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Cholesterol-dependent increases in glucosylceramide synthase activity in Niemann-Pick disease type C model cells: Abnormal trafficking of endogenously formed ceramide metabolites by inhibition of the enzyme

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ABSTRACT

Sphingolipids such as sphingomyelin and glycosphingolipids (GSLs) derived from glucosylceramide (GlcCer), in addition to cholesterol, accumulate in cells/neurons in Niemann-Pick disease type C (NPC). The activities of acid sphingomyelinase and lysosomal glucocerebrosidase (GCase), which degrade sphingomyelin and GlcCer, respectively, are down-regulated in NPC cells, however, changes in GlcCer synthase activity have not yet been elucidated. We herein demonstrated for the first time that GlcCer synthase activity for the fluorescent ceramide, 4-nitrobenzo-2-oxa-1,3-diazole-labeled C6-ceramide (NBD-ceramide) increased in intact NPC1$^{(-/-)}$ cells and cell lysates without affecting the protein levels. In NBD-ceramide-labeled NPC1$^{(-/-)}$ cells, NBD-fluorescence preferentially accumulated in the Golgi complex and vesicular specks in the cytoplasm 40 and 150 min, respectively, after labeling, while a treatment for 48 h with the GlcCer synthase inhibitors, *N*-butyldeoxynojirimycin (NB-DNJ) and 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, accelerated the appearance of vesicular specks emitting NBD-fluorescence within 40 min. The treatment of NPC1$^{(-/-)}$ cells with NB-DNJ for 48 h additionally increased the levels of cholesterol, but not those of sphingomyelin. Increases in the activity of GlcCer synthase and formation of vesicular specks emitting NBD-fluorescence in NPC1$^{(-/-)}$ cells were dependent on cholesterol. LacCer taken up by endocytosis, which accumulated in the Golgi complex in normal cells, accumulated in vesicular specks after 10 and 40 min in NPC1$^{(-/-)}$ cells, and this response was not accelerated by the NB-DNJ treatment, but was restored by the depletion of cholesterol. The cellular roles for enhanced GlcCer synthesis and increased levels of cholesterol in the trafficking of NBD-ceramide metabolites in NPC1$^{(-/-)}$ cells have been discussed.

1. Introduction

Most cases of Niemann-Pick disease type C (NPC) result from mutations in the *NPC1* gene encoding the NPC1 protein, which is involved in the transport and/or processing of unesterified cholesterol. In addition to cholesterol, sphingolipids including sphingomyelin (SM) and glycosphingolipids (GSLs) such as glucosylceramide (GlcCer), lactosylceramide (LacCer), and gangliosides accumulate in the late endosomes/lysosomes of cells in the livers and spleens of NPC patients and animal models (Vanier, 1983, 2010; Mukherjee and Maxfield, 2004; Vance and Karten, 2014). GlcCer levels are known to be markedly higher than those of LacCer and gangliosides in the liver and spleen, and, among GSLs, GlcCer is

Abbreviations: NPC, Niemann-Pick disease type C; SM, sphingomyelin; GSLs, glycosphingolipids; GlcCer, glucosylceramide; LacCer, lactosylceramide; GCase, glucocerebrosidase; NB-DNJ, *N*-butyldeoxynojirimycin; NPC1$^{(-/-)}$, NPC1-null; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; NBD-ceramide, 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine; BODIPY-LacCer, boron dipyrromethene difluoride-labeled lactosylceramide; FBS, fetal bovine serum; LPDS, lipoprotein-deficient serum; PPMP, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; HPβCD, 2-hydroxypropyl-β-cyclodextrin; CHO, Chinese hamster ovary; NBD-specks, specks emitting NBD-fluorescence; NB-DGJ, *N*-butyldeoxygalactonojirimycin.

