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Differential signaling of sphingosine derivatives in U937 human monocytes depends on the degree of *N*-methylation

Hyo-Lim Kim [1], Mijin Han [1], Dong-Soon Im *

Laboratory of Pharmacology, College of Pharmacy (BK21 Project) and Longevity Life Science and Technology Institutes, Pusan National University, Busan 609-735, Republic of Korea

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ABSTRACT

Previously, we studied *N*,*N*-dimethyl-D-erythro-sphingosine (DMS)-induced cell death and signaling in U937 human monocytes; we found that DMS-induced sphingosine kinase- and PKC-independent apoptosis. In the present study, we studied apoptotic responses by three *N*-methyl derivatives of sphingosine: *N*-monomethyl-D-erythro-sphingosine (MMS), *N*,*N*,*N*-trimethyl-D-erythro-sphingosine (TMS), and D-erythro-sphingosine (SPH). The potency order in the apoptotic response was DMS ≥ MMS > TMS > SPH. We compared cellular responses to the derivatives in terms of activities of MAPK signaling molecules, mitochondrial membrane potential ($\Delta\Psi_m$), and reactive oxygen species (ROS) generation. Our results suggest that the degree of *N*-methylation affects the apoptosis-inducing capacity and other related responses including MAPK modulation, $\Delta\Psi_m$, and ROS generation. Dimethylation and monomethylation on the C2 amine of sphingosine enhance the apoptotic response; however, trimethylation induces differential modulation of signaling molecules and less cytotoxicity. Our investigation will be useful for understanding the actions of sphingolipids in apoptosis and for developing chemotherapeutics based on DMS structure.

1. Introduction

Sphingolipids are structural components of cell membranes that can be metabolized to ceramide, sphingosine (SPH), and sphingosine-1-phosphate (S1P) [1]. These metabolites are involved in cell differentiation and apoptosis [2–4] and are implicated in signaling mechanisms such as Ca^{2+} influx [5–7] and kinase activation [8–10].

The functions of *N*,*N*-dimethyl-D-*erythro*-sphingosine (DMS), a natural sphingolipid metabolite [11–13], have been studied in PKC inhibition [14], EGFR augmentation [9], src kinase inhibition [14] and SPH kinase inhibition. DMS has been used as a PKC inhibitor, along with H7 or staurosporin, in apoptosis studies [15,16]. After discovery of its inhibition of SPH kinase, however, DMS effects were interpreted by inhibition of SPH kinase and subsequent accumulation of SPH [17–19]. However, the anti-neoplastic mechanism of DMS remains poorly understood. We recently reported that DMS increases the intracellular pH and cytosol Ca^{2+} concentrations in a PKC-independent manner in the U937 human monocyte cell line [6] and also suggested that DMS-induced cell death in U937 cells is mediated through concerted modulation of many signaling molecules in a SPH kinase- and PKC-independent manner [20].

In the present study, we studied apoptotic responses to three *N*-methyl derivatives of SPH: *N*-monomethyl-D-erythro-SPH (MMS), *N*,*N*,*N*-trimethyl-D-erythro-SPH (TMS), and D-erythro-SPH (SPH). We compared cellular responses to the derivatives in terms of activities of signaling molecules, mitochondrial membrane potential ($\Delta\Psi_m$), and reactive oxygen species (ROS) generation to determine the molecular mechanism of action of sphingolipids and to provide a fundamental basis for the development of DMS as a chemotherapeutic agent.

2. Materials and methods

2.1. Reagents

MMS, DMS, TMS, and SPH were purchased from Avanti Polar Lipids (Alabaster, AL, USA). JC-1 was purchased from Biotium (Hayward, CA, USA). Antibodies for phospho-ERK (Thr202/Tyr204), phospho-p38 MAP kinase (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), and phospho-Akt (Ser 473) were from Cell Sig-

Abbreviations: SPH, D-*erythro*-sphingosine; MMS, *N*-monomethyl-D-*erythro*-sphingosine; DMS, *N*,*N*-dimethyl-D-*erythro*-sphingosine; TMS, *N*,*N*,*N*-trimethyl-D-*erythro*-sphingosine; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential; S1P, sphingosine 1-phosphate; PTX, pertussis toxin; PKC, protein kinase C; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide.

* Corresponding author at: Laboratory of Pharmacology, College of Pharmacy, Pusan National University, San 30, Jang-Jun-dong, Geum-Jung-gu, Busan 609-735, Republic of Korea. Tel.: +82 51 510 2817; fax: +82 51 513 6754.
E-mail address: imds@pusan.ac.kr (D.-S. Im).

[1] Both authors contributed equally to this work.

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naling Technology (Beverly, MA, USA). All other materials were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sphingolipids were dissolved in ethanol as a stock (10 mM). The stock or diluted sphingolipid was directly added to culture media or HEPES-buffered media.

2.2. Cell culture

U937 human monocytes were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, and 1 mM pyruvate sodium at 37 °C in a humidified 5% CO_2 incubator.

