N,*N*-Dimethylsphingosine Is a Potent Competitive Inhibitor of Sphingosine Kinase but Not of Protein Kinase C: Modulation of Cellular Levels of Sphingosine 1-Phosphate and Ceramide†

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Received April 2, 1998

ABSTRACT: Sphingosine 1-phosphate (SPP), a lipid second messenger formed by the action of sphingosine kinase, has been implicated in regulating diverse biological processes, including growth, survival, and differentiation. *N*,*N*-Dimethylsphingosine (DMS) inhibits sphingosine kinase and has been used to investigate the biological roles of SPP; however, little is known of the mechanism of inhibition of sphingosine kinase by DMS. In addition, DMS has been shown to inhibit protein kinase C in vitro. Here we report that DMS is a competitive inhibitor of sphingosine kinase from U937 monoblastic leukemia cells, Swiss 3T3 fibroblasts, and PC12 pheochromocytoma cells. DMS decreases basal levels of SPP and prevents increases in SPP in response to physiological stimuli known to activate sphingosine kinase. DMS also effectively increases cellular levels of ceramide in a variety of cell types, and resetting of the ceramide/SPP rheostat may account for the pro-apoptotic effects of DMS. Moreover, DMS, at concentrations which effectively inhibit sphingosine kinase, has no effect on protein kinase C activity or its membrane translocation. Thus, DMS acts as a specific competitive inhibitor of sphingosine kinase in diverse cell types and is a useful tool to elucidate the role of SPP as an intracellular second messenger.

Sphingolipid metabolites, such as ceramide, sphingosine, and sphingosine 1-phosphate (SPP),[1] are lipid second messengers involved in diverse cellular processes (*1*−*4*). Ceramide (*N*-acylsphingosine) has been implicated as an inducer of programmed cell death, known as apoptosis, and cell cycle arrest (*2*, *5*−*7*), whereas a closely related metabolite, SPP, is emerging as lipid second messenger in calcium mobilization, cellular proliferation, and survival induced by various physiological stimuli (*8*−*12*). Stress stimuli, including Fas ligand, TNF-α, IL-1, growth factor withdrawal, anticancer drugs, oxidative stress, heat shock, and ionizing radiation, stimulate sphingomyelinase (SMase), leading to increased ceramide levels (*2*, *6*, *7*), while PDGF and other growth factors stimulate ceramidase and sphingosine kinase and elevate SPP levels (*4*, *8*, *13*). Moreover, pretreatment of human promyelocytic HL-60 cells, Jurkat T cells, or U937 monoblastic leukemia cells with SPP prevents induction of apoptosis resulting from elevations of ceramide induced by TNF-α and Fas ligation, sphingomyelinase treatment, or cell-permeable ceramide analogues (*9*, *12*). In addition, stimulation of sphingosine kinase and concomitant increases in SPP are responsible for the inhibition of ceramide-induced apoptosis resulting from activation of protein kinase C (PKC) (*9*, *12*). Thus, we have proposed that the dynamic balance between levels of the sphingolipid metabolites, ceramide and SPP, and consequent regulation of opposing signaling pathways is an important factor that determines whether a cell survives or dies (*9*, *11*, *12*). Recently, we demonstrated that this ceramide/SPP rheostat is an evolutionarily conserved stress regulatory mechanism influencing growth and survival of yeast (*14*).

The level of SPP in cells is low and is determined by the relative contributions of its formation, mediated by sphingosine kinase (*15*, *16*), and its degradation, catalyzed by an endoplasmic reticulum pyridoxal phosphate-dependent lyase and a phosphatase (*14*, *17*−*19*). Effective inhibitors of sphingosine kinase have been key tools used to elucidate the intracellular roles of SPP. DL-*threo*-Dihydrosphingosine (DHS) has been used as an inhibitor of sphingosine kinase (*8*, *10*, *15*) to substantiate the role of SPP in cell growth and calcium mobilization. Recently, *N*,*N*-dimethylsphingosine (DMS) has been found to be a more potent inhibitor of sphingosine kinase (*9*, *20*). DMS induces apoptosis in cancer cells of both hematopoietic and carcinoma origin (*21*−*23*) as well as in transformed rat mesangial cells (*23*). However, DMS did not induce apoptosis in primary cultures of human umbilical vein endothelial cells or rat mesangial cells (*23*). DMS also inhibits the growth of various human tumor cell lines in vivo in nu/nu mice (*24*) and blocks neurite extension (*11*, *25*) and platelet aggregation (*26*). Cell surface expression of crucial selectins which promote adhesion of Le^x or

† This work was supported by National Institutes of Health Grants GM43880 and CA61774.

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[1] Abbreviations: DMS, *N*,*N*-dimethylsphingosine; SPP, sphingosine 1-phosphate; DHS, D,L-*threo*-dihydrosphingosine; PDGF, platelet-derived growth factor; NGF, nerve growth factor; PKC, protein kinase C; SMase, sphingomyelinase; DETPAC, diethylenetriaminepentaacetic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TNF-α, tumor necrosis factor-α; BSA, essentially fatty acid-free bovine serum albumin; HRP, horseradish peroxidase.

S0006-2960(98)00744-2 CCC: $15.00
Published on Web 08/28/1998

sialosyl-Le^x expressing cells with platelets and endothelial cells was also inhibited by DMS (*27*). While the diverse actions of DMS may be mediated through inhibition of sphingosine kinase, it has been suggested that some of its effects may be mediated by inhibition of PKC (*28*−*30*), and that induction of apoptosis and decreased tumor growth by DMS may be related to inhibition of PKC activity (*22*, *24*, *31*). In this report, we show that DMS is a potent and competitive inhibitor of sphingosine kinase and does not affect activity or redistribution of PKC.

