N,N-Dimethylsphingosine Inhibition of Sphingosine Kinase and Sphingosine 1-Phosphate Activity in Human Platelets†

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ABSTRACT: Potential sphingosine (Sph) metabolites include phosphorylated, *N*-acylated, and *N*-methylated derivatives. Phosphorylated Sph, i.e., sphingosine 1-phosphate (Sph-1-P), may act as an autocrine stimulator of blood platelets, as it is abundantly stored in platelets and released extracellularly and its exogenous addition induces platelet activation. In this study, we evaluated Sph-1-P formation and its effects in human platelets in the presence of other Sph metabolites. On addition of [^{3}H]Sph to intact platelets, the label was rapidly converted to Sph-1-P. This conversion into [^{3}H]Sph-1-P was inhibited by *N,N*-dimethylsphingosine (DMS) in a dose-dependent manner, but not by other structurally related Sph derivatives, including ceramide. The inhibition of Sph-1-P formation by DMS was reproduced using a cell-free system (Sph kinase obtained from platelet cytosolic fractions) and much stronger than that by DL-*threo*-dihydrosphingosine, which had been considered to be the strongest inhibitor of Sph kinase. Administration of DMS to intact platelets resulted in a decrease in Sph-1-P mass and an increase in Sph mass. Furthermore, DMS inhibited the release of Sph-1-P from platelets stimulated with 12-*O*-tetradecanoylphorbol 13-acetate and inhibited platelet aggregation induced by exogenous addition of Sph-1-P. Collectively, our results indicate that DMS is useful as a Sph kinase inhibitor and that Sph-1-P actions as an autocrine stimulator of platelets are inhibited by DMS.

Sphingosine (Sph)[1] and its metabolites have been implicated as modulators of membrane signal transduction systems and shown to be involved in diverse cellular processes (Hakomori, 1990; Hannun & Bell, 1989; Kolesnick & Golde, 1994; Merrill & Stevens, 1989; Spiegel et al., 1993). Sphingosine 1-phosphate (Sph-1-P), formed from Sph by Sph kinase, has recently been added to the list of these bioactive sphingolipids. Sph-1-P stimulates cell proliferation in Swiss 3T3 fibroblasts, possibly via induction of intracellular Ca^{2+} mobilization, enhanced phosphatidic acid synthesis or mitogen-activated protein kinase activation (Desai et al., 1992; Mattie et al., 1994; Wu et al., 1995), whereas this phosphorylated sphingoid base inhibits cell motility and invasiveness of certain tumor cells (Sadahira et al., 1992) and PDGF-induced chemotaxis of human arterial smooth muscle cells (Bornfeldt et al., 1995). In anucleate blood platelets, which lack a nucleus and hence the ability to proliferate, Sph-1-P is rapidly converted from Sph, abundantly stored intracellularly, and released into the extracellular environment upon stimulation with physiological agonists (Yatomi et al., 1995a). Furthermore, exogenously added Sph-1-P induces platelet aggregation (Yatomi et al., 1995a), suggesting that Sph-1-P acts as an autocrine stimulator of platelets.

Although it is clear that Sph-1-P has important physiologic functions in addition to its role as a metabolite of Sph, Sph-1-P is not the only metabolite of Sph. Sph can also be *N*-acylated to ceramide (Cer) (Goldkorn et al., 1991) and *N*-methylated to *N,N*-dimethylsphingosine (DMS) in some cancer cell lines (Igarashi et al., 1990; Felding-Habermann et al., 1990; Goldkorn et al., 1991) and tissues (Igarashi & Hakomori, 1989; Nudelman et al., 1992). Hence, elucidating mutual interaction or regulation among these Sph metabolites is essential to clarify the biologic sequelae of Sph metabolism. In this study, we show that DMS is a potent inhibitor of Sph kinase and that the levels and effects of Sph-1-P are inhibited by this methyl derivative of Sph.

MATERIALS AND METHODS

Materials. Sph-1-P was prepared from sphingosylphosphocholine with bacterial phospholipase D as previously described (Van Veldhoven et al., 1989). C_2-Cer (*N*-acetylated Sph) (Vunnam & Radin, 1979), DMS (Igarashi et al., 1989), and *N,N,N*-trimethylsphingosine (TMS) (Toyokuni et al., 1991) were synthesized as described previously. [^{14}C]DMS was prepared from Sph by reductive methylation in a solution containing $^{14}CH_2O$, $NaCNBH_3$, sodium acetate, and acetic acid. [^{14}C]TMS was prepared from DMS by treatment with $^{14}CH_3I$ and K_2CO_3 in methanol. DMS-1-P and TMS-1-P were chemically synthesized, which will be described elsewhere.[2] [1-^{3}H]Sph was synthesized as described (Toyokuni et al., 1991) and used for *Sph Kinase Activity Assay* and [^{3}H]C_2-Cer synthesis, which was performed by *N*-acylation of [1-^{3}H]Sph with acetic anhydride. [^{3}H]Sph-1-P was prepared by ATP-dependent phosphorylation of [^{3}H]-

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[1] Abbreviations: Sph, sphingosine; Sph-1-P, sphingosine-1-phosphate; Cer, ceramide; DMS, *N,N*-dimethylsphingosine; TMS, *N,N,N*-trimethylsphingosine; DHS, dihydrosphingosine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TLC, thin-layer chromatography.

[2] F. Ruan, S. Yamamura, S. Hakomori, and Y. Igarashi, manuscript in preparation.

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Sph catalyzed by Sph kinase obtained from Balb/c 3T3 fibroblasts (Mazurek et al., 1994).

