was achieved by staining adjacent sections with an antibody against nestin (data not shown). n = 3 for all genotypes and scale bars are 50 μm.
analysis of myelin proteins confirmed the more severe demyelination in DKO mice as judged by the reduced amounts of MBP and CNPase (Fig. 5I and J and Supplementary Fig. 2).

Neuropathy with demyelination and axonal loss in DKO mice

We next assessed the condition of the peripheral nervous system (PNS) in the mutant mice. Immunostaining with an antibody against MBP in sciatic nerves of 10-month-old mice revealed a severe loss of myelinated axons in DKO mice with a thinning of the myelin sheaths in the remaining myelinated fibers (Fig. 6D). Pex7 KO nerves were not as affected as the nerves of DKO mice, although we observed loss of small myelinated axons (Fig. 6C) and...
decreased thickness of the myelin sheaths (data not shown). In Abcd1 KO mice no major changes were observed (Fig. 6B). The observed changes in the PNS of DKO were age-dependent, since at 3 months of age, sciatic nerves from DKO showed only minor changes that, when compared to aged-matched Pex7 KO nerves (Fig. 6E), consisted of a partial loss of myelinated fibers (Fig. 6F).

The consequences of the observed pathological alterations in sciatic nerves of Pex7 and DKO mice were evident when we measured motor nerve conductance velocities (MNCV) in 10-month-old WT and mutant mice. Increased latencies were observed in Pex7 KO and DKO mice. (H) Motor nerve conductance velocities (MNCV) in 10-month-old mice. Bars represent the average values obtained after bilateral measurements in WT (n=6), Abcd1 KO (n=4), Pex7 KO (n=4) and DKO (n=5) mice. Error bars indicate standard deviation. *P<0.05.

**Discussion**

In this study our aim was to investigate the consequences of VLCFA accumulation and deficiency of plasmalogens and their interrelationship. We show that the combined inactivation of Pex7 and Abcd1 has functional consequences at both the biochemical and pathophysiological level.

We found that the accumulation of VLCFA in Pex7 KO mice was tissue-dependent with normal VLCFA levels in brain and liver but abnormally high VLCFA levels in kidney (Fig. 2). We hypothesized that in the absence of thiolase from peroxisomes, its function can be taken over by the sterol carrier protein x (SCPx), since SCPx can use both straight and branched chain fatty acids as substrates (Seedorf et al., 1994; Wanders et al., 1997). This hypothesis is strengthened by the fact that SCPx expression is upregulated during postnatal development (Huyghe et al., 2001) and that liver peroxisomes contain high amounts of SCPx whereas kidney peroxisomes have very low amounts of SCPx (Mi et al., 2007). Thus, in kidney of Pex7 KO mice the absence of thiolase and SCPx leads to a severe defect in VLCFA β-oxidation but in liver and brain, expression of SCPx is able to compensate for the absence of thiolase. The difference in VLCFA found between brain and spinal cord, coupled with different levels of SCPx could be a contributing factor that results in the variable accumulation of VLCFA in Pex7 KO mice.

The genetic inactivation of Abcd1 in Abcd1 KO mice leads to the accumulation of VLCFA in plasma and tissues and is used as a model for X-ALD (Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997; Pujol et al., 2002). The Abcd1 encoded protein, i.e. ALDP, is thought to function as the peroxisomal transporter of VLCFA (Stefkova et al., 2004; Wanders et al., 2007) and it has been hypothesized that the failure to transport VLCFA into peroxisomes may explain the reduced rate of VLCFA β-oxidation and their subsequent accumulation. Despite the accumulation of...
VLCFA in nervous tissue Abcd1 KO mice fail to develop the hallmarks of X-ALD and only aged Abcd1 KO mice develop some motor abnormalities reminiscent of AMN (Pujol et al., 2002).

In this study, by crossing the mutant Pex7 allele with the mutant Abcd1 allele, we blocked peroxisomal β-oxidation at two different steps, i.e. the transport and the degradation of VLCFA and were able to investigate the consequences and the relationship between VLCFA accumulation and plasmalogen deficiency. The biochemical analyses (Fig. 2) clearly demonstrated that in Pex7:Abcd1 DKO mice there is not only a generalized accumulation of VLCFA in cells and tissues but the extent of VLCFA accumulation is higher than in the Abcd1 KO mice. This supports the conclusion of a synergistic block in peroxisomal β-oxidation in DKO mice rather than a merely additive effect. The compensatory effect of SCPx expression found in Pex7 KO mice (Brites et al., 2003; Mi et al., 2007) was not observed in DKO since the inactivation of ALDP may impair the transport of VLCFA into peroxisomes and thus block peroxisomal β-oxidation at an earlier step.