MATERIALS AND METHODS

Materials. Recombinant human TNF-α was from Boehringer (Indianapolis, IN). SPP, sphingosine, and *N*,*N*-dimethylsphingosine were from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]acetic anhydride (50 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Mouse 2.5S nerve growth factor and anti-protein kinase Cα were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-protein kinase Cδ was from Research and Diagnostic Antibodies (Berkeley, CA). HRP-conjugated secondary antibodies were purchased from Life Technologies (Gaithersburg, MD). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), dihydrosphingosine, diethylenetriaminepentaacetic acid (DETPAC), essentially fatty acid-free bovine serum albumin (BSA), bisindolylmaleimide, and bovine brain ceramides type IV were obtained from Sigma (St. Louis, MO). Cardiolipin was purchased from Avanti Polar Lipids (Birmingham, AL). Octyl β-D-glucopyranoside and *E. coli* diacylglycerol kinase were purchased from Calbiochem (La Jolla, CA). Serum and medium were obtained from Biofluids (Rockville, MD).

Cell Culture. Human monoblastic leukemia U937 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with 2 mM glutamine, penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 atmosphere and routinely subcultured every 2−3 days (*9*), and 10^7 cells were used for each experiment. Swiss 3T3 fibroblasts obtained from ATCC were subcultured at a density of 1.5×10^4 cells/cm^2 in DMEM supplemented with 2 mM glutamine and 10% calf serum, refed with the same medium after 2 days, and used 5 days later when the cells were confluent and quiescent (*8*). PC12 cells were a generous gift from Gordon Guroff (NICHD, NIH, Bethesda, MD) and were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% FBS (*11*). All experiments with PC12 cells were performed in the presence of serum unless otherwise indicated.

Sphingosine Kinase Activity. Sphingosine kinase activity was determined as previously described (*32*). Briefly, cells were washed with cold PBS, centrifuged and resuspended in buffer A [20 mM Tris buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 μg/mL each leupeptin and aprotinin, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine], disrupted by freeze−thawing, and the cytosolic fraction was prepared by centrifugation. Known amounts of cytosolic protein (20−50 μg) were incubated with sphingosine (50 μM), delivered as a sphingosine−BSA complex (1 mM in 4 mg of BSA/mL), unless otherwise indicated for kinetic studies. Buffer A was added to a final volume of 190 μL, and reactions were started by addition of 10 μL of [γ-^{32}P]ATP (10−20 μCi, 20 mM) containing 100 mM $MgCl_2$. Samples were incubated for 30 min at 37 °C, and reactions were terminated by addition of 20 μL of 1 N HCl. Lipids were extracted by addition of 0.5 mL of chloroform/methanol/concentrated HCl (100:200:1, v/v). Phases were separated by addition of 250 μL of chloroform and 250 μL of 2 M KCl. Lipids in the organic phase were resolved by TLC on silica gel G60 using 1-butanol/methanol/acetic acid/water (80:20:10:20, v/v) as solvent, and the radioactive spots corresponding to authentic SPP were quantified with a Molecular Dynamics Storm phosphoimager (Sunnyvale, CA).

Mass Measurements of SPP. SPP levels were determined as previously described (*11*, *33*). Briefly, 10^7 cells were washed with PBS, suspended in 3.5 mL of methanol/chloroform/PBS (2:1:0.5, v/v), and sonicated on ice. Alkaline extraction was performed by addition of 4.1 mL of chloroform/1 M KCl/concentrated NH_4OH (2:2:0.1, v/v). The aqueous phase containing SPP was extracted with 3.2 mL of chloroform/concentrated HCl (3:0.2, v/v), and the organic phases were then evaporated under nitrogen and solubilized in 20 μL of 0.008 N NaOH/methanol. Acetylation reactions proceeded with 20 μL of 10 mM [^{3}H]acetic anhydride (10 μCi) at 37 °C for 2 h, and acetylated SPP was extracted with 2.77 mL of methanol/chloroform/1 M KCl/concentrated HCl (0.75:1:1:0.02, v/v). The chloroform phases were washed twice with 1 mL of chloroform/methanol/water (3:48:47, v/v) plus 10 μL of concentrated HCl and then evaporated under nitrogen. Samples were dissolved in 200 μL of chloroform/methanol (2:1, v/v) and separated by TLC on silica gel G60 using butanol/acetic acid/water (3:1:1, v/v). Bands corresponding to acetylated SPP were scraped from the plates and counted with a scintillation counter.

[^{3}H]SPP Formation. During the final 25 min of treatments with various agents, 10^7 cells were incubated with [^{3}H]sphingosine (2 μCi, 1 μM) added as a BSA (4 mg/mL) complex. Cells were then washed with PBS, harvested in 4 mL of MeOH/chloroform (1:1 v/v), and sonicated briefly on ice. Then 100 μL of concentrated NH_4OH and 2 mL of 1 M KCl were added, and after phase separation, the aqueous layer was collected, acidified with 200 μL of concentrated HCl, and reextracted with 2 mL of chloroform. Organic extracts were dried under nitrogen, resuspended in 100 μL of 0.08 N NH_4OH/MeOH plus 100 μL of chloroform/MeOH (2:1, v/v), and separated by TLC with chloroform/methanol/25 mM Na_2HPO_4 (60:35:8, v/v). The spots corresponding to authentic SPP were scraped from the TLC plates and counted with a scintillation counter.