Platelet Preparation. Preparation of washed platelets was performed as previously described (Yatomi et al., 1994). The platelets were finally resuspended in a buffer containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1.0 mM $MgCl_2$, 1 mg/mL glucose, and 20 mM Hepes (pH 7.4). Unless stated otherwise, the final platelet suspensions were adjusted to 3×10^8/mL. When indicated, 1% bovine serum albumin (fatty acid-free) was added. All experiments using intact platelet suspensions were performed at 37 °C.

For *Sph Kinase Activity Assay*, outdated platelet concentrates obtained from Oregon Red Cross (Portland, OR) were used.

Metabolism of [^{3}H]Sph in Platelets. Platelet suspensions (0.5 mL) were incubated with 1 μM (0.2 μCi) [3-^{3}H]Sph (Du Pont-New England Nuclear, Boston, MA). At the indicated time points, the reaction was terminated by addition of 1.875 mL of ice-cold chloroform/methanol/concentrated HCl (100:200:1), and lipids were extracted and analyzed for [^{3}H]Sph metabolism as described previously (Yatomi et al., 1995a). Portions of lipids obtained from the lower chloroform phase were applied to silica gel HPTLC plates (Merck, Darmstadt, Germany), and the plates were developed in butanol/acetic acid/water (3:1:1), followed by autoradiography. Each autoradiogram shown is a typical one from at least three experiments. When indicated, silica gel areas containing radiolabeled sphingolipids were scraped off and counted by liquid scintillation counting.

In platelet [^{3}H]Sph-1-P release studies, platelet suspensions, to which 1% bovine serum albumin was added, were centrifuged for 15 s at 12000*g*. Lipids were then extracted from the resultant medium supernatant and cell pellet and analyzed as described above. Albumin was included in the medium to prevent released Sph-1-P, a lipophilic molecule, from being nonspecifically attached to the plasma membrane surface and consequently underestimated. In fact, 5.5 ± 0.2% (mean ± range, $n = 2$) and 38.6 ± 4.3% (mean ± SD, $n = 3$) of total Sph-1-P was released from platelets stimulated with 1 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) for 5 min in the absence and presence of albumin, respectively.

Metabolism of [^{14}C]DMS, [^{14}C]TMS, and [^{3}H]C_2-Cer in Platelets. Platelet suspensions (0.5 mL) were incubated with 5 μM [^{14}C]DMS, [^{14}C]TMS, or [^{3}H]C_2-Cer ($2-4 \times 10^4$ cpm). At the indicated times, the reaction was terminated, and lipids were extracted and separated by thin-layer chromatography (TLC) developed in butanol/acetic acid/water (3:1:1), followed by autoradiography as described (Yatomi et al., 1995a).

Sph Kinase Activity Assay. Washed platelets (5×10^{10} cells) were suspended in 15 mL of an ice-cold buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, and 1 mM phenylmethanesulfonyl fluoride. Subsequent operations were performed at 0−4 °C. The suspension was subjected to sonication for 30 s with a Branson sonifier 450, and the sonicate was centrifuged for 60 min at 100000*g*. Sph kinase was recovered in the soluble fraction.

The reaction mixture (200 μL) for the Sph kinase assay contained 16.7 μM [^{3}H]Sph (0.2 μCi), 80 mM Tris-HCl, 20 mM $MgCl_2$, 1 mM ATP, and enzyme preparation (240 μg of protein). After 1 h at 37 °C, the reaction was terminated by the addition of 1.875 mL of ice-cold chloroform/methanol/concentrated HCl (100:200:1), lipids were extracted, and the phases were separated by the method of Bligh and Dyer (1959). Resultant lower chloroform phase samples were analyzed for [^{3}H]Sph-1-P formation from [^{3}H]Sph by TLC developed in butanol/acetic acid/water (3:1:1), followed by autoradiography.

Quantitative Measurement of Sph and Sph-1-P in Platelets. Platelet suspensions were adjusted to $1-2 \times 10^9$/mL, and indicated reactions in 0.5 mL aliquots were terminated by the addition of 3 mL of ice-cold chloroform/methanol (1:2), followed by thorough mixing and sonication for 30 min. Phases were separated by adding 2 mL of chloroform, 2 mL of 1 M KCl, and 100 μL of NH_4OH, and the resultant lower chloroform phase was assayed for quantification of Sph mass, based on conversion of Sph to [^{3}H]C_2-Cer (*N*-[^{3}H]acetylated Sph) by *N*-acylation with [^{3}H]acetic anhydride (Ohta et al., 1994). Recovery of [^{3}H]Sph, similarly treated, in the lower phases was 98%.

The cellular amount of Sph-1-P was measured using the alkaline upper phase, where 94% of [^{3}H]Sph-1-P, similarly treated, was recovered. The assay was based on quantitative conversion of Sph-1-P into [^{3}H]C_2-Cer-1-P (*N*-[^{3}H]acetylated Sph-1-P) by acylation with [^{3}H]acetic anhydride (Yatomi et al., 1995b).

Three milliliters of chloroform and 200 μL of concentrated HCl were added to the upper aqueous phase. Under these new acidic conditions, 47% of [^{3}H]Sph-1-P, similarly treated, shifted to the lower chloroform phases, which were then evaporated under N_2. The dried samples were dissolved in 40 μL of 0.008 N NaOH in methanol/10 mM solution of [^{3}H]acetic anhydride (1:1) by sonication. Acylation reactions proceeded at 37 °C for 2 h. The remaining anhydride was hydrolyzed by addition of 0.2 mL of 0.2 N NaOH in methanol. Following a 1-h incubation at room temperature, the C_2-Cer-1-P formed was extracted by addition of 0.78 mL of methanol, 0.98 mL of chloroform, 0.9 mL of 1 M KCl, and 20 μL of concentrated HCl. The resultant lower chloroform phase was washed twice by 1 mL of chloroform/methanol/water (3:48:47) plus 10 μL of concentrated HCl. Samples from the chloroform phase were evaporated under N_2 and resuspended in small volumes of chloroform/methanol (2:1). Portions of the lipids obtained were applied to HPTLC plates, which were developed in butanol/acetic acid/water (3:1:1), chloroform/methanol/7 N NH_4OH/water (80:20:0.5:0.5), or chloroform/methanol/acetic acid/water (65:43:1:3). After enhancer (Resolution TLC; E. M. Corp., Chestnut Hill, MA) treatment of plates, autoradiography was performed and radioactive spots corresponding to [^{3}H]C_2-Cer-1-P were scraped off and counted by liquid scintillation counting. Amounts of extracted Sph-1-P were calculated by extrapolation from Sph-1-P standards run through the same procedures.

