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N,N-Dimethyl-D-*ribo*-phytosphingosine Modulates Cellular Functions of 1321N1 Astrocytes

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Abstract – *N,N*-Dimethyl-D-*ribo*-phytosphingosine (DMPH) is an N-methyl derivative of sphingosine. In the present paper, we studied effects of DMPH on intracellular Ca^{2+} concentration, pH, glutamate uptake, and cell viability in human 1321N1 astrocytes. DMPH increased intracellular Ca^{2+} concentration and cytosolic pH significantly in a dose-dependent manner. DMPH also inhibited glutamate uptake by 1321N1 astrocytes. Finally, treatment of cells with DMPH for 24 h reduced viability of cells largely and concentration-dependently. In summary, DMPH increased intracellular Ca^{2+} concentration and pH, inhibited glutamate uptake and evoked cytotoxicity in 1321N1 astrocytes. Our observations with DMPH in the 1321N1 astrocytes would enhance understanding of DMPH actions in the brain.

Keywords □ Sphingosine, Calcium, pH, Glutamate, Astrocytes

INTRODUCTION

Sphingolipid metabolites play important roles as cellular modulators. They are involved in cell differentiation and apoptosis (Sakakura *et al.*, 1998). But they are also implicated in signal transduction mechanisms as Ca^{2+} influx (Mathes *et al.*, 1998) or kinase phosphorylation (Igarashi *et al.*, 1990; Pushkareva *et al.*, 1992). Sphingosine is a sphingolipid metabolite with multiple intracellular functions. After cellular activation by an agonist such as platelet-derived growth factor or insulin-like growth factor, its cellular level increases (Coroneos *et al.*, 1995; Spiegel and Milstien, 1995). The increase results from activation of ceramidase that converts ceramide to sphingosine.

N,N-Dimethyl-D-*erythro*-sphingosine (DMS) is an N-methylated derivative of sphingosine found naturally in cells and the enzymes that convert sphingosine to DMS have been detected in several tissues including the brain and liver (Igarashi and Hakomori, 1989; Kobayashi *et al.*, 1988; Mano *et al.*, 1997), suggesting that DMS could be a physiological mediator and can be involved in cellular signaling in the brain. DMS has been reported to induce an important phosphorylation in cells by activating a sphingosine-dependent protein kinases (Megidish *et al.*, 1998) or by inhibiting a sphingosine kinase (Meyer zu Heringdorf *et al.*, 1998).

Calcium is a ubiquitous second messenger controlling a broad range of cellular functions and increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) plays an important role in cell functions such as cell proliferation and insulin secretion (Himmel *et al.*, 1998; Lipskaia and Lompre, 2004). The elevation of $[Ca^{2+}]_i$ by DMS has been reported in several cell types including T lymphocytes and monocytes (Alfonso *et al.*, 2003; Lee *et al.*, 2006a). Regulation on intracellular pH is also crucial in a variety of cellular responses (Izumi *et al.*, 2003). Change of intracellular pH has been observed in response to cell growth, tumoral promoters, secretary processes or changes in membrane permeability (Izumi *et al.*, 2003).

Astrocytes are the most common glial cells within the central nervous system and are crucial to the normal homeostatic regulation of the neuronal microenvironment (Benveniste, 1992), in large part because of their ability to selectively regulate extracellular levels of glutamate (Rothstein *et al.*, 1996), which is the primary excitatory neurotransmitter in the brain.

In the previous study, we reported the responses of 1321N1 asctrocytes and PC12 cells to DMS (Lee *et al.*, 2007). DMS

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modulated several cellular responses such as increases of $[Ca^{2+}]_i$ and pH and inhibition of glutamate uptake in 1321N1 astrocytes. In the present paper, we conducted similar experiments with *N,N*-dimethyl-D-*ribo*-phytosphingosine (DMPH), that was reported more potent sphingolipid in Ca^{2+} influx and pH increase in U937 cells (Chang *et al.*, 2006; Lee *et al.*, 2006a; Lee *et al.*, 2006b).

MATERIALS AND METHODS

Materials

N,N-dimethyl-D-*ribo*-phytosphingosine was kindly provided from Doosan Biotech (Yongin, Korea). 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxylmethyl ester (BCECF-AM) was purchased from Biotium (Hayward, CA, USA); Fura 2-AM was from Calbiochem (Darmstadt, Germany); L-[G-^{3}H]Glutamic acid was from Amersham biosciences (Buckinghamshire, UK). All other materials were purchased from Sigma-Aldrich Korea (St. Louis, MO, USA).

