Molecular and Cellular Neuroscience 89 (2018) 42–48

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Anti-GM1 ganglioside antibodies modulate membrane-associated sphingomyelin metabolism by altering neutral sphingomyelinase activity☆

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ARTICLE INFO

Keywords:
Guillain-Barré syndrome (GBS)
Anti-GM1 antibody
Neutral sphingomyelinase
Sphingomyelin
Ceramide

ABSTRACT

Previous studies have shown that patients with Guillain-Barré syndrome express autoantibodies against ganglioside GM1 (GM1), although its pathogenic significance for the development of the disease remains to be elucidated.

nSMase2 is the best characterized neutral sphingomyelinase (nSMase) found in neuronal cells. Activation of this enzyme leads to ceramide production, which is a known second messenger of the cell-death program in neuronal cells.

We have explored the effects of anti-GM1 antibodies on sphingomyelin metabolism of PC12 cells stably transfected with human *trk* cDNA (PCtrk cells) by determining their effects on nSMase2 activity. The data we present here strongly suggest that anti-GM1 caused a significant change in sphingomyelin content of the membrane fraction in PCtrk cells. Both nSMase2 activity and the level of nSMase2 protein were significantly decreased by anti-GM1 treatment of PCtrk cells, while acidic SMase activities remained unchanged.

Our results indicate, for the first time, that anti-GM1 may produce profound impacts on lipid metabolism in neuronal cell membranes.

1. Introduction

Guillain-Barré syndrome (GBS) is an autoimmune peripheral neuropathy for which autoantibodies to a variety of glycosphingolipids, especially acidic glycosphingolipids and gangliosides, have been detected in patients' sera. Notably, many patients with the axonal form of GBS or acute motor axonal neuropathy (AMAN) have been reported to exhibit autoantibodies against ganglioside GM1 (GM1) (Yuki et al., 1990; Visser et al., 1995; Kaida et al., 2000), although the molecular mechanisms of these autoantibodies underlying development of the disease phenotype remain unknown. We previously found that anti-GM1 antibodies (anti-GM1) provoke a change in the localization of Trk neurotrophin receptors, which are essential for neuronal survival and differentiation, from the detergent-resistant membranes (DRMs) fraction to the soluble fraction (Ueda et al., 2010). Moreover, anti-GM1 inhibits nerve growth factor (NGF)-induced Trk autophosphorylation (Ueda et al., 2010). The DRMs fraction does not always mean as lipid rafts or a liquid-ordered lipid phase (Kenworthy et al., 2004; Lichtenberg et al., 2005; Magee et al., 2005). Lipid rafts are thought to form membrane micro-domains in vivo with distinct compositions of lipids and proteins, such as cholesterol, glycosphingolipids, sphingomyelin, and signaling molecules such as receptor-type tyrosine kinase (e.g., Trk). These rafts serve as platforms for protein segregation and signaling, and they can be extracted from plasma membranes by the following standard method. Extraction takes advantage of the resistance of lipid rafts towards non-ionic detergents, such as 1% Triton X-100, at the low temperatures (e.g., 4 °C) employed in our past studies (Mutoh et al., 2000; Ueda et al., 2010).

Sphingomyelin (SM) hydrolysis by sphingomyelinase (SMase) to yield ceramide (Cer) is a major, rapid source of ceramide production in response to various conditions, such as hypoxia, oxidative stress, ultraviolet irradiation, and inflammation (Cogolludo et al., 2009; Caricchio et al., 2003; Levy et al., 2006), although Cer is also a rate-limiting substrate for the synthesis and the metabolite of many glycosphingolipids subspecies. SMases can be divided into several subtypes according to their pH optima and subcellular localization, such as neutral sphingomyelinases (nSMases), which localize to the plasma membrane, and acid sphingomyelinases (aSMases), which localize to

☆ Footnote: anti-GM1, anti-GM1 antibodies; anti-nSMase, anti-neutral sphingomyelinase antibody; anti-Na-K ATPase, anti-Na-K ATPase antibody; GM1, ganglioside GM1; GBS, Guillain-Barré syndrome; NGF, nerve growth factor.