Production of C_2-Cer-1-P under these conditions was identified separately by fast atom bombardment−mass spectrometry (FAB-MS) analysis using nonradioactive acetic anhydride (Yatomi et al., 1995b). Furthermore, the product of *N*-acylation with [^{3}H]acetic anhydride of Sph-1-P (standard Sph-1-P prepared from sphingosylphosphocholine with phospholipase D or Sph-1-P extracted from platelets) coincided with the FAB-MS-identified C_2-Cer-1-P in the TLC mobility under three different solvent systems described above (Yatomi et al., 1995b). TLC shown in this study was developed in butanol/acetic acid/water (3:1:1).

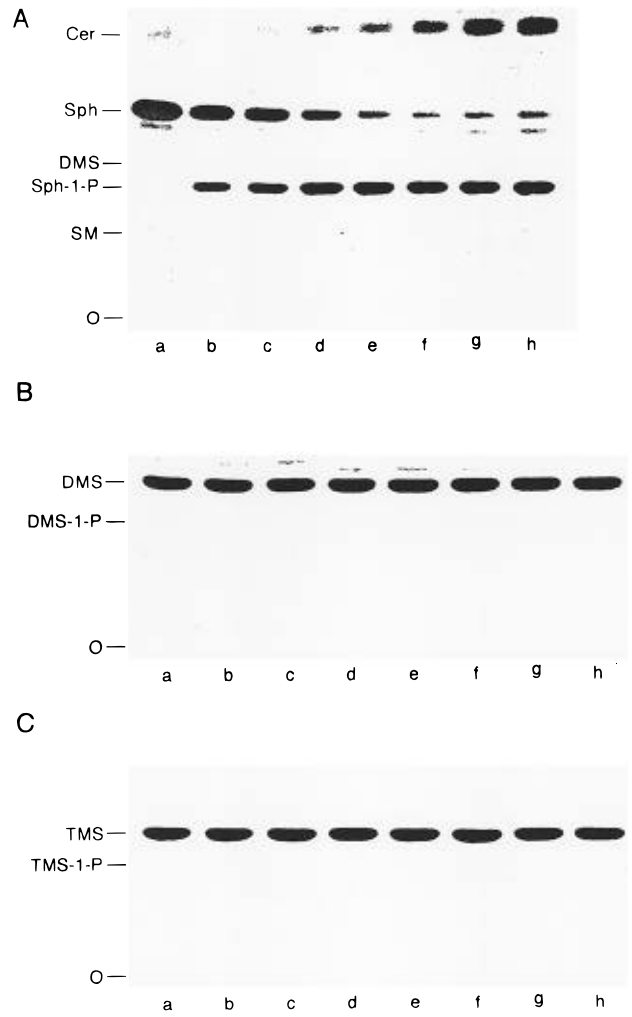

FIGURE 1: Metabolism of [^{3}H]Sph (A), [^{14}C]DMS (B), and [^{14}C]-TMS (C) in human platelets. Platelet suspensions were incubated with [^{3}H]Sph (A), [^{14}C]DMS (B), or [^{14}C]TMS (C) for various durations (lanes a and b, 0 and 10 s; lanes c−e, 1, 5, and 20 min; lanes f−h, 1, 2, and 3 h). Lipids were then extracted and analyzed as described under Materials and Methods. Locations of standard lipids are indicated on the left. SM, sphingomyelin; O, origin.

Platelet Aggregation. Platelet aggregation was determined turbidometrically (Zucker, 1989) in a Platelet Ionized Calcium Aggregometer (Chrono-Log, Havertown, PA). Human fibrinogen (500 μg/mL) was added to platelet suspensions shortly before the addition of stimuli unless stated otherwise. Calibration was performed with zero light transmission defined for platelet suspension and 100% transmission for the buffer, and the increase in light transmission caused by platelet aggregation was continuously recorded.

RESULTS

Metabolic Fates of [^{3}H]Sph, [^{14}C]DMS, [^{14}C]TMS, and [^{3}H]C_2-Cer in Human Platelets. When [^{3}H]Sph was added exogenously to human platelet suspensions, the label was efficiently removed from the medium (see Figure 6Aa); uptake of [^{3}H]Sph was 96.2 ± 0.2% (mean ± range, $n = 2$) at 5 min after the label addition. [^{3}H]Sph incorporated into platelets was rapidly converted to [^{3}H]Sph-1-P (Figure 1A), which is consistent with the fact that platelets possess a highly active Sph kinase (Stoffel et al., 1973a,b; Buehrer & Bell, 1992). Within 5 min, the level of Sph-1-P approached a plateau, with 45% conversion of [^{3}H]Sph to [^{3}H]Sph-1-P. At 3 h (the latest time point observed), 42% of the [^{3}H]Sph originally added remained as [^{3}H]Sph-1-P (Figure 1A), indicating the stability of Sph-1-P in platelets. Under the conditions employed, Sph did not induce platelet activation (shape change, aggregation, intracellular Ca^{2+} mobilization, and protein kinase C activation) (data not shown). Sph kinase, which converts [^{3}H]Sph into [^{3}H]Sph-1-P, is therefore considered to be present as an active enzyme in resting platelets. Furthermore, the rapid conversion of [^{3}H]Sph into [^{3}H]Sph-1-P (Sph kinase activity) was not affected by platelet activators such as thrombin and TPA (data not shown), which activate protein kinase C (Yatomi et al., 1994). [^{3}H]Sph was also converted by *N*-acylation into Cer (Goldkorn et al., 1991) at later time points (Figure 1A); 3%, 7%, and 10% of added [^{3}H]Sph was converted into Cer at 1, 2, and 3 h, respectively. Cer can be utilized in the synthesis of complex sphingolipids such as sphingomyelin and glycosphingolipids (Dressler et al., 1991). However, the incorporation of [^{3}H]-Sph into sphingomyelin was very low with a lag period of 2 h, and no radioactive glycosphingolipids were detected in platelets under our experimental conditions (Figure 1A). Under the conditions employed, conversion from [^{3}H]Sph into [^{3}H]DMS was not observed (Figure 1A).