Cell culture

1321N1 astrocytes were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 50 μg/ml streptomycin, 2 mM of glutamine, and 1 mM of sodium pyruvate at 37°C in a humidified 5% CO_2 incubator. Cells were seeded in 12 well plates. When cell density was ~80% of confluence, the cells were incubated in Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM HEPES, and 10 mM glucose, pH 7.2) for 24 h and then used in the glutamate uptake study (Lee *et al.*, 2007).

Measurement of intracellular pH or Ca^{2+} concentration

Cells were trypsin-digested, sedimented, and resuspended in Hepes-buffered medium (HBM), consisting of 20 mM Hepes (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 15 mM glucose and 0.1% bovine serum albumin (fatty acid free), and then incubated for 40 min with 5 μM BCECF-AM for pH measurement or fura 2-AM for Ca^{2+} measurement. The BCECF fluorescence was recorded at excitation wavelengths of 440 and 490 nm and an emission wavelength of 530 nm by F4500 fluorescence spectrophotometer (Hitachi, Japan). The 490/440 fluorescence ratios were calibrated by nigericin and carnobyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)(James-Kracke, 1992; Thomas *et al.*, 1979). The amount of $[Ca^{2+}]_i$ was estimated from the change in the fluorescence of the fura 2-loaded cells (Yun *et al.*, 2004). Fluorescence emission at 510 nm wavelength from two excitation wavelengths (340 nm and 380 nm) were measured every 0.1 sec by F4500 fluorescence spectrophotometer (Hitachi, Japan), and the ratio of fluorescence intensities from the two wavelengths was monitored as an estimate of $[Ca^{2+}]_i$ (Lee *et al.*, 2007; Yun *et al.*, 2004).

Glutamate uptake

Cells were incubated in Locke's solution supplemented with 0.5 mg/ml fatty acid-free bovine serum albumin (BSA) and designated concentration of DMPH for 1 h. Stock solution of DMPH was prepared in EtOH. Cells were incubated with either 10 nM [^{3}H] glutamate (1 μCi/μl) for the last 7 min of the treatment regimen. The plates were then placed on ice, and the cells were scraped off into cold lysis buffer containing 0.1% NaOH and 0.1% (wt/vol) sodium dodecyl sulfate. Radioactivity in the cell extract was determined by a scintillation counter (Lee *et al.*, 2007).

Cell viability assay

Cells were seeded in 48 well flasks (falcon) and starved in DMEM containing 0.5% FBS for 24 h. Cells were treated with DMPH at concentrations of 1, 3, 10, 30, 50 μM for 24 h. Thirty μl of 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide (MTT, 5 mg/ml) was added to cell cultures in each well and cultured for additional 4 h in a humidified atmosphere. Medium were carefully replaced with 500 μl of DMSO:EtOH (1:1, v/v) solution and shaken for 10 min. The absorbance was measured at 570 nm by SpectraCount microplate reader (Packard Instrument Co., IL, USA). Optical density (OD) of untreated cells was defined as 100% (Chang *et al.*, 2006).

Data presentation

Representative traces for intracellular pH or Ca^{2+} concentration were chosen out of 3 separate experiments and shown in Figs 1 and 2. In Figs 3 and 4, results of 3 independent experiments were shown as % of none-treated control. Student's t test was conducted to analyze significant differences between control group and lipid-treated group.

RESULTS

DMPH increased $[Ca^{2+}]_i$ and cytosolic pH in 1321N1 astrocytes

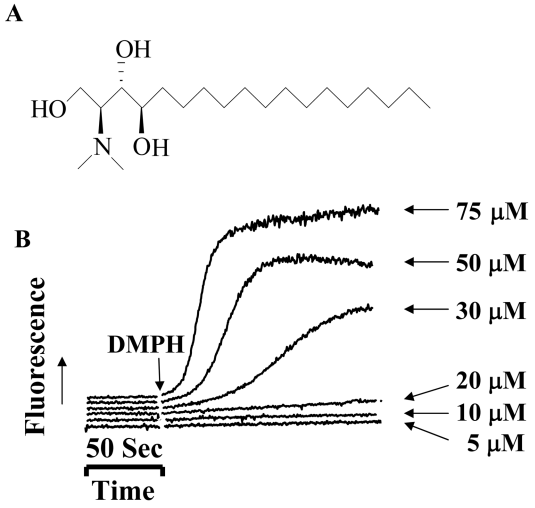

Fig. 1. Chemical structure of DMPH and dose-dependence of DMPH-induced intracellular Ca^{2+} increase. Chemical structure of DMPH is shown (A). Representative Ca^{2+} traces by each concentration of DMPH (B) in 1321N1 astrocytes. Numbers of experiments for each concentration were 3.

