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Structure-Activity Relationships of Dimethylsphingosine (DMS) Derivatives and their Effects on Intracellular pH and Ca^{2+} in the U937 Monocyte Cell Line

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We recently reported that dimethylsphingosine (DMS), a metabolite of sphingolipids, increased intracellular pH and Ca^{2+} concentration in U937 human monocytes. In the present study, we found that dimethylphytosphingosine (DMPH) induced the above responses more robustly than DMS. However, phytosphingosine, monomethylphytosphingosine or trimethylsphingosine showed little or no activity. Synthetic C3 deoxy analogues of sphingosine did show similar activities, with the C16 analogue more so than C18. The following structure-activity relationships were observed between DMS derivatives and the intracellular pH and Ca^{2+} concentrations in U937 monocytes; 1) dimethyl modification is important for the DMS-induced increase of intracellular pH and Ca^{2+}, 2) the addition of an OH group on C4 enhances both activities, 3) the deletion of the OH group on C3 has a negligible effect on the activities, and 4) C16 appears to be more effective than C18. We also found that W-7, a calmodulin inhibitor, blocked the DMS-induced pH increase, whereas, KN-62, ML9, and MMPX, specific inhibitors for calmodulin-dependent kinase II, myosin light chain kinase, and Ca^{2+}-calmodulin-dependent phosphodiesterase, respectively, did not affect DMS-induced increases of pH in the U937 monocytes.

Key words: Dimethylsphingosine, pH, Sphingosine, Calcium, Structure-activity relationship

INTRODUCTION

N,N-dimethyl-D-*erythro*-sphingosine (DMS) is a natural sphingolipid metabolite in mammalian cells (Merrill *et al.*, 1988; Igarashi and Hakomori, 1989; Mano *et al.*, 1997) that was first reported to inhibit protein kinase C (PKC) along with D-*erythro*-sphingosine (Hannun and Bell, 1987; Igarashi *et al.*, 1989; Merrill *et al.*, 1989). Subsequently, DMS was found to specifically inhibit sphingosine kinase, whereas its inhibitory action on PKC could not be reproduced in certain cell types(Edsall *et al.*, 1998; De Jonghe *et al.*, 1999). Furthermore, the anti-cancer effects of DMS such as inhibition of tumor cell migration and cancer cell growth have been reported and have provided a fundamental base for the development of chemotherapies (Endo *et al.*, 1991; Okoshi *et al.*, 1991; Kimura *et al.*, 1992). The anti-neoplastic mechanism of DMS, however, remains poorly understood. We recently reported that DMS increased the intracellular pH and cytosol Ca^{2+} concentrations in the U937 human monocyte cell line, and that the DMS-induced increase of intracellular pH was not stereo-selective (Lee *et al.*, 2006). Furthermore, the DMS-induced increase of cytosol Ca^{2+} was due to a Ca^{2+} influx across the plasma membrane, and the DMS-induced pH increase and Ca^{2+} responses were independent of each other (Lee *et al.*, 2006). In the present study, we have investigated structure-activity relationships of DMS and their derivatives on intracellular pH and Ca^{2+} concentrations in the U937 human monocyte cell line and compared their cytotoxicity.

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MATERIALS AND METHODS

Reagents

N,N-dimethyl-D-*erythro*-sphingosine, *N,N,N*-trimethyl-D-*erythro*-sphingosine (TMS), D-*erythro*-dihydrosphingosine (sphinganine, H2Sph), N-acetyl (C2:0) ceramide (C2-cer) and D-*ribo*-phytosphingosine-1-phosphate (PhyS1P) were purchased from Avanti Polar lipids (Alabaster, AL, U.S.A.); D-*ribo*-phytosphingosine (Phyto), *N*-methyl-D-*ribo*-phytosphingosine (MMPH), *N,N*-dimethyl-D-*ribo*-phytosphingosine (DMPH), and *N*-acetyl (C2:0)-D-*ribo*-phytosphingosine (C2Ph) were kindly provided by Doosan Biotech (Yongin, Korea). 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxylmethyl ester (BCECF-AM) was purchased from Biotium (Hayward, CA, U.S.A.); Fura 2-AM from Calbiochem (Darmstadt, Germany); *N*-(6-amino-hexyl)-5-chloro-1-naphthalensulfonamide (W-7), 1-[*N,O-bis*-(5-isoquinoline sulfonyl)-*N*-methyl-*L*-tyrosyl]-4-phenyl-piperazine (KN62), 1-(5-naphthalenesulfonyl)homopiperazine (ML9), 8-methoxymethyl-3-isobutyl-methylxanthine (MMPX) and 5,7-dihydroxy-3-(4-hydroxyphenyl)-*4H*-1-benzopyran-4-one (genistein) from Tocris Cooksan Ltd (Avonmouth, Bristol, UK). The C3-deoxy sphingosines (C16 and C18) were synthesized by Chung's laboratory (Lim *et al.*, 2004) All other materials were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell culture

The U937 human monocytes were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and maintained in RPMI1640 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.

