Biochemical and Biophysical Research Communications 500 (2018) 597–602

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

The cross roles of sphingosine kinase 1/2 and ceramide glucosyltransferase in cell growth and death

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ARTICLE INFO

Article history:
Received 11 April 2018
Accepted 13 April 2018
Available online 23 April 2018

Keywords:
Sphingosine kinases 1 and 2
Sphingosine
S1P
Ceramide
Ceramide glucosyltransferase
Cell cycle
Anti-cancer

ABSTRACT

Sphingosine-1-phosphate is synthesized by two sphingosine kinases, cytosolic SK1 and nuclear SK2 but SK2 expression was much higher than SK1in mouse skin fibroblasts. However, in SK2$^{-/-}$ cells, SK1 expression was markedly increased to SK2 levels whereas in SK1$^{-/-}$ cells, SK2 expression was unaffected. Ceramide, glucosylceramide and sphingosine levels were all increased in SK1$^{-/-}$ but less so in SK2$^{-/-}$ cells and S1P levels were not significantly reduced in either SK1$^{-/-}$ or SK2$^{-/-}$ cells. Greatly increased Ceramide glucosyltransferase expression was observed in SK1$^{-/-}$ cells but less so in SK2$^{-/-}$ cells suggested a role in drug resistance. SK2$^{-/-}$ cells grew faster than control and SK1$^{-/-}$. The cell division gene PCNA was significantly overexpressed in SK2$^{-/-}$ cells, suggesting a cross regulation between SKs and Ceramide glucosyltransferase.

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1. Introduction

Bioactive sphingolipids such as Sphingosine 1-phospahate (S1P) have demonstrably important physiological functions in the regulation of cell growth, proliferation and differentiation [1]. S1P is a derived from sphingosine by SK1 and SK2. Both kinase isoforms have been characterized and shown to have distinctly different biological functions [2,3]. Previous studies demonstrated that the expression of sphingosine kinase in fibroblasts significantly increased S1P levels and DNA synthesis, promoted cell growth in low-serum media and increased the proportion of cells in the S phase of the cell cycle with increased cell numbers. Subsequent studies have demonstrated that overexpression of SK1 enhances cell survival and growth whereas SK2 suppressed cell growth and promoted apoptosis [4–6]. When SK1 and SK2 knockout mice were generated, they were found to be fertile and normal by several criteria. However, the simultaneous deletion of both enzymes led to a total deficiency of S1P and embryonic lethality, a marked increase in apoptosis was seen resulting in defective neurogenesis, angiogenesis and failure of neural tube closure, suggesting that SK1 and SK2 could be compensating for the deficiency of another in vivo [7].

SK1 is believed to be activated by extracellular signal regulated kinase (Erk) – dependent phosphorylation at Serine-225, leading to its translocation from the cytosol to the plasma membrane and responsible for cell growth [8]. In contrast SK2 is present in nuclei and mitochondria [6]; less is known of its activation [8] although there some reports of its activation by Erk1 and protein kinase D [8]. SphK2 produces S1P that specifically binds to histone deacetylases HDAC1/2 and inhibits their enzymatic activities, linking sphingolipid metabolism and S1P in the nucleus to epigenetic regulation of gene expression [9]. To further clarify the interplay of two sphingosine kinase and their functions we derived cell lines of skin fibroblasts from these mice and investigated the role of these two isoforms. We now report that SK1$^{-/-}$ mouse skin fibroblasts showed significant increased glucosylceramide (GlcCer) compared to WT; but for SK2$^{-/-}$ cells which expressed SK1, only minor increases in GlcCer were observed. This not only implied that the critical step in ceramide glycosylation is regulated by SK1/2, but

Abbreviations: SK1, sphingosine kinases 1; SK2, sphingosine kinases 2; PCNA, proliferating cell nuclear antigen; GSL, glycosphingolipids; Cer, Ceramide; Sph, sphingosine; S1P, sphingosine-1-phosphate; HDAC, histone deacetylases; GlcCer, glucosylceramide; CGlcT, ceramide glucosyltransferase; MU, 4-Methylumbelliferone; ROME, (R)-FTY720 methyl ether; HPTLC, high performance thin-layer chromatography; ERK, extracellular signal regulated kinase.

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https://doi.org/10.1016/j.bbrc.2018.04.110
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also indicated that SK1 is the key enzyme in the regulation of GlcCer synthesis. SK1 is significantly elevated (>5X) in $SK2^{-/-}$ fibroblasts together with the increased expression of the cell division marker proliferating cell nuclear antigen (PCNA) leading fast growth of $SK2^{-/-}$ fibroblasts. SK1 and SK2 are intimately involved in regulating the cell cycle, suggesting a key regulatory role not only based on S1P/ceramide ratios, but also GlcCer signaling and S1P/SK2 regulated HDAC related gene expression.

