Biochemical Pharmacology 82 (2011) 287–294

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

The effect of sphingomyelin synthase 2 (SMS2) deficiency on the expression of drug transporters in mouse brain

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ARTICLE INFO

Article history:
Received 26 February 2011
Accepted 19 April 2011
Available online 30 April 2011

Keywords:
Sphingomyelin synthase 2
Drug transporters
Brain
ERM proteins

ABSTRACT

Sphingomyelin synthase (SMS), the last enzyme involved in the biosynthesis of sphingomyelin (SM), plays a critical role in the constitution of cell membrane and has impact on the expression of membrane proteins. SMS2, one of two SMS enzymes, is predominantly located in the plasma membrane, and is mainly expressed in the brain. Therefore, it is conceivable that SMS2 deficiency may have impact on expression of some membrane proteins, such as membrane-bound drug transporters. Using SMS2 gene deficient mouse brain tissues, we studied the gene and protein expression profiles of drug transporters, ERM proteins (ezrin/radixin/moesin) and the cytoskeleton protein, β-actin, in mouse brain by RT-PCR, western blot and immunohistochemistry analysis. We found that the mRNA expression of Mdr1 rather than the other drug transporters was significantly decreased in the SMS2 deficient brain. Accordingly, the expression and the function of Pgp (Mdr1/P-glycoprotein) were significantly downregulated in brain. In addition, the substantially downregulated expression of ezrin and β-actin was also observed in the SMS2 deficient brain. The immunohistochemistry analysis further revealed the suppressed expression of Pgp, ezrin and β-actin in both cortex and paraventricular areas of SMS2 knockout mice. Furthermore, both Pgp and β-actin were found to be co-immunoprecipitated with ezrin from the total brain lysate, suggesting the association between Pgp, ezrin and β-actin in the brain. These results indicate that SMS2 participates in the expression regulation of drug transporters, particularly Pgp, and suggest that SMS2 may be a potential target for enhancing drug access to the brain.

1. Introduction

In recent years, there has been remarkable progress in determining the pharmacological and toxicological impacts of drug transporters, which play an important role in modulating drug absorption, distribution, and elimination [1]. Drug transporters, a special family of membrane proteins, belong to two superfamilies of SLC (solute carrier) and ABC (ATP-binding cassette). While SLC transporters, characterized by 12 putative transmembrane domains, are driven by an electrochemical gradient of inorganic (or organic) solutes; ABC transporters, comprised of homologous ATP-binding and large multispanning transmembrane domains, are powered by ATP hydrolytic energy [2].

Understanding the regulation of drug transporter expression has become of great help in predicting pharmacokinetics. Recent results show that altered drug transporters expression occurs in response to signals that activate specific transcription factors, including pregnane-X receptor (PXR), constitutive androstane receptor (CAR), nuclear factor-κB and activator protein-1 [3–5]. Several other studies have demonstrated that the expression of transporters is affected by cellular stress and stress responses induced by various chemical substances and pathophysiological conditions [6–9]. Being integral membrane proteins, drug transporters are dependent on their lipid environment for their expression and optimal functions [10]. However, the effect of membrane lipids on drug transporters expression has been remained relatively unexplored.

Sphingomyelin (SM), one of the major lipids on the plasma membrane, plays an important role in determining the physical properties and biological functions of cellular membranes [11]. The last step of SM biosynthesis is catalyzed by sphingomyelin synthase (SMS) that has two isoforms of SMS1 and SMS2, which are located at golgi apparatus and plasma membrane, respectively [12]. Recent report has revealed that SMS2 deficiency inhibits sphingomyelin synthesis; thus SMS2 deficiency changes intracellular sphingomyelin accumulation and plasma membrane lipid organization [13]. SMS2 deficiency is also found to be responsible

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doi:10.1016/j.bcp.2011.04.009

to significantly upregulated protein expression of ABC transporters of ABCA1 and ABCG1 in macrophage [14]. On the other hand, transporter ABCA2 deficiency caused abnormalities in the metabolism of SM in mouse brain [15]. However, the effect of SM/SMS2 on the expression of drug transporters in mouse brain has not been established.

There are increasing evidences showing that membrane-cytoskeletal linking proteins of ERM (ezrin, radixin and moesin) and the cytoskeleton protein, β-actin, are involved in the regulation of drug transporters [16,17]. The ERM deficiency was reported to be responsible to the expression and localization changes of drug transporters, such as ABCC2 in both animals and human colorectal carcinoma Caco-2 cells [18,19].

Given the brain is the organ in which the main activity of SMS and the predominant expression of SMS2 are found [12], we choose SMS2 deficient (SMS2 knockout and heterozygous) and wild type mouse brains to investigate the effect of SMS2 deficiency on drug transporters. We studied the gene expression profiles of some drug transporters, and examined the protein expression and the function of Pgp (Mdr1/P-glycoprotein) which showed significant change on its mRNA level. We also studied possible association between Pgp, ERM and the β-actin in the brain.

2. Materials and methods

2.1. Ethics statement

The Ethics Committee for Animal Experiments of Fudan University approved all animal work (permit number: SYXK 2007-0002) and the experimental protocols strictly complied with the institutional guidelines and the criteria outlined in the "Guide for Care and Use of Laboratory Animals".