We also examined metabolic fates of Sph derivatives in a similar fashion. When [^{14}C]DMS, [^{14}C]TMS, and [^{3}H]C_2-Cer (a synthetic cell-permeable Cer analog) were incubated with platelet suspensions, 97.0 ± 0.1%, 84.2 ± 0.4%, and 89.4 ± 0.9% (mean ± range, $n = 2$) of added radioactivities were incorporated into platelets in 5 min, respectively. In striking contrast with [^{3}H]Sph, [^{14}C]DMS (Figure 1B), [^{14}C]-TMS (Figure 1C), or [^{3}H]C_2-Cer (data not shown) incubated with platelets was unchanged regardless of incubation time, up to 3 h. The fact that methyl derivatives of Sph are metabolically stable is consistent with our previous findings using neutrophils (Kimura et al., 1992) and mouse melanoma cells (Sadahira et al., 1992) and indicates that they cannot be a substrate for Sph kinase.

Inhibition by DMS of [^{3}H]Sph Conversion into [^{3}H]Sph-1-P in Intact Platelets. We next studied the effects of various reagents on the [^{3}H]Sph metabolism. At 5 min after [^{3}H]-Sph addition to platelets, as much as 45% of the label added was converted to [^{3}H]Sph-1-P, but practically no conversion into [^{3}H]Cer occurred (Figure 1A). At this time point, preincubation of DMS, which is a poor substrate for Sph kinase and metabolically stable (Figure 1B), caused very strong inhibition of the [^{3}H]Sph conversion into [^{3}H]Sph-1-P, as revealed by both the decrease of [^{3}H]Sph-1-P radioactivity and the increase of [^{3}H]Sph radioactivity (Figures 2 and 3). The effect of DMS was concentration-dependent, with the IC_{50} value around 10 μM (Figure 3). The inhibition by DMS was specific; structurally related Sph derivatives such as C_2-Cer, TMS, DMS-1-P, and TMS-1-P did not affect the [^{3}H]Sph conversion into [^{3}H]Sph-1-P (Figure 2). Furthermore, the inhibitory effect of DMS was much stronger than that of DL-*threo*-DHS (Figure 2), which reportedly had been the most effective inhibitor of Sph kinase (Buehrer et al., 1992; Olivera & Spiegel, 1993). We also examined the effects of staurosporine, a potent protein kinase inhibitor (Tamaoki, 1991; Yatomi et al., 1994), and R59022 (de Chaffoy de Courcelles et al., 1985; Ohtsuka et al., 1990) and R59949 (de Chaffoy de Courcelles et al., 1989), established diacylglycerol kinase inhibitors, but they had little inhibitory effect (Figure 2).

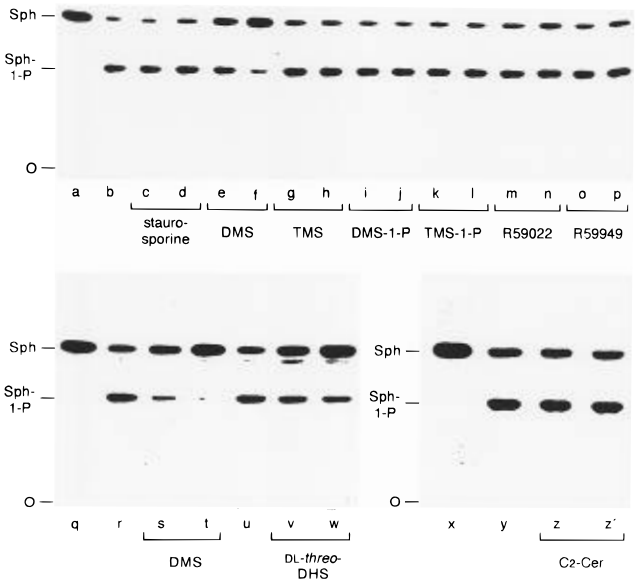

FIGURE 2: Effects of various agents on platelet [^{3}H]Sph conversion into [^{3}H]Sph-1-P. Platelet suspensions were pretreated without (b, r, u, and y) or with one of the following agents (c−p, s, t, v, w, z, and z′) for 5 min and then incubated for another 5 min with [^{3}H]-Sph. Lanes: c, 500 nM staurosporine; d, 2 μM staurosporine; e, 5 μM DMS; f, 20 μM DMS; g, 5 μM TMS; h, 20 μM TMS; i, 5 μM DMS-1-P; j, 20 μM DMS-1-P; k, 5 μM TMS-1-P; l, 20 μM TMS-1-P; m, 2 μM R59022; n, 10 μM R59022; o, 2 μM R59949; p, 10 μM R59949; s, 10 μM DMS; t, 25 μM DMS; v, 10 μM DL-*threo*-DHS; w, 25 μM DL-*threo*-DHS; z, 10 μM C_2-Cer; z′, 25 μM C_2-Cer. Added [^{3}H]Sph without interaction with intact platelets was run on lanes a, q, and x. Locations of standard Sph and Sph-1-P are indicated on the left.