Measurement of intracellular pH and Ca^{2+} concentrations

Cells were sedimented, 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 incubated for 40 min with 5 μM BCECF-AM for pH measurement or with fura 2-AM for Ca^{2+} measurements. 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 using nigericin and carnobyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)(Thomas *et al.*, 1979; James-Kracke, 1992). The amount of intracellular $[Ca^{2+}]_i$ 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 using a F4500 fluorescence spectrophotometer (Hitachi, Japan), and the ratio of the fluorescence intensities from the two wavelengths monitored as an estimate of intracellular Ca^{2+} concentration(Yun *et al.*, 2004).

MTT cytotoxicity assay

Approximately 2×10^5 cells per well were plated in 48 well flasks (Falcon) and starved for 24 h in RPMI1640 media containing 0.5% FBS. The cells were treated with sphingolipids at concentrations of 1, 3, 10, 30, or 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 the cell cultures and cultured for an additional 4 h in a humidified atmosphere. The cell culture media containing cells were collected and centrifuged, supernatants carefully removed, and the pellets resuspended with 0.5 mL of DMSO:EtOH = 1:1 solution and shaken for 10 min. Absorbance was measured at 570 nm by a SpectraCount microplate reader (Packard Instrument Co., IL, U.S.A.); optical density (OD) of untreated cells was defined as 100%.

Data presentation

Representative traces for intracellular pH or Ca^{2+} concentrations were chosen from 3–5 separate experiments and are shown in Figs. 2-5. Results from two separate experiments, shown as the percent of control level, are shown in Fig. 6.

RESULTS

Structure-activity relationships of DMS and DMS derivatives with increased pH

DMS-induced increases of intracellular pH and Ca^{2+} concentration have recently been studied by employing a series of structurally-related stereo-isomers of sphingolipids in U937 monocytes(Lee *et al.*, 2006). Low stereo-specificity was found with four stereo-isomers of sphingosine (Lee *et al.*, 2006). In the present study, we tested derivatives of sphingosine and phytosphingosine having various modifications on the C2 amino group and C3 deoxy of sphingosine with different chain lengths (Fig. 1). Fig. 2 indicates that phytosphingosine and phytosphingosine-1-phosphate did not increase the pH. However, dimethylphytosphingosine (DMPH) induced a greater pH increase than DMS and that monomethylphytosphingosine (MMPH) had a lesser response than DMS; trimethylsphingosine (TMS) was without effect on intracellular pH. These results indicate that a dimethyl modification of the C2 amino group is important for DMS-induced pH increases. A pH increase was not observed with *N*-acetyl