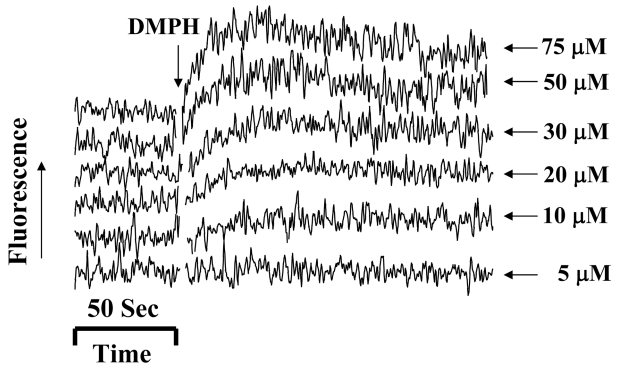

Fig. 2. Dose-dependence of DMPH-induced cytosolic pH increase. Representative pH traces by each concentration of DMPH in 1321N1 astrocytes. Numbers of experiments for each concentration were 3.

Because DMS was detected in the brain and has been shown to modulate cellular functions of 1321N1 astrocytes, it was of interest to determine effects of DMPH on cytosolic Ca^{2+} concentration and pH in 1321N1 astrocytes. DMPH increased $[Ca^{2+}]_i$ eminently and dose-dependently (Fig. 1). DMPH also induced elevation of cytosolic pH in 1321N1 astrocytes in a concentration-dependent manner (Fig. 2). DMPH showed similar potency to DMS in increasing cytosolic Ca^{2+} concentration and intracellular pH (Figs 1 and 2) (Lee *et al.*, 2007).

DMPH inhibited glutamate uptake in 1321N1 astrocytes.

It is well known that one of the major roles of astrocytes is to terminate neurotransmission by the uptake of extracellular glutamate through high affinity glutamate transporters. We estimated effects of DMPH on astrocytic glutamate uptake activity. [^{3}H]glutamate was used as labeled precursors for monitoring glutamate uptake by 1321N1 astrocytes. When cells were incubated with 30 μM DMPH for 1 h, DMPH impeded uptake of glutamate by 1321N1 astrocytes at 90.4% as compared with control (Fig. 3). DMPH was most efficacious in inhibiting glutamate uptake, although DMS and sphingosine also showed concentration-dependent inhibition on glutamate uptake (Fig. 3) (Lee *et al.*, 2007).

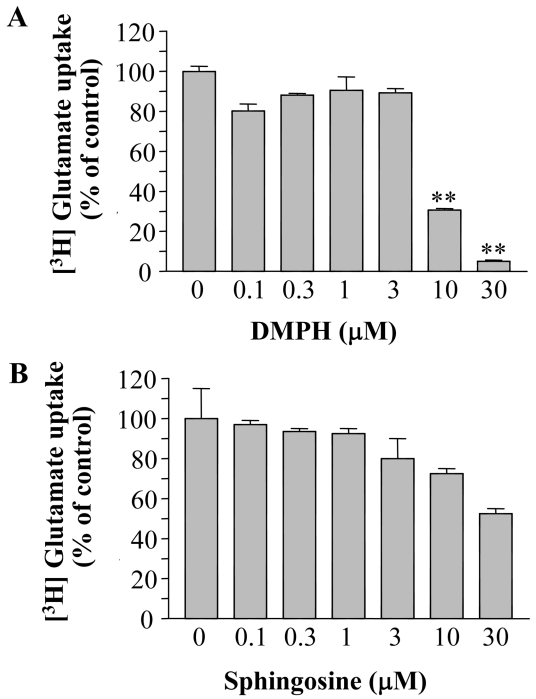

Fig. 3. Effects of DMPH and sphingosine on [^{3}H]glutamate uptake. [^{3}H]Glutamate uptakes by 1321N1 astrocytes in the presence of each concentration of DMPH (A) or sphingosine (B) are shown as % of control without lipid addition.

DMPH induced cytotoxicity in 1321N1 astrocytes

DMPH has been considered as a possible candidate for anticancer therapy. In 1321N1 astrocytes, when cells were exposed to DMPH for 24 h, DMPH reduced cell viability effectively and significantly in a dose-dependent manner (Fig. 4).