When the incubation time was extended to 2 h after [^{3}H]-Sph addition, a substantial percentage of the label was converted into not only [^{3}H]Sph-1-P but also [^{3}H]Cer (see Figure 1A). Under these conditions (2-h [^{3}H]Sph incubation), increased level of radioactivity into Cer, as well as Sph, was detected by DMS; control Cer radioactivity without DMS addition (100%) increased to 159 ± 5% (mean ± SD, $n = 3$) by 20 μM DMS. It is most likely that Sph, increased by inhibition of its conversion into Sph-1-P, was converted by *N*-acylation into Cer (Goldkorn et al., 1991), which appears not to be sensitive to DMS inhibition. Therefore, DMS inhibition is considered to be specific to [^{3}H]Sph conversion into [^{3}H]Sph-1-P (through Sph kinase) and not due to its nonspecific cytotoxic effect.

Inhibition by DMS of Sph Kinase Activity in a Cell-Free System. In agreement with the previous report that Sph kinase is a soluble, cytoplasmic enzyme in platelets (Stoffel et al., 1973a,b), the kinase was present in the 100000*g* supernatant as a soluble enzyme. The activity of Sph kinase was inhibited by addition of DMS in a concentration-dependent manner, with the IC_{50} value around 5 μM (Figure 4A). The structurally related TMS did exert an inhibitory effect, but the effect was significantly weaker than that of DMS (Figure 4B); control Sph kinase activity (100%) was inhibited by 20 μM DMS and TMS to 25.4 ± 3.6% and 57.9 ± 2.4% (mean ± SD, $n = 3$), respectively. Furthermore, the inhibitory effect of DMS was much stronger than that of the established Sph kinase inhibitor DL-*threo*-DHS (Figure 4B); control Sph kinase activity (100%) was inhibited by 20 μM DL-*threo*-DHS to 48.0 ± 7.3% (mean ± SD, $n = 3$) under the conditions employed. Almost no inhibitory effects were observed when staurosporine or diacylglycerol kinase inhibitor (R59022 or R59949) was included in the assay mixtures (Figure 4B).

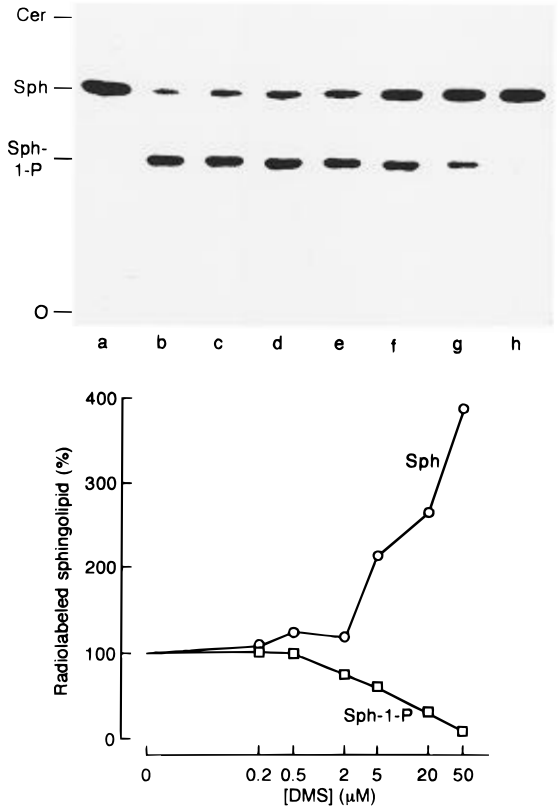

FIGURE 3: Effects of various concentrations of DMS on the [^{3}H]-Sph metabolic changes. (Upper) Platelet suspensions were pretreated without (b) or with the following concentrations of DMS (c−h) for 5 min and then incubated with [^{3}H]Sph for 5 min. Lanes: c, 0.2 μM; d, 0.5 μM; e, 2 μM; f, 5 μM; g, 20 μM; h, 50 μM. Added [^{3}H]Sph without interaction with intact platelets was run on lanes a. (Lower) Following the autoradiography shown in the upper panel, silica gel areas containing radiolabeled sphingolipids were scraped off and counted by liquid scintillation counting. Sph, m; Sph-1-P, o. Radioactivity was expressed as a percentage of the values obtained in lane b (no DMS addition). The counts for 100% were as follows: Sph, 12 878 cpm; Sph-1-P, 27 888 cpm.

Sph Mass Increase and Sph-1-P Mass Decrease in Platelets by the Administration of DMS. To determine the effect of DMS on cellular Sph mass, platelets were incubated with DMS, and Sph was extracted and quantitated by its conversion to [^{3}H]C_2-Cer (*N*-[^{3}H]acetylated Sph) by *N*-acylation with [^{3}H]acetic anhydride. The mass of Sph in untreated platelets was 354 ± 72 pmol/10^9 platelets (mean ± SD, $n = 6$). The administration of DMS (20 μM, 5 min) to platelets resulted in about a 2-fold increase of Sph mass (Figure 5A).