Mass Measurements of Ceramide. Lipids were extracted, and mass amounts of ceramide in cellular extracts were measured by the DAG kinase enzymatic method (*34*). Briefly, an aliquot (10−50 nmol of total phospholipid) of the chloroform phase from cellular lipid extracts or standard bovine brain type IV ceramides was resuspended in 40 μL of 7.5% (w/v) octyl β-D-glucopyranoside/5 mM cardiolipin in 1 mM DETPAC/10 mM imidazole (pH 6.6) and solubilized by freeze−thawing and subsequent sonication. The enzymatic reaction was started by the addition of 20 μL of DTT (20 mM), 10 μL of *E. coli* diacylglycerol kinase (0.88

unit/mL), 20 μL of [γ-^{32}P]ATP (10−20 μCi, 10 mM), and 100 μL of reaction buffer [100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM EGTA]. After incubation for 1 h at room temperature, lipids were extracted with 1 mL of chloroform/methanol/concentrated HCl (100:100:1, v/v) and 0.17 mL of 1 M KCl. Labeled phosphatidic acid and ceramide 1-phosphate were resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) and quantified with a Molecular Dynamics Storm phosphoimager.

Measurement of Total Cellular Phospholipids. Total phospholipids present in cellular lipid extracts used for sphingolipid analyses were quantified as previously described (*35*) with minor modifications. Briefly, to dried aliquots of cellular lipid extracts was added 40 μL of a mixture of concentrated H_2SO_4/70% perchloric acid (1:3, v/v), and samples were incubated for 30 min at 210 °C. After cooling, 75 μL of water and 400 μL of 4.2% ammonium molybdate in 4 N HCl/0.045% (w/v) malachite green (1:3, v/v) were added. Samples were incubated at 37 °C for 15 min and absorbances measured at 660 nm.

Protein Kinase C Assay. After various treatments, cells were washed twice with PBS, scraped from dishes in kinase buffer [0.1 M Tris-HCl (pH 7.4) containing 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM Na_3VO_4, 15 mM NaF, 10 mg/mL each leupeptin and aprotinin, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine], and lysed by freeze−thawing 3 times. After centrifugation at 14000*g* for 20 min, supernatant was saved as the cytosolic fraction, and the pellet was resuspended by passing through a 27 gauge needle 10 times in kinase buffer containing 0.1% Triton X-100. After centrifugation for 20 min at 14000*g*, supernatant from this preparation was collected and designated as the membrane fraction. Equal amounts of protein from cytosolic and membrane fractions were assayed for PKC activity, using a protein kinase C assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) according to manufacturer's specifications. In some cases, cytosolic and membrane preparations of PKC were partially purified on DEAE-cellulose columns as previously described (*36*).

Western Blotting. Aliquots (5−10 μg) of protein from cytosolic and membrane fractions were separated on 10% SDS−PAGE. After transblotting to nitrocellulose (0.45 μm), the membrane was blocked overnight in 5% nonfat dry milk in PBS containing 0.05% Tween 20 (PBS−Tween), and then incubated with primary antibody (1:2000) for 5 h. After five washes in PBS−Tween, the membrane was incubated for 1 h with goat anti-mouse IgG HRP-conjugate (1:10 000 dilution). After five additional washes, the blot was incubated for 1 min using the ECL detection system (Amersham) according to the manufacturer's instructions, and bands were visualized by exposure to X-ray film. Blots were reprobed for different PKC isoforms after stripping in 0.1 M glycine (pH 2.9) for 30 min with agitation.

Statistics. Data were analyzed by the Student's *t*-test, with p values <0.05 considered to be statistically significant.

RESULTS

In Vitro Inhibition of Sphingosine Kinase by DMS. DMS potently inhibited sphingosine kinase activity present in cytosolic extracts of U937, Swiss 3T3, and PC12 cells in a dose-dependent manner (Figure 1A−C). Sphingosine kinase activity was inhibited approximately 50% by 5 μM DMS, and was maximally inhibited by 20−25 μM DMS. As previously described (*8*), DHS also inhibited sphingosine kinase in U937 and Swiss 3T3 cell extracts (Figure 1A,B), with a similar potency to DMS. However, DHS only slightly inhibited sphingosine kinase activity in PC12 cells at low concentrations, but had no apparent effect at 10 μM (Figure 1C), whereas DMS inhibited sphingosine kinase with similar potency to that seen in Swiss 3T3 and U937 extracts. At higher concentrations, DHS acted as an effective substrate for sphingosine kinase from PC12 cells, producing dihydrosphingosine 1-phosphate (data not shown). Since SPP and dihydrosphingosine 1-phosphate differ only by the presence of one double bond, they cannot be separated by our thin-layer chromatographic technique, which accounts for the increase in phosphorylated product formed with increasing concentrations of DHS used (Figure 1C). In contrast to DHS, DMS is not a substrate for sphingosine kinase. Therefore, while DHS is an effective inhibitor in some cell types, such as Swiss 3T3 cells (*8*, *37*), DMS generally appears to be a more potent and effective inhibitor of sphingosine kinase.

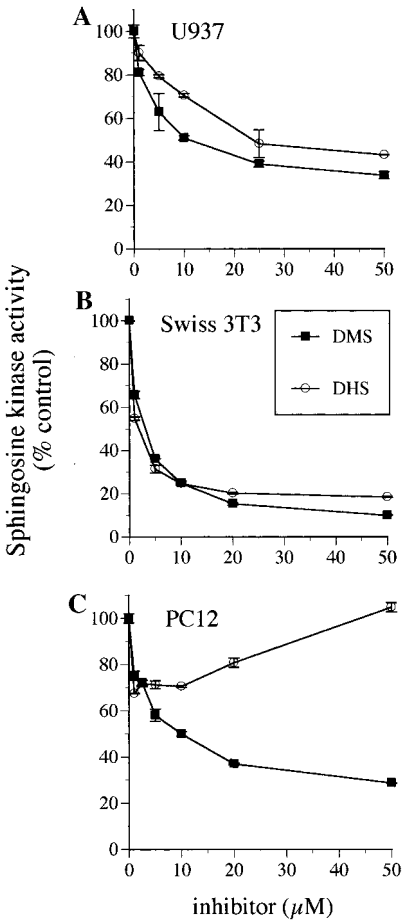

FIGURE 1: Inhibition of sphingosine kinase by DHS and DMS in vitro. Sphingosine kinase activity in cytosolic extracts of U937 (A), Swiss 3T3 (B), and PC12 (C) cells was measured in the presence of the indicated concentrations of DMS (filled squares) or DHS (open circles) using 10 μM D-*erythro*-sphingosine as substrate. Results are expressed as percent of the activity measured in the absence of inhibitors and are representative of three independent experiments. Error bars represent standard deviations of duplicate determinations.