DISCUSSION

In the previous study, treatment of 1321N1 astrocytes and PC12 neuronal cells with DMS resulted in increases of $[Ca^{2+}]_i$

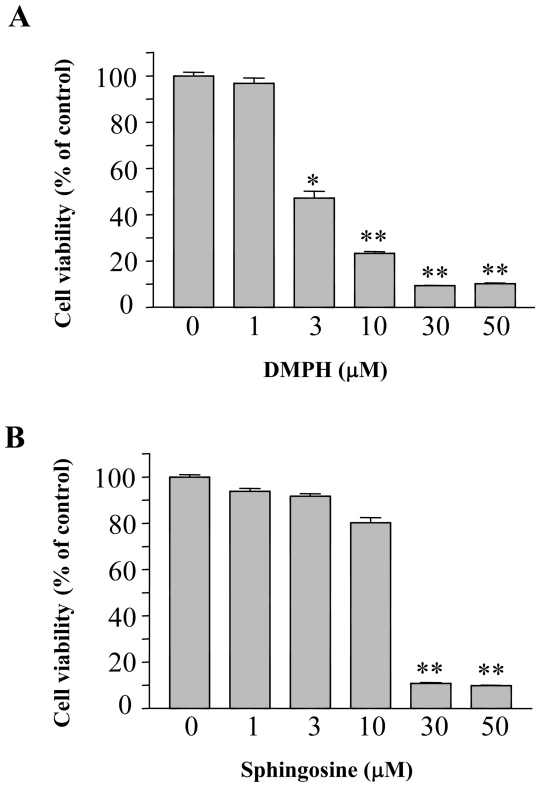

Fig. 4. Effects of DMPH and sphingosine on cell viability. Cell viability in the presence of each concentration of DMPH (A) or sphingosine (B) are shown as % of control without lipid addition in 1321N1 astrocytes.

and pH in a concentration-dependent manner (Lee *et al.*, 2007). It is quite possible that DMS may spawn excitotoxic processes by impairing glutamate uptake and Ca^{2+} and cytosolic pH could be potential regulators. Our present results on DMPH in the 1321N1 astrocytes extend the previous results in terms of intracellular Ca^{2+} and pH regulation. The results showed that DMPH is more efficacious than DMS. DMPH has an additional hydroxy group on the C4 of DMS structure, in place of a double bond between C4 and C5. DMPH showed faster and more efficient response of pH and Ca^{2+} increases than DMS in U937 monocytes also, possibly implying similar action modes between two cell lines (Chang *et al.*, 2006; Lee *et al.*, 2006a). The result on cell viability suggests DMPH as an anti-cancer candidate. Our present results showed that DMPH prevented glutamate uptake of 1321N1 astrocytes. Glutamate competitively inhibits neuronal uptake of cysteine, which is one of the precursors for glutathione (Piani and Fontana, 1994), and high extracellular level of glutamate may render neurons more vulnerable to oxidative injury. Furthermore, modulation of glutamate uptake can directly affect energy metabolism of astrocytes, since glutamate uptake stimulates glucose uptake (Pellerin and Magistretti, 1994). Further study is needed to identify precise mechanism of DMPH-evoked inhibition of glutamate uptake by 1321N1 astrocytes.

DMS was first described as a PKC inhibitor, although subsequent studies have shown to have different effects, such as to strongly increase kinase activity of epidermal growth factor receptor (Igarashi *et al.*, 1990), Src kinase activity (Abdel-Ghany *et al.*, 1992), sphingosine-dependent protein kinase activation (Megidish *et al.*, 1998), and calcium mobilization (Alfonso *et al.*, 2003). In addition, DMS-elicited increase of $[Ca^{2+}]_i$ has been reported in hematopoietic cells such as HL-60 leukemia (Shin *et al.*, 2000), human T lymphocytes (Alfonso *et al.*, 2003) and monocytes (Lee *et al.*, 2006a). Previously, the effects of DMS on astrocytic cells and neuronal cells have been reported by us (Lee *et al.*, 2007). Effects of DMPH on intracellular Ca^{2+} and pH was shown in U937 monocytes (Chang *et al.*, 2006; Lee *et al.*, 2006a) and its modulation of Ca^{2+} influx in the cells was characterized (Lee *et al.*, 2006b). Involvement of $G_{i/o}$ proteins in T lymphocytes was reported, possibly indicating that the effect of DMS is partly due to it's interaction with some membrane structure which is coupled to G-proteins (Alfonso *et al.*, 2003). However, we could not observe any involvement of $G_{i/o}$ proteins and phospholipase C in astrocytes (Lee *et al.*, 2007).

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