We also measured the mass of Sph-1-P in platelets by its conversion to [^{3}H]C_2-Cer-1-P (*N*-[^{3}H]acetylated Sph-1-P) by *N*-acylation with [^{3}H]acetic anhydride. The mass of Sph-1-P in resting platelets was 1.37 ± 0.07 nmol/10^9 platelets (mean ± SD, $n = 4$) and hence is about 4 times higher than that of Sph. It is most likely that the higher cellular amount of Sph-1-P than that of Sph results from the uniqueness of platelets in metabolism involving Sph-1-P: (i) platelets possess a very active Sph kinase (Stoffel et al., 1973a,b; Buehrer & Bell, 1992); (ii) platelets contain no lyase activity for degradation of Sph-1-P to ethanolamine phosphate and fatty aldehyde,[3] as suggested by previous studies (Stoffel et al., 1970, 1973a). Sph-1-P mass decreased with time after

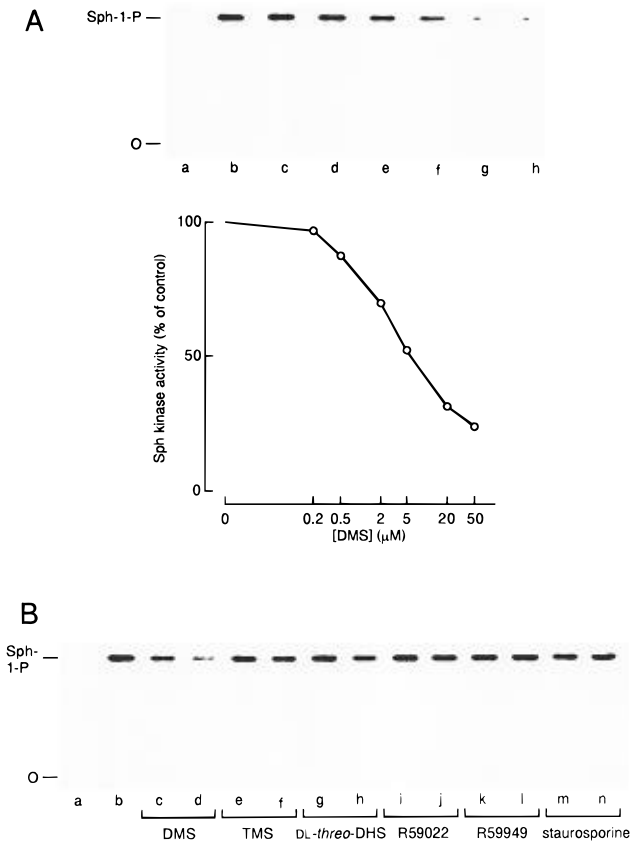

FIGURE 4: Inhibition by DMS of the activity of Sph kinase obtained from platelet cytosolic fraction. (A) (Upper) Sph kinase activity was assayed by [^{3}H]Sph-1-P formation from [^{3}H]Sph (b−h) in the presence of various concentrations of DMS. Lanes: b, 0 μM; c, 0.2 μM; d, 0.5 μM; e, 2 μM; f, 5 μM; g, 20 μM; h, 50 μM. Lane a shows the basal activity (no enzyme activation). (Lower) Following the autoradiography shown in the upper panel, silica gel areas containing radiolabeled Sph-1-P were scraped off and counted by liquid scintillation counting. Radioactivity was expressed as a percentage of the value obtained in lane b (no DMS addition). (B) Sph kinase activity was assayed in the absence (b) or the presence of the following agents (c−n). Lanes: c, 5 μM DMS; d, 20 μM DMS; e, 5 μM TMS; f, 20 μM TMS; g, 5 μM DL-*threo*-DHS; h, 20 μM DL-*threo*-DHS; i, 2 μM R59022; j, 10 μM R59022; k, 2 μM R59949; l, 10 μM R59949; m, 500 nM staurosporine; n, 2 μM staurosporine. Lane a shows the basal activity (no enzyme activation).

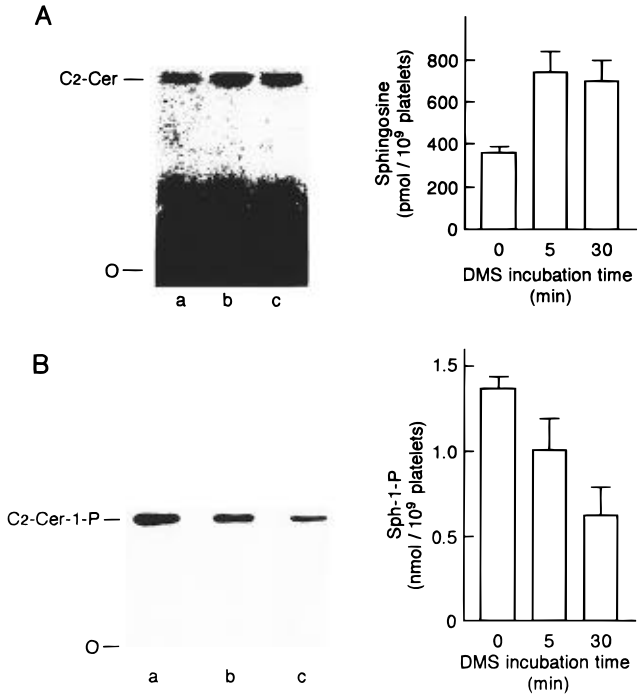

FIGURE 5: Sph mass increase (A) and Sph-1-P mass decrease (B) in platelets treated by DMS. (A) Platelets were incubated with 20 μM DMS and assayed for Sph mass by its *N*-acylation into *N*-[^{3}H]-acetylated Sph ([^{3}H]C_2-Cer) with [^{3}H]acetic anhydride. (Left) A representative autoradiogram shows the increase of Sph mass in platelets, as revealed by enhancement of the [^{3}H]C_2-Cer formed. DMS was incubated for 0 (a), 5 (b), or 30 (c) min. (Right) Sph levels in platelets were calculated by extrapolation from Sph standards which were run through the same procedures. Columns and error bars represent mean ± SD of three experiments. (B) Platelets were incubated with 20 μM DMS and assayed for Sph-1-P mass by its *N*-acylation into *N*-[^{3}H]acetylated Sph-1-P ([^{3}H]-C2-Cer-1-P) with [^{3}H]acetic anhydride. (Left) A representative autoradiogram shows the decrease of Sph-1-P mass in platelets, as revealed by reduction of the [^{3}H]C_2-Cer-1-P formed. DMS was incubated for 0 (a), 5 (b), or 30 (c) min. (Right) Sph-1-P levels in platelets were calculated by extrapolation from Sph-1-P standards which were run through the same procedures. Columns and error bars represent mean ± SD of three experiments.

the addition of 20 μM DMS (Figure 5B); the DMS treatment for 30 min resulted in a 55% decrease in the cellular amount of Sph-1-P.