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stored at high levels in these tissues in NPC patients, 10–40-fold more than those in controls (Vanier, 2010, 2015). Cellular levels of GlcCer are directly controlled by the activities of GlcCer synthase and glucocerebrosidases (GCases, β-glucosidases). Complex GSLs derived from GlcCer are the main storage lipids in the brains of NPC patients and animal models, whereas the accumulation of cholesterol and SM is limited (Elleder, 1989; Vanier, 1999, 2010; Zervas et al., 2001a; Taniguchi et al., 2001; Stein et al., 2012). GSLs are essential components of the pre- and post-synaptic machineries regulated by neurons and glial cells in the brain. A treatment with *N*-butyldeoxynojirimycin (NB-DNJ, miglustat, BRAZAVES®, an inhibitor of GlcCer synthase) has been shown to decrease the accumulation of gangliosides in the brain, such as in the cerebral cortex and cerebellum, delay the onset of neurological dysfunction, and increase the average life span of NPC patients and animal models (Zervas et al., 2001b; Patterson et al., 2010; Stein et al., 2012). Thus, changes in GSL levels appear to be involved in neuronal dysfunction in NPC; however, the exact roles of GSLs in the neuropathology of NPC currently remain unknown (Lee et al., 2014; Marques et al., 2015). The accumulation of complex GSLs in NPC cells has been, at least partially, attributed to decreases in the activity of lysosomal GCase (Besley and Moss, 1983; Salvioli et al., 2004). In contrast, a recent study reported that the activity and expression of a non-lysosomal GCase were increased in the brains of *NPC1*$^{-/-}$ mice (NPC1-null mice, Marques et al., 2015); therefore, the roles of GCase in the accumulation of GSLs including GlcCer in NPC cells have yet to be determined. To the best of our knowledge, changes in GlcCer synthase activity have not been examined in NPC cells.

The NPC1 protein is essential for the transport of cholesterol from late endosomes/lysosomes to the endoplasmic reticulum. In NPC1$^{(-/-)}$ cells cultured with normal serum, cholesterol accumulated in various endosomes including late and recycling endosomes (Salvioli et al., 2004; Wojtanik and Liscum, 2003; Pipalia et al., 2007; Sztolsztener et al., 2012). Furthermore, NPC1 and cholesterol have been reported to regulate vesicular trafficking for the sorting of cargo including GSLs bi-directionally between late endosomes/lysosomes, and the recycling of cargo to various cellular organelles including the plasma membrane via many types of endosomes (Puri et al., 1999; Choudhury et al., 2002; Cheng et al., 2006). However, the effects of NPC1 and cholesterol on the transport of sphingolipids such as SM and GlcCer endogenously formed in the Golgi complex have not been elucidated in detail. In addition, the effects of the inhibition of GlcCer synthase on the trafficking of sphingolipids formed in the Golgi complex in NPC1$^{(-/-)}$ cells remain unknown. In the present study, we focused on changes in GlcCer synthase activity in NPC1$^{(-/-)}$ cells and the trafficking of 4-nitrobenzo-2-oxa-1,3-diazole (NBD)-fluorescence derived from the metabolites of NBD-labeled C6-ceramide (NBD-ceramide) in NPC1$^{(-/-)}$ cells treated with and without GlcCer synthase inhibitors. We demonstrated for the first time that the formation of NBD-GlcCer was enhanced in cells and cell homogenate without changing the protein levels of GlcCer synthase in NPC1$^{(-/-)}$ cells, suggesting an upregulation of synthase activity. The possible cellular roles for enhanced GlcCer synthesis and the effects of GlcCer synthase inhibitors on the trafficking of ceramide metabolites in NPC1$^{(-/-)}$ cells have been discussed.