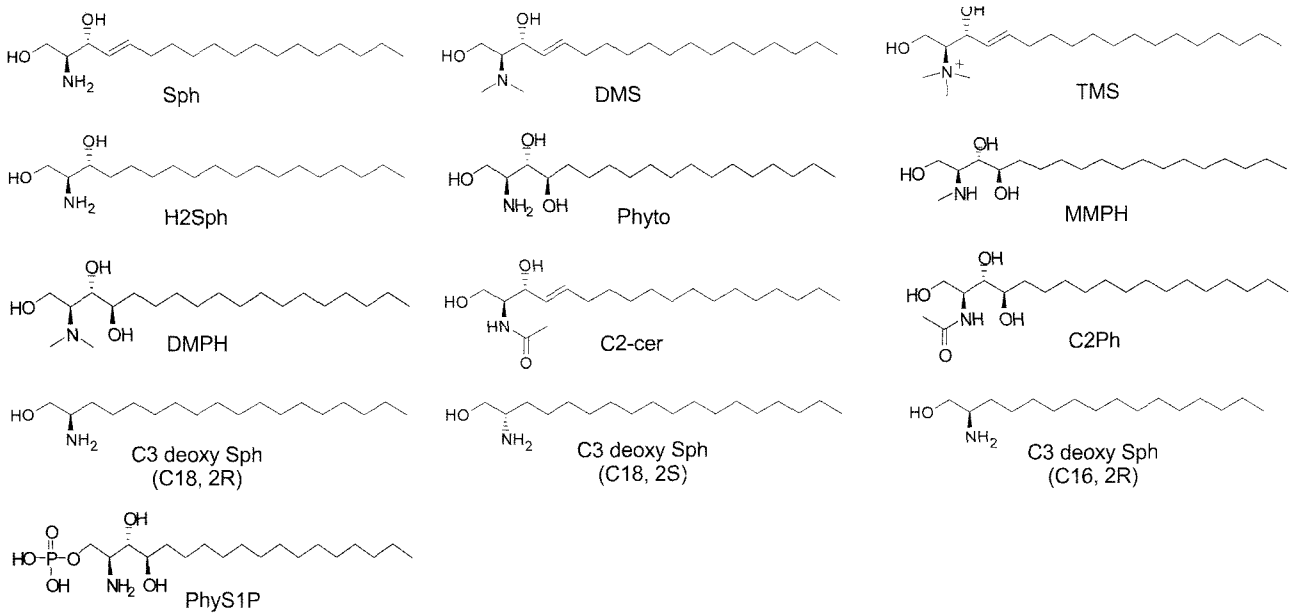

Fig. 1. Structures of DMS and other sphingolipids. DMS dimethylsphingosine; Sph sphingosine; H2Sph dihydrosphingosine; Phyto phytosphingosine; MMPH monomethylphytosphingosine; DMPH dimethylphytosphingosine; TMS trimethylsphingosine; C3 deoxy Sph C3 deoxysphingosine; C2-cer *N*-acetyl ceramide; C2Ph *N*-acetyl phytosphingosine; PhyS1P phytosphingosine-1-phosphate.

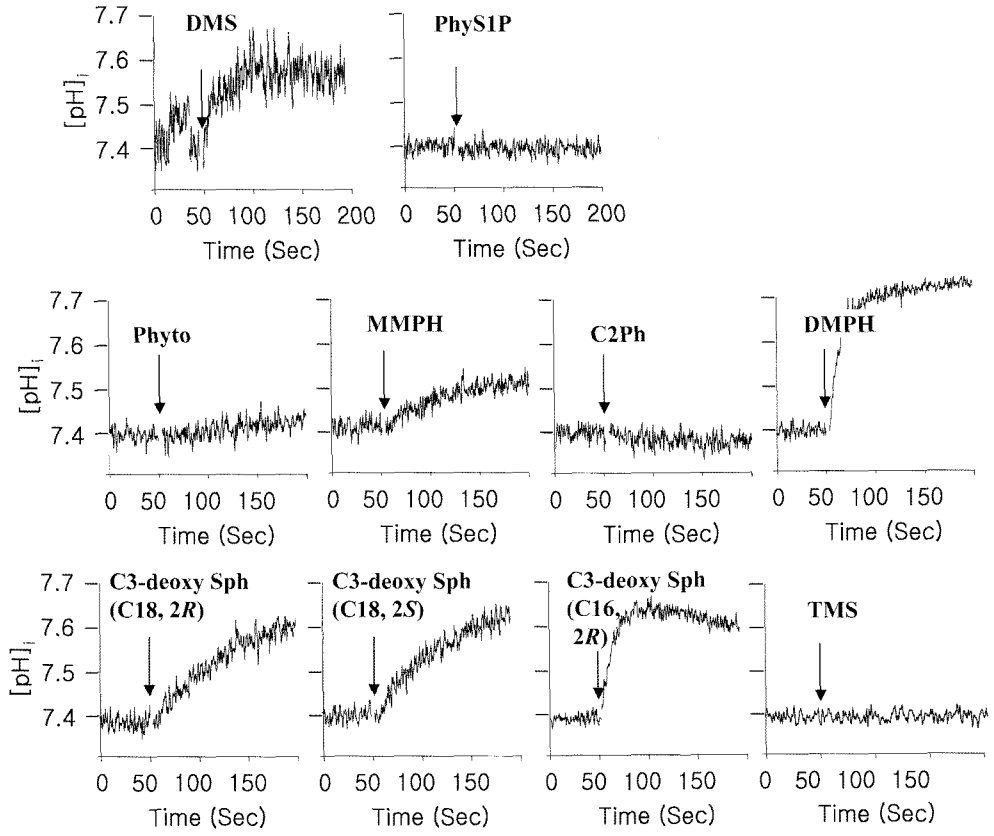

Fig. 2. Structure-activity relationships of DMS on pH increases. Representative pH traces with 30 μM each of sphingolipids in the U937 cells. PhyS1P phytosphingosine-1-phosphate; Phyto phytosphingosine; C2Ph *N*-acetyl phytosphingosine.

phytosphingosine (Fig. 2). To assess whether the C3 hydroxyl group is important for DMS-induced pH increases, we synthesized and tested C3 deoxy sphingosine analogues with C16 and C18 backbones (naturally