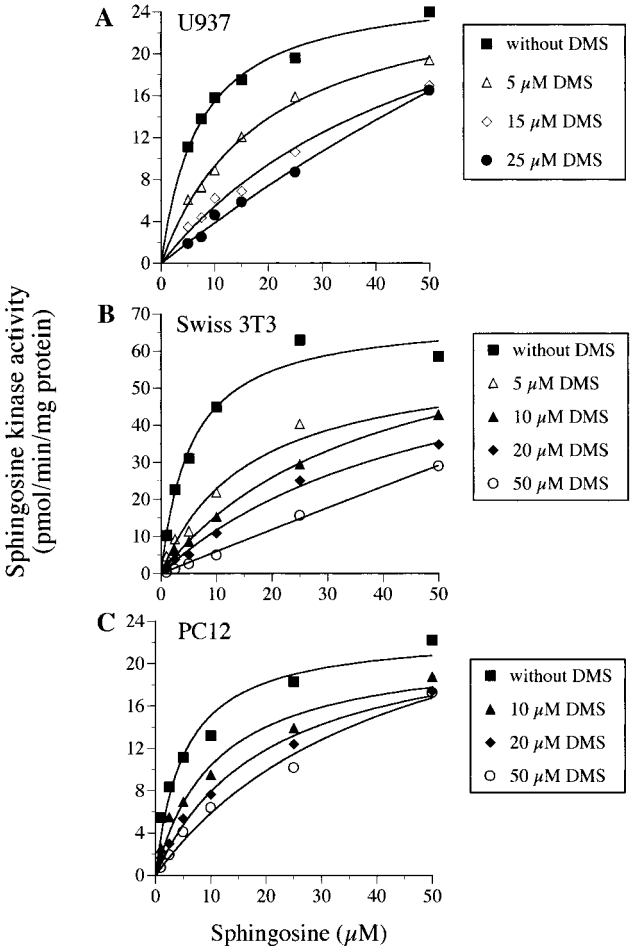

FIGURE 2: Competitive inhibition of sphingosine kinase by DMS in vitro. Sphingosine kinase activity in cytosolic extracts from U937 (A), Swiss 3T3 (B), and PC12 (C) cells was measured in the presence of increasing concentrations of sphingosine and the indicated concentrations of DMS. Sphingosine kinase activity is expressed as picomoles of SPP formed per minute per milligram of protein and is the mean of quadruplicate determinations.

DHS is a competitive inhibitor of sphingosine kinase in platelets (*15*, *38*). However, the mechanism of sphingosine kinase inhibition by DMS has not previously been characterized. Cytosolic preparations of sphingosine kinase from Swiss 3T3, PC12, and U937 cells were incubated with various concentrations of sphingosine in the presence or absence of increasing concentrations of DMS (Figure 2A−C). In the absence of inhibitor, sphingosine kinase activity showed typical Michaelis−Menten kinetics with K_M values of approximately 5 μM, in agreement with previous reports (*32*, *39*). This was similar to the K_M for sphingosine kinase purified from rat kidneys (*40*). The V_{max} was 3-fold greater in Swiss 3T3 fibroblasts than in U937 or PC12 cells (Table 1). By hyperbolic curve-fitting, apparent K_M values in U937, Swiss 3T3, and PC12 cells increased to 98.9, 51.5, and 43.5 μM, respectively, in the presence of 50 μM DMS, while V_{max} values did not significantly change, indicating that DMS is a competitive inhibitor of sphingosine kinase with a K_i of approximately 5 μM.

Effect of DMS on Sphingosine Kinase Activity in Intact Cells and on Levels of SPP and Ceramide. The effect of DMS on sphingosine kinase activity was assessed in intact U937 cells by measuring the formation of [^{3}H]SPP in cells labeled with [^{3}H]sphingosine. Treatment with 50 nM TPA for 30 min stimulated sphingosine kinase activity 1.3-fold, which is comparable to previously reported effects of TPA in these cells (*9*). DMS potently decreased formation of [^{3}H]-SPP by 60% both in untreated and in TPA-stimulated cells. Similar results were obtained with PC12 cells in the absence or presence of NGF (data not shown). If DMS is an effective inhibitor of sphingosine kinase in intact cells, treatment of cells with DMS should decrease cellular levels of SPP. Pretreatment of U937 cells with DMS decreased basal SPP levels more effectively than TNF-α (Figure 3A), which has previously been shown to decrease SPP levels in these cells (*9*). In addition, for DMS to be a useful tool to explore the role of sphingosine kinase in signal transduction, it should prevent agonist-stimulated increases in SPP. TPA treatment of U937 cells, which stimulates sphingosine kinase activity (*9*), increased SPP levels, and this response was completely prevented by pretreatment with DMS (Figure 3A).

Table 1: Kinetic Parameters for Sphingosine Kinase in Cytosolic Extracts of U937, Swiss 3T3, and PC12 Cells[a]

cells	K_m (μM)	V_{max} (pmol min^{-1} mg^{-1})	K_i (μM)
U937	5.6	24.1	3.1
PC12	5.2	22.0	6.8
Swiss 3T3	5.5	69.6	2.3

[a] K_m and V_{max} values were determined by hyperbolic curve-fitting of the kinetic data in Figure 2 with Delta Graph 4.0 for Macintosh. K_i values for DMS were determined using the Michaelis−Menten equation modified for competitive inhibition.