2. Materials and methods

2.1. Materials

The materials used in this study and their sources were as follows: NBD-ceramide from Molecular Probes (Eugene, OR, USA); boron dipyrromethene difluoride-labeled LacCer (BODIPY-LacCer) and BODIPY-TR-ceramide were from Invitrogen (Carlsbad, CA); fetal bovine serum (FBS) from Thermo Trace Ltd. (Nobel Park, Australia); lipoprotein-deficient serum (LPDS) from fetal calf, cholesterol and conduritol B-epoxide were from Sigma-Aldrich (St. Louis, MO); NB-DNJ hydrochloride was from Enzo Life Sciences (Farmingdale, NY); U18666A from Cayman Chemical (Ann Arbor, MI); 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP, Biomol, Plymouth Meeting, PA). NB-DNJ hydrochoride, U18666A, and PPMP were used at similar concentrations as those in previous reports including ours (Platt et al., 1994; Delgado et al., 2006; Tada et al., 2010; Nakamura et al., 2012). NBD-ceramide and PPMP were dissolved in dimethyl sulfoxide, and the reagents were diluted in medium prior to experiments. The final concentration of dimethyl sulfoxide in culture dishes was less than 0.5%, and the vehicle had no effect on the NBD-ceramide metabolism or trafficking of NBD-fluorescence derived from metabolites. For preparing a complex of cholesterol and 2-hydroxypropyl-β-cyclodextrin (HPβCD), cholesterol dissolved in chloroform:methanol (2:1) was dried under nitrogen. An appropriate volume of 100 mM HPβCD was added to the dried film at a molar ratio of 10:1 (HPβCD:cholesterol) and vortexed to suspend the film. Suspensions were bath sonicated for 20 min, then incubated at 37 °C with shaking. Finally, suspensions were filtered through 0.2 μm filters to clarify solutions.

2.2. Cell cultures

A control Chinese hamster ovary (CHO) cell line (CHO–K1 cells named JP17 cells) and cells lacking NPC1 (NPC1$^{(-/-)}$ cells named A101 cells) were established and kindly provided by Professor H. Ninomiya (Tottori University). These two cell lines have been shown to be a useful tool to study the regulation of cellular cholesterol homeostasis and the pathogenesis of NPC (Higaki et al., 2001; Sugimoto et al., 2001). Cells were cultured in Ham's F12 medium containing 10% FBS as described previously (Nakamura et al., 2012). In order to measure NBD-ceramide metabolites in cells, control and NPC1$^{(-/-)}$ cells in 6-well plate at approximately 50–60% confluence were further cultured in Ham's F12 medium with 10% FBS or 10% LPDS for 1 or 2 days. In some experiments, 2 μM U18666A was further supplemented in the medium. Regarding fluorescence microscopy in living cells, cells were seeded on the cover slips (12 mm in diameter) of glass-bottomed dishes (Iwaki, Tokyo, Japan), and images of fluorescence derived from NBD-ceramide and BODIPY-LacCer were examined when cells achieved 60% confluence.

2.3. Measurement of NBD-ceramide metabolites in cells

Cells were labeled with 10 μM NBD-ceramide at 37 °C for 30 min. Labeled cells were washed with NBD-ceramide-free Hank's solution, and further incubated at 37 °C for 10 min or 120 min with Ham's F12 medium. Ceramide metabolites were extracted by the addition of chloroform and methanol, and were analyzed on a TLC silica gel-60 plate (#105724, Merk, Darmstadt, Germany) using 1-butanol:acetic acid:water (3:1:1) as the mobile phase, as described previously (Tada et al., 2010; Makiyama et al., 2015) with minor modifications. For a quantitative analysis, various amounts of standard NBD-ceramide (0.1–4 pmol) were spotted in the upper area of the plate after separation by TLC. The fluorescence intensity was linear to 10 pmol NBD-ceramide, as previously reported (Tada et al., 2010). We previously confirmed that more than 80% of NBD-fluorescence was native NBD-ceramide and approximately 5–10% of NBD-fluorescence was derived from NBD-SM and NBD-GlcCer, respectively, 30–40 min after NBD-ceramide labeling in various cells including CHO cells (Tada et al., 2010; Makiyama et al., 2015). The levels of NBD-labeled molecules in cells 150 min after labeling were as follows; 40–55% in NBD-GlcCer, 20–25% in NBD-SM,