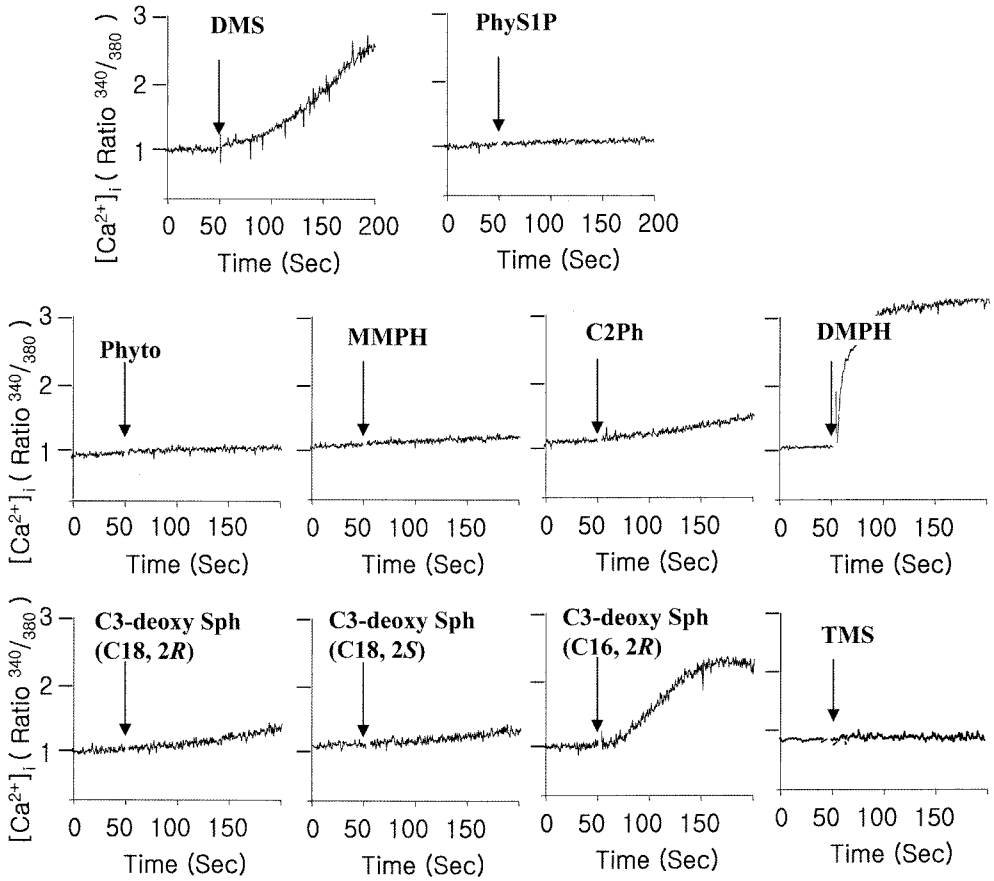

Fig. 3. Structure-activity relationships of DMS on Ca^{2+} increases. Representative Ca^{2+} traces with 30 μM each of sphingolipids in the U937 cells. PhyS1P phytosphingosine-1-phosphate; Phyto phytosphingosine; C2Ph *N*-acetyl phytosphingosine.

occurring sphingosine has a backbone of 18 carbons). As seen in the Fig. 2, C16 and C18 sphingosine analogues without a C3 hydroxyl group induced a pH increase. A (2*S*) C3 deoxy sphingosine analogue (C18) showed the same activity on pH increase as (2*R*) C3 deoxy sphingosine (C18), supporting our previous finding on the absence of stereo-selectivity(Lee *et al.*, 2006). The (2*R*) C16 analogue, however, yielded greater responses than the (2*R*) C18 analogue.

Structure-activity relationships of DMS and DMS derivatives with intracellular Ca^{2+} increases

The DMS-induced increase of intracellular Ca^{2+} concentration was also studied with the same set of sphingolipids described in this report. In our recent study, we identified a difference in stereo-selectivity between the DMS-induced Ca^{2+} increase and the DMS-induced pH increase i.e. the L-*threo* isomer induced a much greater response than the other stereo-isomers in terms of a Ca^{2+} increase(Lee *et al.*, 2006). As shown in Fig. 3, phytosphingosine, MMPH, *N*-acetyl phytosphingosine, TMS and phytosphingosine-1-phosphate did not alter the cytosol Ca^{2+} concentrations, however, DMPH did increase Ca^{2+} more so than DMS. The C3 deoxy sphingosine analogues with a C16 backbone robustly increased Ca^{2+}, but analogues with C18 backbone did not, confirming our previous results that different signals operate for DMS-induced pH and Ca^{2+} increases (Lee *et al.*, 2006).

Effects of specific inhibitors on DMS-induced actions

We have previously excluded the possibility that PKC was an upstream signaling molecule for DMS-induced responses (Lee *et al.*, 2006). In the present study, we have tested specific pharmacologic inhibitors of several signaling proteins, including genistein, fumonisin B_1, and W-7. It is known that in the human T lymphocyte, genistein, a tyrosine kinase inhibitor, partially inhibits DMS-induced pH increases (Alfonso *et al.*, 2003). As shown in Fig. 4, however, genistein did not influence a DMS-induced pH increase in the U937 monocyte. We also tested fumonisin B_1, a specific inhibitor of ceramide synthase, to examine whether metabolites of DMS induced a pH increase and found no effect of fumonisin B_1. We also studied W-7, a calmodulin (CaM) antagonist, and found that while W-7 treatment blocked DMS-induced increases of pH and Ca^{2+}, W-7 by itself increased the intracellular pH and Ca^{2+}