2.2. Animals

Original SMS2 knockout (KO) mice were donated by Prof. XC Jiang at the Department of Cell Biology, State University of New York Downstate Medical Center, Brooklyn, and were maintained at Fudan University. The establishment of SMS2 KO mice had been previously reported by Hailemariam et al. [20]. The abbreviations for wild type, heterozygous, and SMS2 KO mice are WT, SMS2+/−, and SMS2−/−, respectively. The animals used in this study were 10–12-week-old littermates. The tissues were frozen on liquefacient nitrogen and stored at −80 °C until further use.

2.3. Reagents

SMase (S-8889), alkaline phosphatase (P5521), choline oxidase (C5896), peroxidase (P1432) and 4-aminoantipyrine (A4382) as well as phosphatidylcholine (P6638), NBD-C6-ceramide (N8278) were purchased from Sigma (St. Louis, MO, USA). DAOS (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, sodium salt) was purchased from Dojindo Molecular Technologies, Inc. in USA. Rhodamine123 was purchased from J&K Chemical in China. Other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) or from Invitrogen (Carlsbad, CA, USA), and were of analytical grade. Antibodies were obtained from the following sources: monoclonal mouse anti-P-glycoprotein C219 antibodies from Abcam, Cambridge, UK; ACTB antibody from ProteinTech Group, USA; monoclonal anti-ezrin antibody from Thermo, USA; moesin Ab-1 (Clone 38/87) monoclonal antibody from Lab Vision, USA; monoclonal anti-GAPDH antibody from Beyotime, China; and secondary antibody labeled with alkaline phosphatase from Vecter Laboratories Inc., USA.

2.4. SMS activity assay

SMS activity was measured as described previously [21,14]. Brain tissues were homogenized in a buffer containing 50 mM Tris–HCl, 1 mM EDTA, 5% sucrose, and a cocktail of protease inhibitors (Sigma, USA). The homogenate was centrifuged at 5000 rpm for 10 min and the supernatant was mixed in assay buffer containing 50 mM Tris–HCl (pH 7.4), 25 mM KCl, NBD-C6-ceramide (0.1 μg/μL), and phosphotidylcholine (0.01 μg/μL). The mixture was incubated at 37 °C for 2 h. Lipids were extracted in $CHCl_3$:MeOH (2:1), dried under nitrogen gas, and separated by thin layer chromatography (TLC) using $CHCl_3$:MeOH:NH_4OH (14:6:1). The plate was scanned with a PhosphorImager, and the intensity of NBD-SM band was measured by Gel-Pro Analyzer 6.0 (MediaCybernetics, Inc.).

2.5. SM measurement

Mouse brain tissues (100 mg) were homogenized in 1 mL of 1 N NaOH, and the total lipids were extracted twice from the tissues with 1 mL of a $CHCl_3$:MeOH (2:1) solution. The organic layer was dried under nitrogen gas and resolubilized in 0.2 mL of $CHCl_3$ containing 2% Triton X-100. The lipid extract was dried and resuspended in 0.2 mL of water to achieve a final concentration of 2% Triton X-100. Prior to the assay for SM measurement, an enzyme solution was prepared in 50 mL of reaction buffer (Tris–HCl 0.05 M with calcium chloride 0.66 mM, pH 8) to the final concentrations of SMase 25 U, Alkaline phosphatase 500 U, Choline oxidase 25 U, Peroxidase 1000 U, DAOS 0.73 mM and 4-aminoantipyrine 0.73 mM. To 100 μL reaction buffer added 10 μL of the lipid extracts. After the reaction at 37 °C for 45 min, the absorption was measured at 595 nm on a spectrophotometric plate reader (Thermo scientific, USA) [22,23].

2.6. Reverse transcriptase-polymerase chain reaction analysis

Total RNA was isolated from the whole brain tissue using Trizol reagent (Invitrogen, Japan) according to the manufacturer's instructions. Subsequently, reverse transcription was performed using 2 μg of the RNA in a final reaction mixture (20 μL) containing random primers (12.5 ng, Invitrogen, USA), RNase inhibitor (RNaseOUT, 20 U, Takara, Japan), 0.5 mM deoxynucleotides (dNTPs, Promega, WI, USA), and 100 U of RNA reverse transcriptase (ReverTra Ace, TOYOBO, Japan). The target genes of Mdr1, Mrp1, Mrp2, Mrp3, Mrp4, Bcrp, Oatp1, Oatp2, β-actin, ezrin, radixin, and moesin as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified with specific primers (Table 1). PCR amplifications were conducted under the following conditions: denaturation at 96 °C for 60 s, annealing at 54–60 °C for 60 s (depending on the primer), and elongation at 72 °C for 60 s (30 cycles). The PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining.

To calculate the percentage changes of $mRNA_{SMS2+/-}$ or $mRNA_{SMS2-/-}$ relative to $mRNA_{WT}$ in mouse brains: the optical density of target genes was normalized with the amount of housekeeping gene GAPDH.

$$\%\ \mathrm{mRNA_{Target}} = 100\% \left\{ \frac{(\mathrm{mRNA_{Target}}/\mathrm{mRNA_{GAPDH}})_{\mathrm{SMS2\ Deficient}}}{(\mathrm{mRNA_{Target}}/\mathrm{mRNA_{GAPDH}})_{\mathrm{WT}}} \right\}$$

2.7. Western blot analysis

Whole brain tissues were resuspended in RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% nonidet P-40,