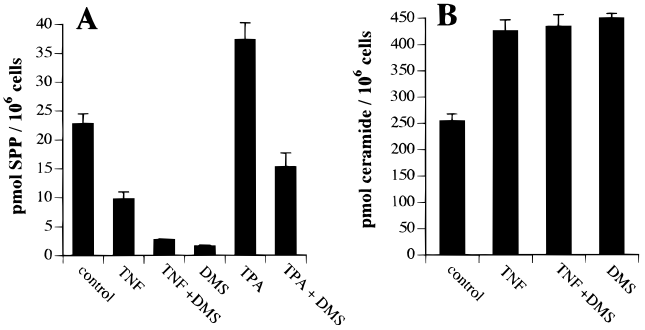

FIGURE 3: Effect of DMS on sphingosine kinase and levels of SPP and ceramide in U937 cells. U937 cells were treated in serum-free medium without or with DMS (10 μM) for 30 min and then stimulated with TNF-α (1000 units/mL) or TPA (50 nM) as indicated for 15 min, and SPP (A) and ceramide (B) levels were measured. Data are representative of three independent experiments, and all results were statistically significant ($p < 0.05$) compared to controls.

DMS is a potent inducer of apoptotic cell death (*9*, *21*−*23*). As ceramide has been implicated as a mediator of apoptosis, it was of interest to determine whether DMS also affects cellular levels of this sphingolipid metabolite. Indeed, ceramide levels were elevated in U937 cells treated with 10 μM DMS (Figure 3C) to levels similar to that seen with TNF-α, which has been shown to stimulate SMase resulting in increased ceramide levels (*1*, *41*−*43*).

The effect of DMS on sphingolipid metabolite levels is not limited to U937 cells, as similar results were seen in PC12 cells. Although DMS did not significantly affect basal levels of SPP, it completely prevented NGF- or TPA-induced increases in SPP (Figure 4A). In addition, DMS elevated ceramide levels in these cells with a maximal increase after

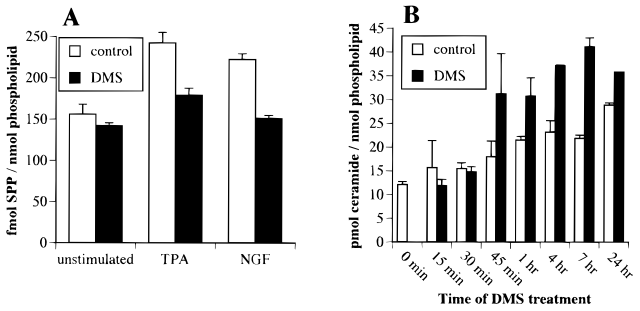

FIGURE 4: Effect of DMS on SPP and ceramide levels in PC12 cells. (A) PC12 cells were pretreated with 10 μM DMS for 1 h and then stimulated with NGF (100 ng/mL, 3 h) or TPA (100 nM, 30 min), and SPP levels were subsequently measured. (B) PC12 cells (2×10^6) cultured in serum-free medium were treated without or with DMS (10 μM) for the indicated time, and ceramide levels were determined. Data are means $\pm$ SD of triplicate determinations, and treatments with NGF or TPA in the absence or presence of DMS were statistically significant ($p < 0.05$). In (B), elevation of ceramide levels after serum withdrawal or treatment with DMS was statistically significant ($p < 0.05$) compared to controls after 1 h.

7 h of treatment (Figure 4B). It should be noted that serum deprivation also results in increased ceramide levels in PC12 cells as previously described in other cell types (*2*). Thus, DMS effectively decreases the ratio of SPP to ceramide in a variety of cell types, which may account for its pro-apoptotic effects.

Lack of Effect of DMS on PKC Activity. In addition to its role as an inhibitor of sphingosine kinase, DMS has been reported to inhibit PKC in vitro (*24*, *30*, *44*). However, the effect of DMS on PKC in intact cells has not yet been determined. Sphingosine has also been shown to be an in vitro inhibitor of PKC [reviewed in (*45*, *46*)]. However, numerous studies have shown that sphingosine has diverse biological actions which are clearly unrelated to its effect on PKC activity [reviewed in (*47*)]. We compared the effects of DMS on the activities of sphingosine kinase and PKC in PC12 cells. DMS pretreatment prevented TPA- and NGF-stimulated sphingosine kinase activation (Figure 5A). However, DMS had no effect on activation of PKC by either TPA or NGF (Figure 5B). In contrast, the pan-specific PKC inhibitor, bisindolylmaleimide, reduced activation of sphingosine kinase by TPA, but had no effect on NGF-stimulated sphingosine kinase activity. These results are in agreement with previous studies in other cells types demonstrating that activation of PKC results in the stimulation of sphingosine kinase (*9*, *15*, *48*). As expected, bisindolylmaleimide, which is a potent and selective inhibitor of PKC (*49*), effectively blocked PKC activation by either TPA or NGF.

We also examined PKC activity in cytosolic and membrane fractions as well as the subcellular localization and PKCα and PKCδ in PC12 cells treated with TPA, DMS, or both. As shown in Figure 6A, DMS treatment had no effect on basal levels of either membrane-associated or cytosolic PKC activity in PC12 cells. Moreover, 10 μM DMS had no effect on the increased membrane-associated PKC activity in TPA-treated PC12 cells. Similar results were seen in U937 cells and Swiss 3T3 fibroblasts (data not shown). As expected, Western blotting of cytosolic and membrane fractions from TPA-treated PC12 cells showed translocation of PKCα and PKCδ to membrane fractions (Figure 6B). This TPA-induced translocation of PKCα or PKCδ in PC12 cells