2.3. MTT cytotoxicity assay

Approximately 4×10^5 cells per well were plated in 48-well flasks (Falcon) and starved for 24 h in RPMI 1640 media containing 0.5% FBS. The cells were treated with sphingolipids at the indicated concentrations for 4 h. Thirty microlitres of 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide (MTT, 5 mg/mL) was added to the cell cultures and cultured for an additional 4 h in a humidified atmosphere. The cell culture media, which contained cells, were collected and centrifuged, the supernatants were carefully removed, and the pellets were resuspended in 0.5 mL of DMSO:EtOH (1:1) solution and shaken for 20 min. Absorbance was measured at 570 nm by a SpectraCount microplate reader (Packard Instrument Co., Downers Grove, IL, USA); the optical density (OD) of untreated cells was defined as 100%.

2.4. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using JC-1. This cationic mitochondrial dye is lipophilic and becomes concentrated in the mitochondria in proportion to the membrane potential; more dye accumulates in mitochondria with greater $\Delta\Psi_m$ and ATP generating capacity. The dye exists as a monomer at low concentrations (emission at 530 nm, green fluorescence), but at higher concentrations forms 'J' aggregates (emission at 590 nm, red fluorescence). Cells were labeled for 20 min with 1 μM JC-1 at 37 °C, washed, and resuspended in HEPES-buffered medium. Fluorescence was then monitored at two different wavelengths. The ratio of the reading at 590 nm to the reading at 530 nm (590:530 ratio) was considered the relative $\Delta\Psi_m$ value [20].

2.5. Measurement of ROS

The cells were sedimented and resuspended in HEPES-buffered medium without BSA, and incubated for 20 min with 10 μM DCFDA. Fluorescence was measured in DCFDA-loaded cells. Fluorescence emission at 520 nm wavelength following excitation at 488 nm was measured every 0.1 s. Individual histograms were constructed from the percent of control level, and ROS measurements were conducted at least two times.

2.6. Western blotting

Cells were starved in media containing 0.5% FBS for 24 h and further incubated for the indicated times with sphingolipids. The cells were cooled by transferring to conical tubes containing 10 times volume of ice-cold PBS. After centrifugation at 2000 rpm for 5 min, the cell pellets were lysed in Intron Biotechnology Protein Extraction Solution (Intron Biotechnology, Seongnam, Korea). The protein concentration was determined using the Pierce BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL). Proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated with specific rabbit antibodies that recognized the active-phosphorylated forms of ERK, p38 MAP kinase, JNK, or Akt. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. Signals were developed using an enhanced chemiluminescence system (Pierce Biotechnology Inc., Rockford, IL, USA) [21,22].

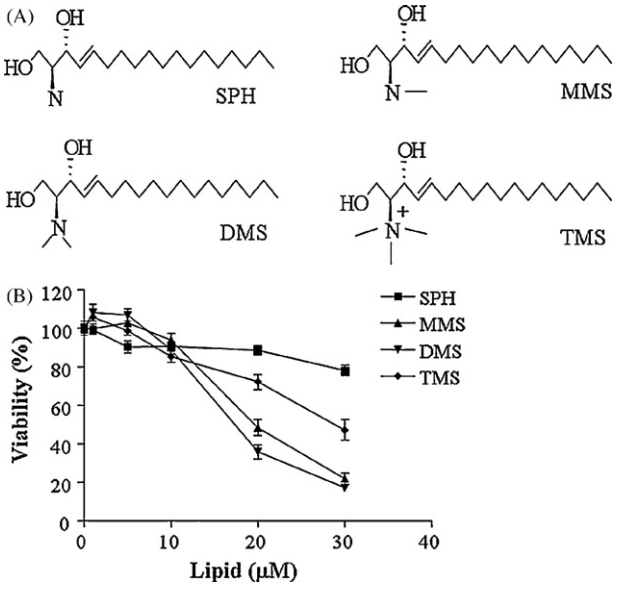

Fig. 1. Cytotoxicity of SPH and *N*-methyl derivatives. (A) Chemical structures of sphingolipids. Sphingosine (SPH), *N*-monomethyl-D-erythro-SPH (MMS), *N,N*-dimethyl-D-erythro-SPH (DMS), *N,N,N* -trimethyl-D-erythro-SPH (TMS). (B) Cell viability was measured by the MTT assay after treatment with the indicated concentrations of SPH, MMS, DMS, or TMS for 4 h.

2.7. Data presentation

Results from three separate experiments, shown as the percent of control level, are shown in Figs. 1, 3 and 4. Representative results for $\Delta\Psi_m$ and Western blotting were chosen from 2 to 3 separate experiments and are shown in Figs. 2 and 3.

3. Results

3.1. Effects of sphingosine derivatives on cell viability in U937 monocytes

We previously found that DMS induces cell death in U937 monocytes [20]. To examine whether the degree of *N*-methylation of SPH affects the potency and efficacy, we tested SPH, MMS, and TMS. MMS- and TMS-induced cell death in a concentration-dependent manner in U937 cells (Fig. 1). The competence of DMS and MMS to induce cell death was similar, but TMS was less potent and less efficacious than DMS (Fig. 1). The potency order in the apoptotic response was DMS ≥ MMS > TMS > SPH.

3.2. Effects of sphingosine derivatives on signaling molecules

Activity changes of several signaling molecules involved in cell proliferation and apoptosis, such as JNK, p38 MAPK, ERK, and Akt, were investigated in the previous report on DMS-induced apoptosis [20]. DMS modulated the MAPK signaling molecules, activating JNK and p38 MAPK and inactivating Akt and ERK [20]. We thus compared the modulation of MAPK signaling molecules by three