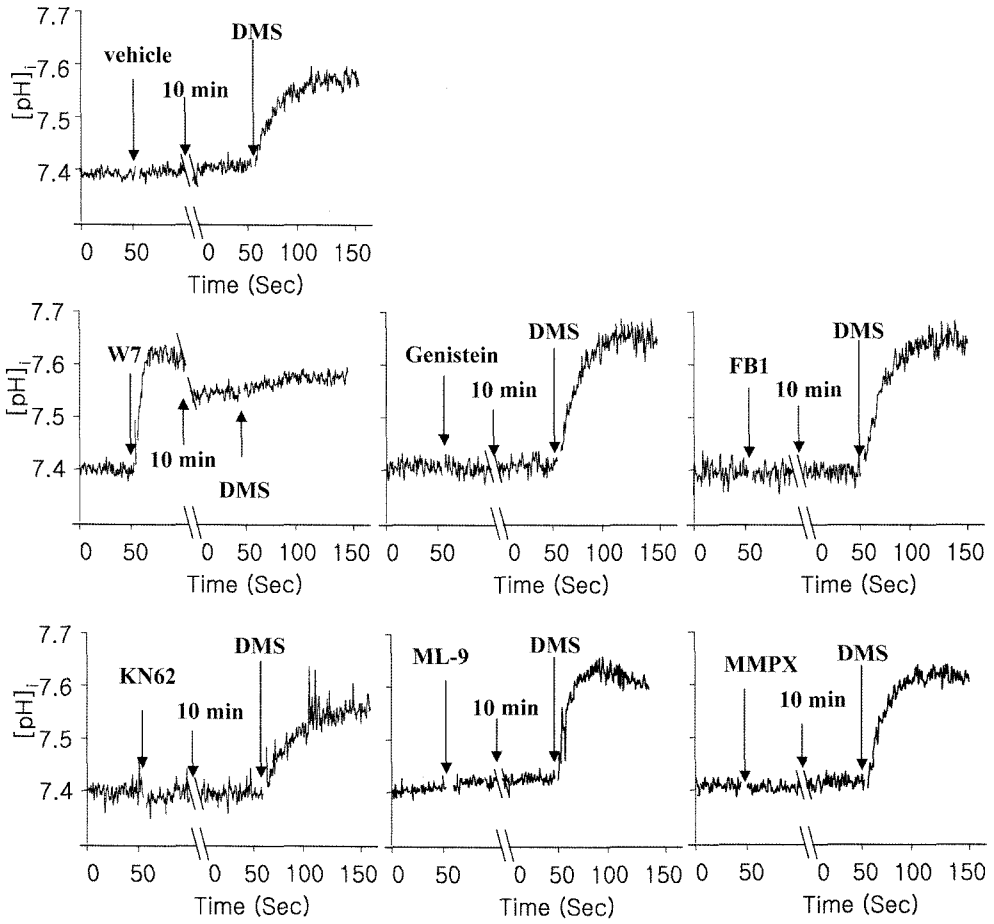

Fig. 4. Effects of pharmacologic inhibitors on DMS-induced intracellular pH increases. Representative pH traces with 30 μM DMS in the U937 cells in the presence of W-7 (500 μM), KN-62 (10 μM), genistein (10 μM), ML9 (4 μM), MMPX (10 μM), or FB1 (fumonisin B_1, 10 μM). Each inhibitor was added at the first arrow (50 sec), at 10 min later (second arrow), the DMS was added as indicated by the third arrow.

(Figs. 4 and 5). Therefore, KN62, a CaM kinase II inhibitor, was tested to determine if W-7 affects CaM kinase II. Neither intracellular pH and Ca^{2+}, nor DMS-induced pH increases were found to be influenced by KN62 (Figs. 4 and 5). It has been reported that W-7 inhibits Ca^{2+}-CaM-dependent phosphodiesterase (PDE) and myosin light chain kinase (MLCK). To evaluate the possible effects of W-7 on pH and Ca^{2+} due to inhibition of MLCK or Ca^{2+}-CaM-dependent PDE, we tested ML9 and MMPX, specific inhibitors for MLCK and a Ca^{2+}-CaM-dependent isozyme of PDE (PDE1), respectively. Neither compound influenced DMS-induced pH and Ca^{2+} responses (Figs. 4 and 5).

Involvement of G proteins and phospholipase C in DMPH-induced actions

We had previously tested whether G proteins and phospholipase C were involved in DMS-induced increases of pH and Ca^{2+}, and found no involvement (Lee *et al.*, 2006). However, since DMPH induced a more rapid and more robust action than DMS (as described above) we reexamined the possible involvement of G proteins and phospholipase C by including pertussis toxin and 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione (U73122), specific inhibitors of $G_{i/o}$ proteins and phospholipase C, respectively. Treatment with pertussis toxin or U73122, however, did not inhibit DMPH-induced Ca^{2+} or pH increases (data not shown).