2. Materials and methods

2.1. Standards and reagents

Multiple standard sphingolipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). [^{3}H]Palmitic acid (43 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Silica gel high performance thin-layer chromatography (HPTLC) plates were obtained from Whatman (Clifton, NJ, USA) and the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Chloroform, methanol, and acetic acid used for HPTLC were of ACS grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). RT-PCR primers for *SphK1, SphK2, Ugcg* and 18s rRNA was obtained from Integrated DNA Technologies (Coralville, IA, USA). The antibodies for ceramide glucosyltransferase (CGlcT) and Proliferating Cell Nuclear Antigen (PCNA) were obtained from cell signaling Technology, Inc. (Beverly, MA, USA), beta-actin antibody and secondary antibodies anti-mouse, anti-goat and anti-rabbit were purchased from Sigma (St. Louis, MO, USA). Sphingosine kinase1 (SK1) inhibitor SKI-178 was purchased from Calbiochem (Billerica, MA, USA), Sphingosine kinase2 (SK2) inhibitor (R)-FTY720 methyl ether (ROME) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 4-Methylumbelliferone (MU) was purchased from Koch-Light Laboratories Ltd (Haverhill, Suffolk, UK). Ceramide glucosyltransferase (CGlcT) inhibitor D-*threo*-1-3′, 4′-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol (Et-DO-P4) was a generous gift from Dr. J. Shayman, University of Michigan School of Med (Ann Arbor, MI, USA).

2.2. Generation of $SK1^{-/-}$ and $SK2^{-/-}$ mutant mouse fibroblast and G26-24 cell lines

Mouse skin fibroblasts were isolated from sterilized ears of newly euthanized 2-week-old postnatal mice generously donated by Dr. R. Proia (NIDDK, NIH). Tissue fragments were plated in 20% bovine FCS in DMEM containing Gentamycin and colonies of fibroblasts were trypsinized and sub-cultured. Cell line G26-24 was isolated from glioma G26 induced by methycholanthrene treatment in the C57BL/6 inbred mouse and has been classified as an immature glial cell with oligodendroglial and some astrocyte features [10].

2.3. Analysis of lipid synthesis by HPTLC

Cells were labeled with [^{3}H] palmitate and lipids were extracted as described previously [11]. Typical labeling experiments were carried out in 100-mm Petri dishes containing 8 ml of serum-free medium for 24 h. Cells (3×10^6/100 mm plate) were harvested and washed three times with phosphate buffered saline, lipids were extracted by Chloroform-Methanol-water (2:1:0.6 v/v) partition and samples were subjected to alkaline methanolysis to remove phosphoglycerides. Lipids were applied to HPTLC plates (10×10 cm; LHP-K TLC plates, Whatman, Inc) and developed in chloroform: methanol: glacial acetic acid: water (70: 25: 8.8: 4.5 v/v). Lipids were visualized through film explosion.

2.4. Lipid extraction and sample preparation for lipid quantification by LC/MS/MS and analysis of sphingoid bases, sphingoid base 1-phosphates and ceramides

Cellular lipids were extracted by a modified Bligh and Dyer [12] procedure with the use of 0.1 N HCl for phase separation. C_{17}-S1P (40 pmol), C_{17}-Sph (30 pmol), and 17:0-Cer (30 pmol) were used as internal standards and were added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v) and aliquots were taken to determine the total phospholipid content. Analyses of sphingolipids were performed by combined LC/MS/MS using an automated Agilent 1100 series liquid chromatograph. Analysis of sphingoid bases and the molecular species of ceramides used ESI in positive ions with MRM analysis [13].

2.5. RT-PCR for and mRNA expression in fibroblasts

Total RNA was extracted from fibroblasts, RT-PCR was executed with Qiagen one-step RT-PCR kit and primer pairs specific to mouse *SphK1, SphK2, Ugcg* and 18S rRNA were used for PCR. Briefly, the reaction mixture was prepared in PCR tubes according to the kit menu and put into a PerkinElmer GeneAMP PCR System 2400 (PerkinElmer Life Sciences). The programming RT-PCR procedure consisted of reverse transcription (50 °C for 30 min), initial PCR activation (95 °C for 15 min), then 35 cycles of 94 °C for 30 s, 55 °C-60 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min, annealing temperature may change according to primer T_m. The RT-PCR amplified samples were visualized on 1.5% agarose gels using ethidium bromide.

2.6. Western blot analysis

Cell lysates from fibroblast cell cultures were subjected to SDS-gel electrophoresis. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), and Western blotting carried out with antibodies to CGlcT and PCNA according to the manufacturer's instructions. Positive bands were detected with a chemiluminescence kit from Fisher Scientific (Pittsburgh, PA). The Western blot bands were scanned with a Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA).

2.7. Cell viability assay

Cells were plated in 24-well culture plates at a density of 1×10^5 cells/cm^2 per well. After treatment, MTT (5 mg/ml, Sigma Chem. Co.) was added to each well of the monolayer cultures, and the cultures were incubated in a humidified 5% CO_2 incubator at 37 °C. Following 2 h of incubation the cells were dissolved in 10% SDS-HCl for 6 h or overnight, and the optical density proportional to cell death was measured at 595 nm by using a microplate reader (Elx800; BioTek).

2.8. Cell-cycle analysis

Single cells were gated using PI-Width and Area parameters as well as FSC and SSC gates. DNA content measurements were calculated using FlowJo's cell cycle platform and the Watson (Pragmatic) model. Percent of cells in G1, S-phase, and G2 was recorded for each sample.

2.9. Statistical analysis

The results of LC-MS/MS analyses are from duplicate experiments run in triplicate. Statistical analyses were performed by

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