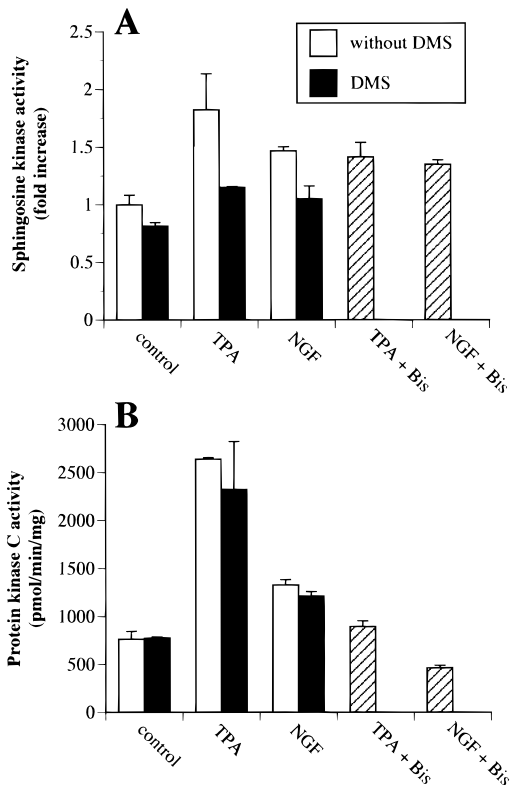

FIGURE 5: DMS inhibits sphingosine kinase but not PKC in intact PC12 cells. PC12 cells (4×10^6) were incubated in the absence or presence of DMS (10 μM) for 3 h or bisindolylmaleimide (5 μM) for 30 min, treated without or with TPA (200 nM) or NGF (100 ng/mL) for 15 min, and then (A) cytosolic sphingosine kinase activity or (B) membrane-associated PKC activity was measured. Inhibition by DMS of stimulation of sphingosine kinase by TPA and NGF was statistically significant ($p < 0.05$), whereas there were no statistically significant effects of DMS on PKC activity.

was not significantly affected by DMS (Figure 6B), even at concentrations as high as 50 μM (Figure 6C). DMS was able to completely reverse TPA-induced activation of sphingosine kinase in PC12 cells, with inhibition occurring at concentrations as low as 1 μM, whereas membrane-associated PKC activity was not significantly affected and remained elevated approximately 4−5-fold in cells treated with up to 50 μM DMS (data not shown). Similarly, DMS did not affect PKC activity or TPA-stimulated membrane translocation of either PKCα or PKCδ in Swiss 3T3 cells (data not shown). Thus, DMS potently inhibits sphingosine kinase activity in a variety of different cell types without significantly affecting PKC activity or translocation.

DISCUSSION

Previously, DHS has been used as an inhibitor of sphingosine kinase to examine the role of SPP as an intracellular second messenger (*8*, *10*, *37*). DHS effectively inhibited mitogenesis and activation of two cyclin-dependent kinases ($p34^{cdc2}$ kinase and Cdk2 kinase) induced by PDGF, but not by EGF (*37*). DHS not only prevented PDGF-induced increases in SPP levels but also markedly reduced PDGF-stimulated, but not EGF-stimulated, MAP kinase activity and DNA binding activity of activator protein-1 (AP-1). Examination of the early signaling events of PDGF action revealed that DHS did not affect PDGF-induced autophosphorylation of the PDGF receptor or phosphorylation of the

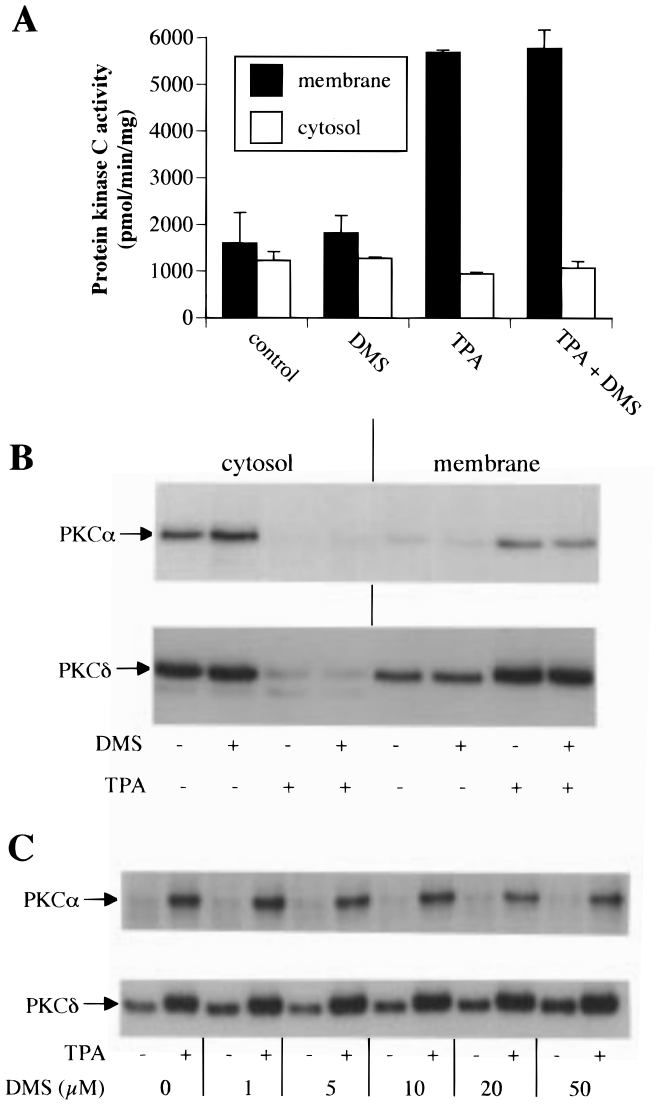

FIGURE 6: Lack of effect of DMS on PKC activity and membrane translocation in PC12 cells. PC12 cells (4×10^6) were incubated in the absence or presence of DMS (10 μM) for 3 h and then stimulated without or with TPA (200 nM) for 15 min, and PKC activity was measured in both membrane and cytosolic fractions (A) or analyzed by Western blotting using antibodies specific for PKCα and PKCδ (B). In (C), PC12 cells were treated with the indicated concentrations of DMS for 3 h and then stimulated without or with TPA (200 nM) for 15 min. Membrane fractions were resolved on SDS−PAGE gels and analyzed by Western blotting using antibodies specific for PKCα and PKCδ. Data are representative of four independent experiments. No statistically significant differences were observed between controls and TPA-stimulated cells treated with DMS (A).

