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Improvement of biochemical and behavioral defects in the Niemann–Pick type A mouse by intraventricular infusion of MARCKS

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ABSTRACT

Niemann–Pick disease type A (NPDA) is a fatal disease due to mutations in the acid sphingomyelinase (ASM) gene, which triggers the abnormal accumulation of sphingomyelin (SM) in lysosomes and the plasma membrane of mutant cells. Although the disease affects multiple organs, the impact on the brain is the most invalidating feature. The mechanisms responsible for the cognitive deficit characteristic of this condition are only partially understood. Using mice lacking the ASM gene (ASMko), a model system in NPDA research, we report here that high sphingomyelin levels in mutant neurons lead to low synaptic levels of phosphoinositide PI(4,5)P2 and reduced activity of its hydrolyzing phosphatase PLCγ, which are key players in synaptic plasticity events. In addition, mutant neurons have reduced levels of membrane-bound MARCKS, a protein required for PI(4,5)P2 membrane clustering and hydrolysis. Intracerebroventricular infusion of a peptide that mimics the effector domain of MARCKS increases the content of PI(4,5)P2 in the synaptic membrane and ameliorates behavioral abnormalities in ASMko mice.

Introduction

Patients with types A and B Niemann–Pick disease (NPD) have an inherited deficiency of acid sphingomyelinase (ASM) activity. The clinical features of this disorder vary from the infantile, neurological form, with almost no ASM residual activity that results in death by 3 years of age (type A NPD) to the non-neurological form (type B NPD), where the remaining activity of the enzyme secures brain function allowing patients to survive into adulthood (Schuchman, 2009). Type A NPD patients exhibit profound organomegaly within the first 3 months of life and developmental milestones are rarely achieved, because of a rapid psychomotor decline. The brain is usually atrophic due to cell death, some areas of the white matter display demyelination and profound gliosis has been shown in all brains. As for several lysosomal disorders, the 'golden standard' therapy approach is enzyme replacement therapy (ERT). This therapy has been tested in mice lacking ASM (ASMko), which mimic the symptoms of the human disease. In affected animals, the disease has a rapid neurodegenerative course and death occurs at seven months of age. Atrophy of the cerebellum and marked deficiency of Purkinje cells is evident at early stages (Horinouchi et al., 1995). Intravenous administration of the human recombinant enzyme (rhASM) to ASMko mice significantly rescued the lipid storage in the reticuloendothelial system (RES) in a dose-dependent manner (Miranda et al., 2000). Despite dramatic improvements in the RES there was no effect on the progression of neurological disease and no extended survival was documented after treatment. Gene replacement by intracranial injection of viral vectors expressing the hASM, as well as intraventricular administration of the human recombinant enzyme have also been tested in ASMko mice, alleviating storage pathology in the brain and motor deficits (Bu et al., 2012). However, gene therapy still raises many concerns for human use and alternative strategies to treat the disease have been proposed. In a recent study, Arroyo et al. (2014) showed that certain brain structural, cellular and behavioral defects present in ASMko mice are prevented by enhancing neutral sphingomyelinase activity through the systemic administration of the corticosteroid dexamethasone. These results suggest that the pharmacological, non-genetic, venue of intervention is a potentially simpler strategy to ameliorate CNS-deficits in patients affected by NPDA. Understanding the molecular alterations caused by ASM deficiency in the brain is necessary to find new pharmacological targets. Alterations in sphingolipid content have been linked to pre- and postsynaptic anomalies in ASMko mice (Arroyo et al., 2014; Camoletto et al., 2009). However, little is

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known about the possible involvement of other lipids in synaptic deficits.

The metabolism of the phosphoinositide PI(4,5)P2 is a key event in synaptic plasticity events that underlie learning and memory abilities. Thus, hydrolysis of PI(4,5)P2 by receptor-activated phospholipase C (PLCγ) generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) leading to the release of calcium from intracellular stores and activation of the calcium/calmodulin (CaM) pathway (Bliss and Collingridge, 1993; Inagaki et al., 2000; Fitzjohn and Collingridge, 2002). This in turn induces phosphorylation of the cAMP response element-binding protein (CREB), triggering the expression of genes involved in learning and memory (West et al., 2001). We have recently demonstrated that the protein myristoylated alanine-rich C kinase substrate (MARCKS) is required in the control of PI(4,5)P2 levels at the synaptic plasma membrane and that MARCKS is sufficient to restore PI(4,5)P2 deficits (Camoletto et al., 2009). In the present study we show that part of the synaptic plasticity deficits of ASMko mice could be due to SM-induced alterations in PI(4,5)P2 spatial availability for PLCγ-mediated signaling. Importantly, we show that addition of MARCKS is sufficient to regulate phosphoinositide content at the neuronal plasma membrane, rescuing cognitive defects in ASMko mice.