Cell death and DMS actions

The regulation of intracellular pH is critically important for a variety of cell responses (Izumi *et al.*, 2003; Putney and Barber, 2003). Thus, a change of intracellular pH has been observed in response to cell growth, tumor promoters, secretary processes and membrane permeability (Izumi *et al.*, 2003; Putney and Barber, 2003). Furthermore, sustained increase of intracellular Ca^{2+} can induce either cell proliferation or cell death (Himmel *et al.*, 1998; Lipskaia and Lompre, 2004). Increases of intracellular pH and Ca^{2+} by DMS may reflect the mechanisms underlying DMS-induced inhibition of the growth of tumor cells. However, TMS, phytosphingosine-1-phosphate, phytosphingosine,

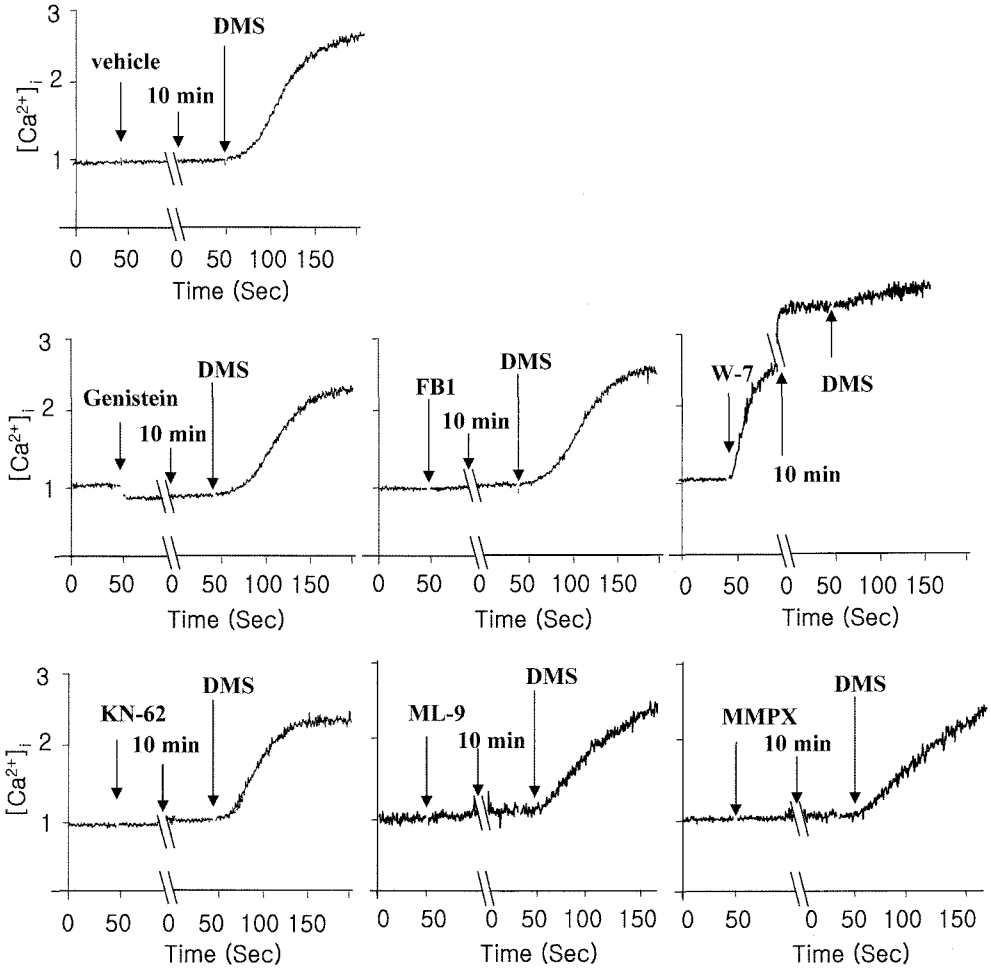

Fig. 5. Effects of pharmacologic inhibitors on intracellular DMS-induced Ca^{2+} increases. Representative Ca^{2+} traces with 30 μM DMS in the U937 cells in the presence of W-7 (500 μM), KN-62 (10 μM), genistein (10 μM), ML9 (4 μM), MMPX (10 μM), or FB1 (fumonisin B_1, 10 μM). Each inhibitor was added at the first arrow (50 sec), at 10 min later (second arrow), the DMS was added as indicated by the third arrow.