SH2/SH3 adapter protein Shc and its association with Grb2 (*37*). These experiments allowed us to conclude that regulation of sphingosine kinase activity defines divergence in signal transduction pathways of PDGF and EGF receptors leading to MAP kinase activation. In addition, DHS has also been used to demonstrate an important role of sphingosine kinase-generated SPP in calcium mobilization induced by cross-linking of the FcϵRI in mast cells (*10*).

In this study, we found, in agreement with previous studies (*8*, *15*, *37*), that while DHS is an effective sphingosine kinase inhibitor in some cell types, such as Swiss 3T3 cells, DMS generally appears to be a more potent and effective inhibitor of sphingosine kinase. Previously, it has been shown that the K_i for inhibition of platelet and mast cell sphingosine kinase by DHS is 5−18 μM (*10*, *15*), while our studies indicated that the K_i for inhibition of sphingosine kinase from U937, Swiss 3T3, and PC12 cells by DMS was 2−5 μM. Thus, 10 μM DMS is sufficient to inhibit sphingosine kinase activity. Higher concentrations of DMS ($\geq$50 μM) should be avoided as they have nonspecific toxic effects. Another disadvantage of DHS is that in some cell types it may be phosphorylated by sphingosine kinase to dihydrosphingosine 1-phosphate, which could have other biological effects. In contrast, DMS is not a substrate for sphingosine kinase from any of the cell types that were examined in this study. Furthermore, the lower solubility of DHS compared to DMS reduces its efficacy.

It has been shown that DMS is a potent and specific pharmacological inhibitor of PKC activity in vitro, and it was suggested that some of its effects, particularly its pro-apoptotic effect, may be mediated by inhibition of PKC (*28*−*30*). However, our results cast doubt on this conclusion. We found that DMS effectively inhibited endogenous and NGF- or TPA-stimulated sphingosine kinase activity, without significantly affecting PKC activity. Stimulation of conventional and novel PKC isoforms by TPA is associated with translocation from the cytosol to the membrane fraction, and this translocation event is often used as an indicator of PKC activation (*50*). In this study, TPA-induced translocation of PKC was also not influenced by DMS. In this regard, it is interesting to compare the effects of DMS and the PKC inhibitor, bisindolylmaleimide, on sphingosine kinase activity and PKC activations induced by NGF or TPA. Whereas bisindolylmaleimide inhibited activation of PKC induced by TPA or NGF, it only inhibited TPA-stimulated but not NGF-stimulated sphingosine kinase activity, suggesting that NGF stimulates sphingosine kinase activity by a mechanism which is independent of PKC. The relationship between sphingosine kinase and PKC is not completely understood. Although known stimulators of PKC, such as TPA, and diacylglycerol stimulate sphingosine kinase (*8*−*11*, *15*, *20*), growth factor induced sphingosine kinase activation has been shown to occur in PKC down-regulated cells (*8*), suggesting that activation of sphingosine kinase can occur independently of PKC.

DMS prevented increases in SPP levels in response to TPA or NGF, agents which are known to activate sphingosine kinase in U937 and PC12 cells, respectively. However, while DMS markedly reduced basal levels in U937 cells, it only had a small effect on basal SPP levels in PC12 cells. This differential effect could be due to different rates of turnover of SPP in these two cell lines. Interestingly, DMS also induced increases in ceramide levels in both cell lines. Since cellular levels of ceramide are determined by the balance between synthesis and degradation, further studies are necessary to determine the site of action of DMS in regulating ceramide levels. However, it is intriguing to note that DMS effectively increases the ratio of ceramide to SPP. We have previously shown that SPP protects cells from apoptosis induced by ceramide, and proposed that a ceramide/SPP rheostat is an important factor for regulation of cell survival (*9*). Thus, resetting of this rheostat by DMS could explain its pro-apoptotic effects.

In summary, we have shown that DMS is a potent competitive inhibitor of sphingosine kinase and prevents the

formation of SPP in response to known activators of sphingosine kinase in intact cells without affecting PKC activity or redistribution. Thus, DMS is an effective and selective tool to investigate the biological functions of sphingosine kinase and SPP and enables us to discriminate between sphingosine kinase dependent and independent signaling pathways downstream of PKC.

ACKNOWLEDGMENT

We thank Dr. Ana Olivera for helpful suggestions and discussion.