We observed marked phenotypic consequences of the VLCFA accumulation in several target organs. Tests from Pex7 and DKO mice showed distinct degenerative alterations. Disorganized seminiferous tubules showing abnormalities in spermatocytes were evident in Pex7 KO mice leading to infertility. This pathology is similar to the one found in plasmalogen-deficient testis of Gnpat KO mice (Rodemer et al., 2003). Our findings suggest a crucial role of plasmalogens for normal spermatocyte development and in protecting spermatocytes from damage caused by VLCFA accumulation.

Although it is well established that nervous tissues are enriched in plasmalogens, their function(s) within myelin have remained elusive (Brites et al., 2004b). Our results indicate that plasmalogens are not required for nervous tissue myelination per se but they may play a role in myelin remodeling, since aged Pex7 KO mice show thinning of myelin sheaths (Fig. 6; Brites et al., unpublished results). Furthermore, based on the results obtained in our DKO mouse, plasmalogens may also have a protective role against the effects of VLCFA accumulation. In plasmalogen-deficient nervous tissues, the damage caused by VLCFA accumulation was evident and marked by demyelination, axonal loss and reactive gliosis. Recently, Kassmann et al. (2007) showed that a mouse model with peroxisome-deficient oligodendrocytes, developed demyelination, axonal loss and inflammation. But, since the lack of peroxisomes in these mice yields abnormalities in all the metabolic functions normally performed by peroxisomes (Wanders and Waterham, 2006), it is difficult to establish which metabolic pathways are responsible for the observed pathology. The results described in the present study provide important information on the question of which peroxisomal dysfunctions mediate these neuropathological consequences, highlighting the biosynthesis of plasmalogens and VLCFA β-oxidation as key players for normal nervous system functioning.

From the pathological standpoint, our results on the Pex7:Abcd1 DKO mice and those of the mice with peroxisome-deficient oligodendrocytes (Kassmann et al., 2007), recapitulate many aspects of the pathology observed in patients with X-ALD (Powers et al., 1992, 2000; Powers, 1995; Moser, 1997), whereas the Abcd1 KO mice do not (Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997; Powers et al., 2005). The complexity of the X-ALD disorder emerges from a common defect in VLCFA β-oxidation that can present either as a severe and rapidly progressive inflammatory demyelination or a slow non-inflammatory axonopathy (Powers et al., 1992, 2000). These different presentations of X-ALD have long been taken to indicate the existence of modifier(s) that may play a role in the pathology (Smith et al., 1999). Our results suggest that plasmalogens could be one such modifier, as their deficiency augments the damage to the nervous tissue caused by VLCFA accumulation. Measurement of plasmalogen levels in samples from X-ALD patients is scarce but decreased plasmalogen levels have been described both in red blood cells and in white matter samples of X-ALD patients (Wilson and Sargent, 1993; Moser et al., 1999; Khan et al., 2008). Interestingly, decreases in plasmalogen content have also been found in several neuropathological conditions including Alzheimer’s disease, Gaucher’s disease, dementia and ischemia (Ginsberg et al., 1998; Guan et al., 1999; Brites et al., 2004b; Goodenowe et al., 2007; Moralet et al., 2008) and it has been proposed that plasmalogens may modulate the severity and progression of the disease. Although it is still unknown which mechanism, i.e. impaired biosynthesis or increased degradation of plasmalogens is responsible for the loss of plasmalogens in these neurological disorders or in X-ALD, our results suggest that regardless of the mechanism involved, the deficiency of plasmalogens may exacerbate the pathology.

Taken together, our findings demonstrate the crucial importance of peroxisomes in normal development and highlight the relevance of cellular plasmalogens for normal functioning and their possible involvement in the VLCFA-induced damage characteristic of X-ALD. Plasmalogens have been implicated in a variety of cellular processes suggesting that these phospholipids may have both structural and functional roles in membrane and cellular stability, fluidity and protection (Brites et al., 2004b). With the increasing in the number of non-peroxisomal disorders that show abnormalities in plasmalogen content, our study highlights the importance of investigating the role of plasmalogens in modulating pathophysiological changes that may contribute to the progression of the disorder.

Supplementary material

Supplementary material is available at Brain online.

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