Materials and methods

Mice

A breeding colony of ASM heterozygous C57BL/6 mice (Horinouchi et al., 1995), kindly donated by Dr. E. Schuchman (Mount Sinai School of Medicine, New York), was established. The experiments were performed by comparing littermates of wild-type (wt) or ASMko mice (4 months of age), which genotype was determined from genomic DNA in a PCR reaction.

ASMko mice were group-housed (four per cage) on a 12-h light/dark cycle. Food and water were available ad libitum. Mice were randomly assigned to experimental groups for all types of experiments. Therapeutic experiments: both groups n = 17 (ko: 9 M–8 F; ko-MARCKS: 10 M–7 F).

All experiments were in accordance with the rules and regulations approved by the Institutional Animal Care and Use Committee of KU Leuven.

SM addition

Sphingomyelin (bovine brain, ≥97.0%; Sigma-Aldrich) was dissolved in ethanol (50 mM stock solution) and added to the neuronal medium at a final concentration of 50 μM. Cells were incubated overnight at 37 °C (Ledesma et al., 1998).

Synaptosomes preparation and treatments

Functional synaptosomes were prepared with an isosmotic Percoll/sucrose discontinuous gradient as previously described (Pilo Boyl et al., 2007; Napoli et al., 2008; Sodero et al.). They were resuspended in HEPES-Krebs buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM KCl, 10 mM glucose, 2 mM $MgSO_4$, and 2 mM $CaCl_2$), equilibrated at 37 °C for 5 min.

Synaptosomes were incubated at 37 °C with SM 50 μM for 30 min in gentle agitation.

Immuno-blotting and antibodies

Proteins separated by PAGE were transferred to nitrocellulose membranes at 100 V for 1 h and probed with specific antibodies, pPLCγ-1 (Cell Signaling), PLCγ-1 (BD Transductions), MARCKS (Santa Cruz, CA). Species-specific peroxidase-conjugated secondary antibodies were subsequently used to perform enhanced chemiluminescence detection (Amersham, Little Chalfont, United Kingdom). The signals were developed using Fuji film LAS-3000 (Fuji). Quantification was performed using the ImageJ software and the AIDA software (Raytest Isotopenmeßgeräte GmbH).

Phosphoinositides measurements

Mass ELISA Kit K-4500 and K-2500s (Gray et al., 2003) from Echelon Biosciences Inc. were used to determine phosphoinositides PI(4,5)P2 and PI(3,4,5)P3 content, respectively. Phosphoinositides were extracted and detected according to the manufacturer's instructions. The values are normalized per μg of proteins.

Immunofluorescence microscopy

To analyze PI(4,5)P2, neurons grown on glass coverslips were washed 2 times in pre-warmed HBSS, treated with 0.005% saponin in MBS buffer for 20 s, washed in PBS and fixed with 4% paraformaldehyde/sucrose. Immunofluorescence was then performed according to the procedure for staining phosphoinositides in cultured cells provided by Echelon Biosciences Inc.

Lipid determination

For a qualitative SM determination, lipid extracts were subjected to a double development (first solvent: chloroform/acetone/acetic acid/methanol/water (50:20:10:10:5) to separate phospholipids; second solvent: hexane/ethyl acetate (5:2)). After drying, the plate was sprayed with 7% sulfuric acid, heated at 150 °C, followed by scanning of the areas corresponding to SM.

Behavioral tests

Pump implantation

MARCKS peptide was infused continuously i.c.v. using Alzet® minipumps and brain infusion kit (Alzet® minipumps 1004, 11 μl/h for 28 days, Brain Infusion Kit 3). MARCKS peptide (Biomatik, Wilmington, Delaware USA) was dissolved in sterile saline (75 μg/ml). Pumps and catheter were filled with either MARCKS or vehicle solution and primed for 48 h at 37 °C. For implantation, mice were sedated (chloral hydrate 50 mg/ml at 1% b.w. i.p.) and locally anesthetized (s.c. xylocaine ~0.05 ml). The cannula was stereotactically placed into the right lateral ventricle (1 mm posterior to bregma, 1 mm lateral to midline, 2 mm from skull surface) and glued to the skull using cyanoacrylate, while the pump was inserted subcutaneously on the back. Pre- and postsurgical analgesia included administration of paracetamol in the drinking water (1.6 mg/ml) for 48 h. Animals were allowed to recover from these surgical procedures for 6 days to avoid influences on subsequent behavioral assessment. These behavioral tests were conducted over the course of 2 weeks during continuous i.c.v. MARCKS treatment.

Accelerating rotarod

Mice were first trained to maintain balance for 2 min at a constant speed of 4 rpm. This training trial was followed by four test trials, during which the rod accelerated from 4 to 40 rpm in 5 min. Consecutive trials were separated by a 10 min intertrial interval. Latency to falling off the rod was recorded up to 5 min.

Grip strength

Grip strength was measured using a device consisting of a 10 cm long, T-shaped bar connected to a digital dynamometer (Ugo Basile, Comerio, Italy). Mice were placed before the bar, which they usually grabbed spontaneously, and gently pulled backwards until they released