REFERENCES

1. Kolesnick, R., and Golde, D. W. (1994) *Cell 77*, 325−328.
2. Hannun, Y. (1996) *Science 274*, 1855−1859.
3. Spiegel, S., and Milstien, S. (1995) *J. Membr. Biol. 146*, 225−237.
4. Spiegel, S., Foster, D., and Kolesnick, R. (1996) *Curr. Opin. Cell Biol. 8*, 159−167.
5. Obeid, L. M., Linardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) *Science 259*, 1769−1771.
6. Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. N. (1996) *Nature 380*, 75−79.
7. Santana, P., Peña, L. A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, E. H., Fuks, Z., and Kolesnick, R. (1996) *Cell 86*, 189−199.
8. Olivera, A., and Spiegel, S. (1993) *Nature 365*, 557−560.
9. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996) *Nature 381*, 800−803.
10. Choi, O. H., Kim, J.-H., and Kinet, J.-P. (1996) *Nature 380*, 634−636.
11. Edsall, L. C., Pirianov, G. G., and Spiegel, S. (1997) *J. Neurosci. 17*, 6952−6960.
12. Kleuser, B., Cuvillier, O., and Spiegel, S. (1998) *Cancer Res. 58*, 1817−1824.
13. Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) *J. Biol. Chem. 270*, 23305−23309.
14. Mandala, S., Thornton, R., Tu, Z., Kurtz, M., Nickels, J., Broach, J., Menzeleev, R., and Spiegel, S. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 150−155.
15. Buehrer, B. M., and Bell, R. M. (1992) *J. Biol. Chem. 267*, 3154−3159.
16. Olivera, A., Rosenthal, J., and Spiegel, S. (1996) *J. Cell. Biochem. 60*, 529−537.
17. Van Veldhoven, P. P., and Mannaerts, G. P. (1991) *J. Biol. Chem. 266*, 12502−12507.
18. Van Veldhoven, P. P., and Mannaerts, G. P. (1994) *Biochem. J. 299*, 597−601.
19. Saba, J. D., Nara, F., Bielawska, A., Garrett, S., and Hanun, Y. A. (1997) *J. Biol. Chem. 272*, 26087−26090.
20. Yatomi, Y., Ruan, F., Megidish, T., Toyokuni, T., Hakomori, S., and Igarashi, Y. (1996) *Biochemistry 35*, 626−633.
21. Park, Y. S., Ruan, F., Hakomori, S., and Igarashi, Y. (1995) *Int. J. Oncol. 7*, 487−494.
22. Ohta, H., Yatomi, Y., Sweeney, E. A., Hakomori, S., and Igarashi, Y. (1994) *FEBS Lett. 355*, 267−270.
23. Sweeney, E. A., Sakakura, C., Shirahama, T., Masamune, A., Ohta, H., Hakomori, S., and Igarashi, Y. (1996) *Int. J. Cancer 66*, 358−366.
24. Endo, K., Igarashi, Y., Nisar, M., Zhou, Q. H., and Hakomori, S. (1991) *Cancer Res. 51*, 1613−1618.
25. Uemura, K., Hara, A., and Taketomi, T. (1993) *J. Biochem. (Tokyo) 114*, 610−614.
26. Okoshi, H., Hakomori, S., Nisar, M., Zhou, Q. H., Kimura, S., Tashiro, K., and Igarashi, Y. (1991) *Cancer Res 51*, 6019−6024.
27. Handa, K., Igarashi, Y., Nisar, M., and Hakomori, S. (1991) *Biochemistry 30*, 11682−11686.
28. Felding-Habermann, B., Igarashi, Y., Fenderson, B. A., Park, L. S., Radin, N. S., Inokuchi, J., Strassmann, G., Handa, K., and Hakomori, S. (1990) *Biochemistry 29*, 6314−6322.
29. Igarashi, Y., and Hakomori, S. (1989) *Biochem. Biophys. Res. Commun. 164*, 1411−1416.
30. Khan, W., Dobrowsky, R., Touny, S., and Hannun, Y. (1990) *Biochem. Biophys. Res. Commun. 172*, 683−691.
31. Sakakura, C., Sweeney, E. A., Shirahama, T., Hakomori, S., and Igarashi, Y. (1996) *FEBS Lett. 379*, 177−180.
32. Olivera, A., Rosenthal, J., and Spiegel, S. (1994) *Anal. Biochem. 223*, 306−312.
33. Yatomi, Y., Ruan, F., Ohta, H., Welch, R. J., Hakomori, S., and Igarashi, Y. (1995) *Anal. Biochem. 230*, 315−320.
34. Olivera, A., Buckley, N. E., and Spiegel, S. (1992) *J. Biol. Chem. 267*, 26121−26127.
35. Van Veldhoven, P. P., and Mannaerts, G. P. (1987) *Anal. Biochem. 161*, 45−48.
36. Olivera, A., Romanowski, A., Rani, C. S., and Spiegel, S. (1997) *Biochim. Biophys. Acta 1348*, 311−323.
37. Rani, C. S., Berger, A., Wu, J., Sturgill, T. W., Beitner-Johnson, D., LeRoith, D., Varticovski, L., and Spiegel, S. (1997) *J. Biol. Chem. 272*, 10777−10783.
38. Stoffel, W., Hellenbroich, B., and Heimann, G. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 1311−1316.
39. Rius, R. A., Edsall, L. C., and Spiegel, S. (1997) *FEBS Lett. 417*, 173−176.
40. Olivera, A., Kohama, T., Tu, Z., Milstien, S., and Spiegel, S. (1998) *J. Biol. Chem. 273*, 12576−12583.
41. Dressler, K. A., Mathias, S., and Kolesnick, R. N. (1992) *Science 255*, 1715−1718.
42. Hannun, Y. A. (1994) *J. Biol. Chem. 269*, 3125−3128.
43. Heller, R. A., and Kronke, M. (1994) *J. Cell Biol. 126*, 5−9.
44. Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., el-Ghendy, K., and Racker, E. (1989) *Biochemistry 28*, 6796−6800.
45. Hannun, Y. A., and Bell, R. M. (1989) *Science 243*, 500−507.
46. Merrill, A. H., Jr., Hannun, Y. A., and Bell, R. M. (1993) *Adv. Lipid Res. 25*, 1−24.
47. Spiegel, S., and Merrill, A. H., Jr. (1996) *FASEB J. 10*, 1388−1397.
48. Mazurek, N., Megidish, T., Hakomori, S.-I., and Igarashi, Y. (1994) *Biochem. Biophys. Res. Commun. 198*, 1−9.
49. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., and Loriolle, F. (1991) *J. Biol. Chem. 266*, 15771−15781.
50. Nishizuka, Y. (1995) *FASEB J. 9*, 484−496.

BI980744D