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https://doi.org/10.1016/j.mcn.2018.03.012
Received 9 August 2017; Received in revised form 24 January 2018; Accepted 25 March 2018
Available online 27 March 2018
1044-7431/

endosomal and lysosomal compartments (Pavoine and Pecker, 2009; Wu et al., 2010). Among the nSMase subtypes, nSMase2 is considered an important candidate for ceramide production in the neural membrane (Liu et al., 1988; Haughey et al., 2010).

Heretofore, nSMase2 activity has often been studied in glial cells such as astrocytes and oligodendrocytes, and has been implicated as a positive signal transducer for neuronal death by ceramide production in glial cells. Mounting evidence already indicates that the formation of ceramide from sphingomyelin is implicated in apoptotic cell death (Gu et al., 2013; Testai et al., 2004). A recent study, however, suggested that cell viability promotion by NGF, as demonstrated on PC12 cells, shows that neurotrophin-exerted cell viability is dependent on nSMase activity in neuronal cells. On the other hand, p75 low affinity NGF receptor (p75LNGFR), originally known as a pro-apoptotic signal transducer when expressed alone in the absence of Trk receptors, is known to be required for nSMase2 activation to induce apoptosis (Brann et al., 2002). Taken together, these previous data indicate that activation of the p75LNGFR in response to NGF promotes an apoptotic nSMase2-dependent signal, while neurotrophin activation of the Trk receptors promotes a pro-survival nSMase2-dependent signal. Thus, nSMase2 can provide either pro- or anti-apoptotic signals depending on their local environments and the cell type (Candalija et al., 2014).

In this study, we explored the effects of anti-GM1 on membrane SM metabolism and nSMase2 activities to determine their biological and other impacts on the PCtrk neuronal cells.

2. Materials and methods

2.1. Cell culture and treatment

Rat pheochromocytoma-derived PCtrk cells were established by the stably transfection with human *trk*-complimentary DNA into parental PC12 cells and were cultured as previously described (Mutoh et al., 2000). The cells were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) containing 7.5% fetal bovine serum (FBS) and horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mutoh et al., 1995, 1998). Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. We used rabbit anti-GM1 (Calbiochem, La Jolla, CA. anti-Ganglioside GM1 Rabbit pAb Cat. No. 345757) in this study.

2.2. Subcellular fractionation

To determine the cellular distribution of nSMase2, subcellular fractionation was performed as described previously (Nakagawa et al., 1998; Hamano et al., 2005). Cells were suspended in homogenization buffer (40 mM Tris-HCl pH 7.4, 250 mM sucrose, 1 mM PMSF, 4 mM CaCl2, and 20 mM MgCl2) and homogenized with a Dounce homogenizer with 10 strokes. Homogenates were centrifuged at 700*g* for 5 min at 4 °C. The resulting pellets were considered a nuclear fraction that contains nuclei and cell debris. The supernatant was further centrifuged at 305,400*g* for 35 min using a TLA-110 rotor in an Optima MAX-XP Ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 4 °C. The resultant pellet was considered a membrane fraction and the supernatant, a soluble cytosolic fraction. Membrane and cytosolic fractions were subjected to an nSMase2 activity assay. The membrane fraction was also subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. We compared the amount of Trk protein in the membrane fraction generated by subcellular fractionation of whole crude cell homogenate. As a result, we determined that the total recovery of proteins along the membrane fraction was 5.2%.