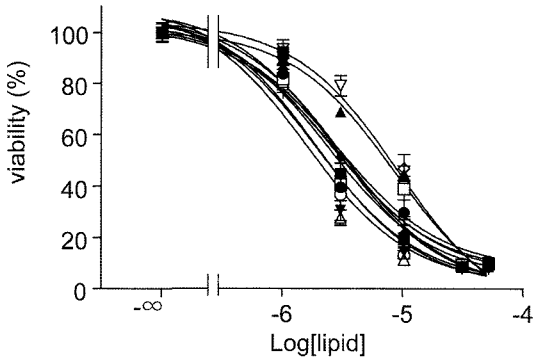

Fig. 6. Cytotoxicity of DMS and other sphingolipids. Concentration-response curves of each sphingolipid on cytotoxicity in the U937 cells were made from two separate experiments using the MTT assay. Key as follows: DMS (filled square), TMS (filled triangle), MMPH (reverse filled triangle), DMPH (filled diamond), (2*R*) C3-deoxy Sph (C18, filled circle), (2*S*) C3-deoxy Sph (C18, open square), (2*R*) C3-deoxy Sph (C16, open triangle), Sph (open reverse triangle), C2Ph (open diamond), PhyS1P (open circle), dihydrosphingosine (X), phytosphingosine (+).

and C2-ceramide, which were without effects on pH and Ca^{2+} responses, yielded significant cytotoxicity and almost all sphingolipids tested had similar cytotoxicity on the U937 cells. Therefore, the cytotoxic effects of DMS have different structure-activity relationships to the pH and Ca^{2+} responses (Fig. 6).

DISCUSSION

By employing a series of DMS derivatives, the present study of structure-activity relationships for DMS with pH and Ca^{2+} responses have been related to cytotoxicity. We also studied several specific inhibitors to help elucidate a mechanism of action for DMS. Our findings are summarized categorically as follows: (1) dimethyl modification is important for DMS-induced increases of pH and Ca^{2+} , (2) deletion of an OH group on C3 has negligible effects on the activities, however, addition of an OH group on C4 enhances activities, (3) a C16 backbone is more effective

than C18, (4) W-7, a CaM inhibitor, blocks DMS-induced pH increases, which are not affected by genistein or fumonisin B_1, and (5) structure-activity relationships for DMS-induced cytotoxicity, pH increases, and Ca^{2+} increases in U937 monocytes differ from one another.

Structure-activity relationships of DMS

In our previously reported study, we characterized DMS-induced increases of intracellular pH and Ca^{2+} concentration in U937 human monocytes and identified different stereo-selectivity's of sphingosine on two responses by using four stereo-isomers of sphingosine and two stereo-isomers of dihydrosphingosine (Table I): stereo-selectivity in the Ca^{2+} response and low stereo-selectivity in the pH response (Lee *et al.*, 2006).

Phytosphingosine has an additional hydroxyl group on the C4 of sphingosine structure in place of the double bond between C4 and C5 (Fig. 1). Considering the increase in pH with sphingosine but not phytosphingosine, the addition of a C4 hydroxyl group seems to influence a pH increase by phytosphingosine, since the only difference in structure is the C4 hydroxyl group; unsaturation between C4 and C5 of sphingosine was without influence on pH and Ca^{2+} responses (Lee *et al.*, 2006). The addition of a methyl group(s) on the C2 amino group of phytosphingosine, however, enabled *N*-methylated phytosphingosines (MMPH and DMPH) to induce pH increases (Table I). DMPH-induced increases of pH and Ca^{2+} were greater than with MMPH, however, TMS had no effect (Table I). Therefore, two methyl groups on the C2 amino moiety may be optimal for a pH increase, although trimethylphytosphingosine was not available for testing. Surprisingly, DMPH showed a more rapid and more efficient response of pH and Ca^{2+} increases compared to DMS, suggesting that the interaction between the C4 hydroxyl group and the methyl group(s) on the C2 amino might enhance DMPH-induced actions.

Employing (2*R*) and (2*S*) C3 deoxy sphingosine analogues, the low stereo-specificity observed previously in pH responses was confirmed in the present study. Different mechanisms of action for DMS on the pH and Ca^{2+} responses were previously suggested and were based on different stereo-selectivity's without an association between pH and Ca^{2+} responses (Lee, 2006). In the present study, the C3 deoxy sphingosine analogues (C18) increased intracellular pH but not Ca^{2+} concentrations, which suggests different signaling mechanisms for the two responses and different molecular targets for DMS in the U937 human monocytes (Table I).

Alternatively, no structural selectivity of sphingolipids could be demonstrated for cytotoxicity in the U937 monocytes. All sphingolipids showed similar degrees of cytotoxicity in contrast to pH and Ca^{2+} responses which suggests that the short-term effects of DMS and its derivatives on pH and Ca^{2+} may not be directly related to cytotoxicity. Therefore, one has to be cautious to interpret DMS-induced cell death, by simply linking pH increase or Ca^{2+} increase to cytotoxicity. Nevertheless, the difference of structural specificity among pH response, Ca^{2+} response and cytotoxicity confirms the specific way, in