To summarize, platelets showed a mass change in Sph (increase) and Sph-1-P (decrease) after incubation of the cells with DMS. Although decrease of Sph-1-P mass by DMS was time-dependent within 30 min (Figure 5B), increase of Sph mass was not; the Sph mass level at 30 min after DMS addition is similar with that at 5 min (Figure 5A). It is most probable that the increased Sph by DMS challenge should undergo *N*-acylation, resulting in enhanced Cer production from Sph, which can be predicted from the above-described platelet [^{3}H]Sph labeling studies.

Inhibition of TPA-Induced Sph-1-P Release by DMS. As described above, Sph-1-P is abundantly stored in platelets. We previously reported that stored Sph-1-P is released extracellularly upon stimulation with physiological agonists (Yatomi et al., 1995a). We consider this Sph-1-P release to be mediated by protein kinase C activation, based on the facts that protein kinase C activators, including phosphoinositide turnover-inducing agonists (thrombin and collagen) (Siess, 1989) and direct activators of the kinase (TPA and 1-oleoyl-2-acetylglycerol) (Nishizuka, 1984), released Sph-1-P and that this release was inhibited by staurosporine, an inhibitor of protein kinases, including protein kinase C.[3] Under our present conditions, 1 μM TPA, a protein kinase C-activating phorbol ester, released as much as 39% of platelet Sph-1-P into the medium (Figure 6). This Sph-1-P release was significantly inhibited by 25 μM DMS, but not by C_2-Cer (Figure 6).

Inhibition of Sph-1-P-Induced Platelet Aggregation by DMS. We previously reported that Sph-1-P is a platelet activator; it induces shape change and aggregation reactions (Yatomi et al., 1995a). As shown in Figure 7A, 40 μM Sph-1-P induced reversible platelet aggregation, which was completely abolished when extracellular fibrinogen was omitted (data not shown). Protein kinase C is involved in exposure of the fibrinogen receptor glycoprotein IIb-IIIa on platelet surfaces and hence in platelet aggregation (Shattil & Brass, 1987; Siess, 1989). This seems true when platelets

[3] Y. Yatomi, S. Yamamura, F. Ruan, S. Hakomori, and Y. Igarashi, manuscript in preparation.

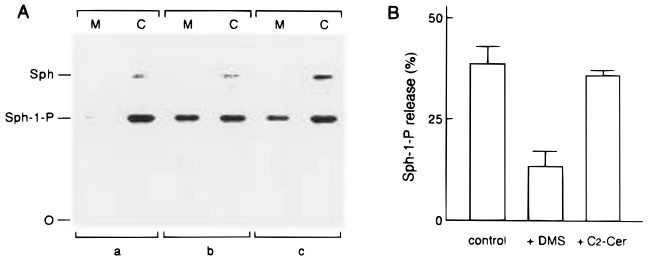

FIGURE 6: TPA-induced release of [^{3}H]Sph-1-P from platelets labeled with [^{3}H]Sph and its inhibition by DMS. (A) Platelet suspensions were incubated with [^{3}H]Sph for 10 min and without (a) or with (b and c) 1 μM TPA for 5 min before termination of reactions. In c, 25 μM DMS was added 1 min prior to TPA addition. Analysis of [^{3}H]Sph and [^{3}H]Sph-1-P in medium (M) and cells (C) were performed to determine platelet Sph-1-P release, as described under Materials and Methods. (B) Percent Sph-1-P release into medium (100 $\times$ [^{3}H]Sph-1-P in medium/total [^{3}H]Sph-1-P in medium plus cell pellet) was measured in platelets treated with TPA only (control), DMS plus TPA (+ DMS), and C_2-Cer plus TPA (+ C_2-Cer). Columns and error bars represent mean $\pm$ SD of three determinations.

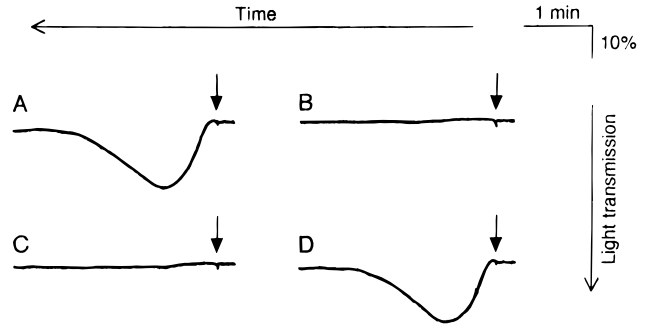

FIGURE 7: Inhibition by DMS of Sph-1-P-induced platelet aggregation. Platelets preincubated without (A) or with 20 μM DMS (B), Sph (C), or C_2-Cer (D) for 1 min were stimulated with 40 μM Sph-1-P, as indicated by arrows. Platelet aggregation was observed turbidometrically.

are activated by Sph-1-P, as well as well-employed platelet stimulators (Siess, 1989) since Sph-1-P-induced platelet aggregation was blocked by the physiological protein kinase C inhibitor Sph (Hannun & Bell, 1989; Merrill & Stevens, 1989) (Figure 7C). This Sph-1-P-induced response was also inhibited by 25 μM DMS (Figure 7B), but not by C_2-Cer (Figure 7D).

