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Combined deficiency of peroxisomal β-oxidation and ether lipid synthesis in mice causes only minor cortical neuronal migration defects but severe hypotonia

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ABSTRACT

The metabolic factors causing cortical neuronal migration defects, hypotonia and malformation of cerebellum in patients and mice with severe peroxisome biogenesis disorders are still not identified. In the present investigation, we tested the hypothesis that the combined inactivity of peroxisomal β-oxidation and ether lipid biosynthesis could be at the origin of these pathologies. Double *MFP2/DAPAT* knockout mice were generated and their postnatal phenotypes were compared with single knockouts and control mice. Cortical neuronal migration was not affected in *DAPAT* knockouts and only mildly in double *MFP2/DAPAT* knockout mice. The latter mice were severely hypotonic and usually died in the postnatal period. Both *DAPAT* and *MFP2* single knockout mice exhibited delays in the formation of cerebellar folia. We conclude that the combined defect of peroxisomal β-oxidation and ether lipid synthesis does not solely account for the typical cortical neuronal migration defect of mice with peroxisome biogenesis disorders but contributes to their hypotonia.

Introduction

Peroxisomes are subcellular organelles that are necessary for normal neurodevelopment. This can be deduced from the pathology in patients with peroxisome biogenesis disorders in which all peroxisomal functions are lacking [1]. The most severe form of these diseases, Zellweger syndrome, is characterized by a typical cortical neuronal migration defect resulting in abnormal gyration including polymicrogyria and pachygyria. Furthermore, cerebellar heterotopias, hypotonia, dys- or demyelination and facial dysmorphisms are common and patients usually do not survive the first year of life. In less severe forms of the Zellweger spectrum of diseases, cortical layering is normal but most of the other pathologies are still present. Several Zellweger syndrome mouse models were generated by inactivating one of the Pex proteins (Pex5p, Pex2p, Pex13p) that are essential components of the peroxisomal import machinery [2]. Cortical neuronal migration defects, malformation of the cerebellum and hypotonia were observed in these animals.

An unresolved question is the molecular interrelationship between metabolic perturbations caused by the absence of functional peroxisomes and cytoarchitectonic abnormalities in the brain. The most important metabolic pathways in peroxisomes, with respect to human health, are α-oxidation of 3-methyl-branched chain fatty acids (e.g. phytanic acid) and 2-hydroxy fatty acids and β-oxidation of a variety of substrates that cannot be chain shortened in mitochondria such as very long chain fatty acids (VLCFA), 2-methyl-branched chain fatty acids (e.g. pristanic acid), bile acid intermediates and eicosanoids [3]. Furthermore, peroxisomes are the site of ether lipid synthesis, a pathway essential for the formation of plasmalogens. The latter are phospholipids that carry an unsaturated bond next to the ether bond and that comprise more than 20% of the phospholipids in brain.

Interestingly, patients with severe deficiency of multifunctional protein 2 (MFP2, also known as D-bifunctional protein), a crucial enzyme of peroxisomal β-oxidation [4], also exhibit cortical neuronal migration defects and hypotonia [5]. Unexpectedly, the *MFP2* knockout mouse model [6] does not recapitulate these migration defects despite similar metabolic abnormalities in mice and in patients, i.e. the accumulation of $C_{26:0}$ levels, a direct consequence of inactivity of peroxisomal β-oxidation.

It is also not clear whether intact ether lipid synthesis is necessary for normal cortical migration. In most Rhizomelic Chondrodysplasia Punctata (RCDP) patients no migratory abnormalities were observed [7] but in a recent case report, neuroimaging revealed pachygyria and polymicrogyria [8]. In *Pex7* knockout mice, a model for RCDP type1, a delay in cortical neuronal migration was observed [9]. Metabolically, these mice showed besides a

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profound reduction of plasmalogen levels, an increased concentration of $C_{26:0}$ in brain at birth.

Taking into account the observations in mice and men, it is plausible that neuronal migration defects are caused by the combined deficiency of peroxisomal β-oxidation and ether lipid synthesis. An interaction between these two peroxisomal metabolic pathways was recently reported with regard to myelination defects, based on findings in *Pex7*$^{-/-}$/*Abcd1*$^{-/-}$ double knockout mice [10]. We investigated this possibility by studying cortical neuronal migration in double *DAPAT*/*MFP2* knockout mice that have besides an ether lipid synthesis defect also a defect in peroxisomal β-oxidation and by comparing them with single *DAPAT* [11] and single *MFP2* [6] knockout mice. Cortical neuronal migration was assessed just before birth and hypotonia and survival in the early postnatal period. Unfortunately, the formation of the cerebellum could only be studied in single knockout mice, because of the early death of the double knockouts.

Methods

Mouse breeding

The generation of *DAPAT* knockout mice (in a C57Bl6 background) and *MFP2* knockout mice (in a Swiss Webster background) was published before. In view of the infertility or subfertility of *DAPAT*$^{-/-}$ and *MFP2*$^{-/-}$ mice, these mouse lines had to be crossbred at the heterozygous stage in order to obtain double knockout mice (expected frequency of 1/16 pups) and single knockout mice (expected frequency 1/4 pups). All the other genotypes (single or double heterozygous or wild type) were considered as controls. Mice were genotyped as described before [6,11].