Table I. Structure-activity relationships of DMS and DMS derivatives

Sphingolipid	Structure[b,c]				Response[d]		
	OH on C3	OH on C4	double bond between C4 and C5	number of methyl group	pH	Ca^{2+}	cytotoxicity
Sph (D-*erythro*)[a]	O	-	O	0	++	+	+
Sph (L-*threo*)[a]	O	-	O	0	++	++	+
H2Sph (D-*erythro*)[a]	O	-	-	0	++	+	+
H2Sph (L-*threo*)[a]	O	-	-	0	++	++	+
DMS[a]	O	-	O	2	++	++	+
TMS	O	-	O	3	-	-	+
Phyto	O	O	-	0	-	-	+
MMPH	O	O	-	1	+	-	+
DMPH	O	O	-	2	+++	+++	+
C3 deoxy (C18, 2*R*)	-	-	-	0	+	-	+
C3 deoxy (C18, 2*S*)	-	-	-	0	+	-	+
C3 deoxy (C16, 2*R*)	-	-	-	0	++	++	+

[a]: stereo-selectivity studied and reported previously(Lee, 2006)
[b]: presence of OH groups on C3 and C4 and the double bond between C4 and C5 in each sphingolipid indicated by (O) mark; absence (-).
[c]: number of methyl groups on C2 amino group shown by Arabic numbers.
[d]: no response indicated by (-); small response (+); modest (++); strong (+++)

Table II. Effects of pharmacologic inhibitors on DMS-induced intracellular responses

Inhibitor	Target of inhibitor	Effect of inhibitor in U937		Effect of inhibitor in T lymphocytes[b]	
		pH	Ca^{2+}	pH	Ca^{2+}
PTX	$G_{i/o}$ proteins	-	-	+	++
Go6976[a]	PKC	-	-		
GF109203X[a]	PKC	-	-		
genistein	protein tyrosine kinase	-	-	+	++
fumonisin B_1	ceramide synthase	-	-		
W-7	CaM antagonist	+	+		
KN62	CaM-dependent kinase II	-	-		
ML-9	myosin light chain kinase	-	-		
MMPX	Ca^{2+}-CaM-dependent PDE	-	-		

[a] : PKC inhibitors studied and reported previously(Lee, 2006).
[b] : Inhibitor study in T lymphocytes from Alfonso et al.(Alfonso, 2003)

which DMS acts on intracellular pH and Ca^{2+}.

Cellular signaling of DMS

Genistein, an inhibitor of tyrosine kinase, modulates DMS-induced pH and Ca^{2+} increases in T lymphocytes (Alfonso, 2003). We did not observe any effects of genistein on DMS-induced pH increases in U937 monocytes (Table II). By applying fumonisin B_1, a specific inhibitor of ceramide synthase, we found that ceramide-related metabolites of DMS also were not involved in DMS-induced actions (Table II). DMS-induced pH increase was abrogated by W-7, a CaM antagonist, however, increases of pH and Ca^{2+} were observed by treatment with W-7 alone, implying that CaM-dependent modulation underlies basal pH and Ca^{2+} homeostasis in U937 cells. KN62, a CaM kinase II inhibitor, by itself did not influence intracellular pH and did not significantly affect DMS-induced pH increase (Table II). Additional studies with ML9 and MMPX further excluded MLCK and PDE1 as targets of W-7 (Table II). Insofar as more than 100 different enzymes are activated by the Ca^{2+}-CaM system, it may be difficult to precisely elucidate a mechanism of W-7 action on intracellular pH and Ca^{2+} in the U937 monocytes. Nevertheless, it is likely that the result obtained with W-7 and its related inhibitors described herein will be useful in further clarifying an action mode for DMS and the physiological significance of DMS.

Abbreviations

Abbreviations: Sph D-*erythro* sphingosine, DMS *N,N*-dimethyl-D-*erythro*-sphingosine, TMS *N,N,N*-trimethyl-D-*erythro*-sphingosine, MMPH *N*-methyl-D-*erythro*-phytosphingosine, DMPH *N,N*-dimethyl-D-*ribo*-phytosphingosine, C3 deoxy Sph C3 deoxy sphingosine, W-7 *N*-(6-amino-hexyl)-5-chloro-1-naphthalensulfonamide, KN62 1-[*N,O-bis*-(5-isoquinoline sulfonyl)-*N*-methyl-*L*-tyrosyl]-4-phenyl-piperazine, ML9 1-(5-naphthalenesulfonyl)homopiperazine, MMPX 8-methoxymethyl-3-isobutyl-methylxanthine, genistein 5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one, CaM calmodulin, PKC protein kinase C, FCCP carnobyl cyanide 4-(trifluoromethoxy)phenylhydrazone, H2Sph D-*erythro*-dihydrosphingosine, C2-cer *N*-acetyl (C2:0) ceramide, PhyS1P D-*ribo*-phytosphingosine-1-phosphate, Phyto D-*ribo*-phytosphingosine, C2Ph *N*-acetyl (C2:0)-D-*ribo*-phytosphingosine, BCECF-AM 2'7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxylmethyl ester, MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, U73122 1-[6-((17-3-methoxyestra-1,3,5(10)-trien-17-yl)amino-hexyl)-1*H*-pyrrole-2,5-dione.

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