2.3. Lipid analysis

Lipids analysis of cultured cells was performed as previously reported (Mutoh et al., 2012). Briefly, PCtrk cells were subjected to crushing under liquid nitrogen, and total lipid extracts (TLEs) were prepared from the crushed cells using tetrahydrofuran/H_2O (4:1). The protein content was normalized using the pellet. The neutral glycosphingolipid (NGL) fraction was isolated from one portion of the TLE for further purification. NGLs were subjected to alkaline methanolysis, and purified with methanol and hexane. NGLs were applied to HPTLC plates, and developed with chloroform/methanol/H_2O (20:7.3:1, v/v/v). These HPTLC plates were stained with anisaldehyde reagent (50 ml acetic acid/1 ml concentrated H_2SO_4/0.5 ml p-anisaldehyde). The aqueous fraction (AF) includes various gangliosides separated from TLE. AF was subjected to the alkaline methanolysis followed by dialyzation against distilled water for four days, lyophilized, and applied to a high-performance thin-layer chromatography plate (HPTLC). HPTLC was developed with chloroform/methanol/0.02% $CaCl_2$ (25:25:3, v/v/v), and stained with resorcinol reagent (10 ml 6% resorcinol/80 ml concentrated HCl/0.25 ml 0.1 M $CuSO_4$/9.75 ml distilled water). To quantify the density of these bands, we used Image J software.

2.4. SMase activity assay

Neutral and acidic SMase activities were determined using the Amplex ® Red Sphingomyelinase Assay Kit (Invitrogen, Carlsbad, CA, USA). In this enzyme-coupled assay, sphingomyelinase activity was monitored indirectly using 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex® Red reagent), a sensitive fluorogenic probe for H_2O_2 (Zhou et al., 1997). First, sphingomyelinase hydrolyzed the sphingomyelin to yield ceramide and phosphorylcholine. After the action of alkaline phosphatase, which hydrolyses phosphorylcholine, choline was oxidized by choline oxidase to yield betaine and H_2O_2. Finally, H_2O_2, in the presence of horseradish peroxidase, reacted with Amplex® Red reagent at 1:1 stoichiometry to generate the highly fluorescent product resorufin (Zhou et al., 1997; Mohanty et al., 1977). The reactions were incubated for 60–120 min at 37 °C, protected from light. The fluorescence was monitored at different times using 550 nm excitation and 590 nm emission in a 2030 ARVO X multi label reader (PerkinElmer, Waltham, MA). SMase activity was calculated from the slope of an intrinsic fluorescence vs. time graph and standardized per mg of protein (mU/h/mg protein).

2.5. Immunoblot analysis

Proteins in the membrane fraction were subjected to SDS-PAGE followed by transfer to polyvinylidene difluoride (PVDF) membranes for immunoblotting. In order to determine the alteration of nSMase2 protein semiquantitatively, nSMase2 was detected by probing with anti-nSMase2 antibodies (anti-nSMase2) (N-SMase2 G-6: sc-166637; Santa Cruz Biotechnology, Dallas, TX, USA). The PVDF membranes were re-probed after stripping with anti-Na, K-ATPase antibody #3010 (Cell Signaling Technology, Danvers, MA, USA) (anti-Na-K ATPase) to determine the amount of input protein in each membrane fraction.

2.6. Preparation of DRMs fraction with sucrose density gradient ultracentrifugation

We prepared DRMs fraction from PCtrk cells as described previously (Mutoh et al., 2000). The cells were collected in phosphate-buffered saline containing *para*-nitrophenyl phosphate and homogenized using a Teflon glass homogenizer in TNE/Triton X-100 buffer (1% Triton X-100, 25 mM Tris-HCl, PH 7.5, 150 mM NaCl, 1 mM EGTA). After centrifugation at 3300*g* for 5 min at 4 °C, the lysates were normalized for protein content and were brought to 1.5 M sucrose. A discontinuous sucrose gradient (1.2 M, 8.5 ml; 0.15 M, 2.5 ml) in TNE buffer without Triton X-100 was layered over the lysates. Gradients were centrifuged at 55,000*g* for 18 h at 4 °C using an SW 32 Ti rotor and an Optima LE-80 K Ultracentrifuge (Beckman Coulter) as described previously (Mutoh et al., 2000; Ueda et al., 2010). One-milliliter fractions and the pellet