Mice were bred under conventional circumstances in the animal housing facility of the University of Leuven, with a 12 h light and dark cycle and permanent access to standard rodent food and water. All animal experiments were approved by the Institutional Animal Ethical Committee of the University of Leuven.

Cortical neuronal migration analysis

Neuronal migration in the neocortex was studied by cresylviolet staining at E18.5 and by pulse-chase experiments with 5′,3′-bromo-2′-deoxyuridine (BrdU) as described before [12,13]. Injections were performed at E13.5 with 40 mg/kg BrdU (Sigma) and BrdU immunocytochemistry was done with a 1/100 mouse monoclonal antibody (Beckton Dickinson, San Jose, CA). Based on previous studies performed in *Pex5* knockout mice (Baes et al. [13]), the density of BrdU-stained cells was measured in the intermediate zone (prospective white matter) (0.05 mm^2) and cortical plate (0.1 mm^2) and was used as an index of the severity of the migration defect. For each experimental group, cells were counted in 18 different fields (6 mice from 3 different litters, 3 non-adjacent sections from comparable anatomical regions in the frontoparietal area of each right hemisphere). Both the intensely and the weakly labeled cells were counted. To avoid regional and experimental variations in labeling, sections from the different experimental groups were treated simultaneously.

Biochemical analysis

Brain tissue was dissected from anesthetized mice and snap frozen in liquid nitrogen. Bligh and Dyer extracts were used to determine plasmalogen levels by HPLC [14] and DHA levels by GC–MS. For the latter, extract corresponding to 20 nmol of phospholipid, was fortified with 10 nmol tricosanoic acid (IS), dried and treated with 0.5 ml 0.5 N HCl in 90% acetonitrile at 100 °C for 1 h [15]. After adding water, released FA were extracted twice with 1 ml hexane, dried, silylated with N-tert butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert butyldimethylchlorosilane (Pierce)/pyridine (1/1, v/v) and analyzed by GC–MS (Trace GC–MS, Thermo Finnigan; EI^+ mode at 35 eV), equipped with an automated cold-on-column injector and high oven temperature device connected to a BPX70 column (30 m × 0.25 mm; 0.25 μm). Identification and quantification was done as described by [16]. Triglycerides, cholesterol and cholesterylesters were isolated by thin-layer chromatography and analyzed with coupled enzymatic assays as described previously [17–19] except for cholesterylesters and triglycerides that were hydrolyzed chemically (5% 5 M KOH in ethanol; 75 °C; 90 min). VLCFA were quantified by GC–MS as previously described [20].

Statistical analysis

Statistical analyses were performed using the Student's *t*-test and the program Graphpad Prism 3.0 (San Diego, CA, USA). Results are expressed as means ± SEM.

Results

Generation of mice with combined peroxisomal β-oxidation and ether lipid synthesis defects

With the purpose to selectively but completely inactivate both peroxisomal β-oxidation and ether lipid synthesis, we chose to inbreed the *DAPAT* [11] and *MFP2* [6] knockout mouse lines. DAPAT is the first committed enzyme of peroxisomal ether lipid synthesis. *DAPAT* knockout mice display profound plasmalogen deficiency and a severe phenotype in the adult central nervous system [21], but they were not yet characterized in the postnatal period. MFP2, is the central enzyme of peroxisomal β-oxidation that is involved in metabolism of all currently known substrates of this pathway [4]. *MFP2* knockouts display the most severe brain phenotype of all mice with peroxisomal β-oxidation gene knockouts [22].

Macroscopical analysis

Embryos of *DAPAT*$^{+/-}$*MFP2*$^{+/-}$ parents were weighed and genotyped at embryonic day 18.5, the day before birth (Fig. 1A). *MFP2*$^{-/-}$ pups had the same weight as control littermates but *DAPAT*$^{-/-}$ mice and to a larger extent *DAPAT*$^{-/-}$*MFP2*$^{-/-}$ mice were significantly growth retarded. Although only a limited number of pups were analyzed and genotyped, double knockout pups seemed to survive fetal development (10/119 pups, which is close to the expected frequency of 6.3%). This is in line with embryonic survival of the Zellweger mouse models (*Pex5, Pex2, Pex13* knockouts) that exhibit the most drastic peroxisomal impairments [2].

In the early postnatal period, double knockout pups could be distinguished from controls and from single knockouts based on their severe hypotonic appearance (Fig. 1C). They never had milk in their stomachs, were not able to support themselves on their paws and could not move forward. Occasionally, single knockout pups were also hypotonic, but the double knockouts were always more affected. Genotyping between 24 and 48 h after birth revealed a slight underrepresentation of *MFP2*$^{-/-}$ and *DAPAT*$^{-/-}$ mice (respectively, 17 and 16 out of 98 pups, expected frequency 1 in 4) but a complete absence of double knockouts. In another cohort of 169 born pups, no surviving double knockouts were found at P7. At this time point, the body weight of both single knockouts was significantly lower in comparison to controls (Fig. 1B). For *MFP2*$^{-/-}$ mice this postnatal growth impairment is likely due to bile acid synthesis problems leading to steatorrhea [23].