DISCUSSION

DMS as a Potent Inhibitor of Sph Kinase. Findings presented in this paper clearly indicate that DMS inhibits Sph kinase, which converts Sph into Sph-1-P. The inhibitory effect was observed in both intact platelets (Figures 2 and 3) and a cell-free system (Figure 4) and was much stronger than the effect by DL-*threo*-DHS (Figures 2 and 4), which had been the most effective widely used inhibitor of Sph kinase (Buehrer & Bell, 1992; Olivera & Spiegel, 1993). This indicates that DMS must be the strongest inhibitor of Sph kinase available now. The action of DMS was specific, as follows: (i) DMS inhibited phosphorylation of Sph to Sph-1-P, but not *N*-acylation of Sph to Cer. Accordingly, incubation of platelets with DMS resulted in increased levels of Cer as well as Sph. (ii) The structurally related Sph derivatives, including Cer, DMS-1-P, and TMS-1-P, did not inhibit Sph kinase (Figures 2 and 4). TMS inhibited Sph kinase in a cell-free system but the effect was weaker than that of DMS. The fact that TMS weakly inhibited Sph kinase in a cell-free system, but not intact platelets, may be related to the finding that the uptake of TMS by intact platelets is low, at least compared with Sph or DMS. Although 84.2% radioactivity of the added [^{14}C]TMS becomes related to platelets at 5 min after its addition, the real percentage incorporated into the cytoplasm must be lower because the calculated value includes that superficially attached to lipid bilayers.

Although DMS also acts as a protein kinase C inhibitor (Igarashi et al., 1989; Khan et al., 1990), its Sph kinase inhibition is most likely independent of its effect on protein kinase C. Sph kinase is present as an active enzyme in resting platelets, whereas protein kinase C is inactive (Nishizuka, 1984; Siess, 1989). Together with the fact that rapid conversion of [^{3}H]Sph into [^{3}H]Sph-1-P cannot be affected by the protein kinase C activator TPA, Sph kinase activity is considered to be independent of protein kinase C activation. Furthermore, we previously reported that, in inhibiting protein kinase C, TMS is more potent than DMS (Endo et al., 1991; Okoshi et al., 1991; Hakomori & Igarashi, 1993), compared with their effects on Sph kinase described here. Staurosporine did not inhibit Sph kinase at all (Figures 2 and 4), whereas it inhibited protein kinase C (essentially under similar conditions using platelets with those in this study) (Yatomi et al., 1994). These facts, indicating that the inhibitory effect of DMS on Sph kinase is not related to its effect on protein kinase C, are consistent with DMS inhibition of Sph kinase in a cell-free system (Figure 4).

Considering the structural resemblance between DMS and the Sph kinase substrate Sph, it is likely that DMS acts as a competitive inhibitor of Sph kinase. In that sense, the fact that positively charged TMS is a poor inhibitor of Sph kinase (Figures 2 and 4) is interesting. Although it is suggested that the dimethyl-N group of DMS is important in inhibiting an interaction between Sph kinase and Sph, further studies are needed to clarify the structural basis for DMS inhibition.

The fact that just administering the Sph kinase inhibitor DMS to intact platelets results in a decrease in Sph-1-P mass and an increase in Sph mass (Figure 5) has important implications. In platelets, which lack Sph-1-P lyase activity, Sph-1-P equilibrium should be determined by the two enzymes, Sph kinase and Sph-1-P phosphatase (Van Veldhoven & Mannaerts, 1994). Accordingly, it is not surprising that inhibition of Sph kinase, but not Sph-1-P phosphatase, by DMS results in a decrease in Sph-1-P mass and an increase in Sph mass. This also suggests a rather rapid interchange between Sph and Sph-1-P in platelets. In view of the finding that DMS fails to inhibit Sph *N*-acylation, DMS does not affect the activities of the enzymes involved in Sph metabolism, other than Sph kinase. We consider that DMS, which is capable of acting on intact cells and exerts the selective inhibitory effect, is very useful as an inhibitor of Sph kinase.

The inhibitory effect of DMS on Sph kinase is interesting and important not only because it is the strongest inhibitor available at present but also because DMS is the methyl derivative of the substrate Sph and can be detected biologically (at least in some systems). Although [^{3}H]DMS was not detected in [^{3}H]Sph-labeled platelets (Figure 1A), *N*-methylation of Sph was observed in brain homogenate (Igarashi & Hakomori, 1989; Nudelman et al., 1992), and labeling corresponding to DMS was observed in A431

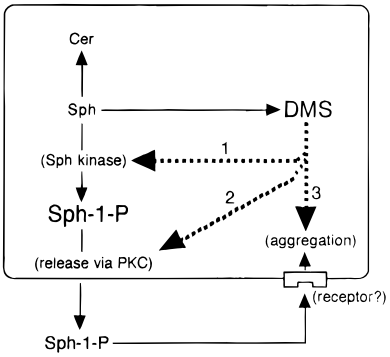

FIGURE 8: DMS inhibits Sph-1-P actions as an autocrine stimulator of platelets. Sph-1-P, which is abundantly stored in platelets, is released upon stimulation [possibly through mediation by protein kinase C (PKC)]. Since exogenous Sph-1-P induces platelet aggregation, Sph-1-P is proposed as an autocrine stimulator of platelets. DMS suppresses Sph-1-P formation (by inhibiting Sph kinase) (broken line 1), Sph-1-P release (line 2), and Sph-1-P-induced platelet aggregation (line 3). Thus, biological effects of phosphorylated Sph (Sph-1-P) are inhibited by *N*-methylated Sph (DMS), but not *N*-acylated Sph (Cer).

(human epidermoid carcinoma) (Igarashi et al., 1990) and CTLL (mouse T cell line) cells (Felding-Habermann et al., 1990) metabolically labeled with [^{3}H]serine. Presumably, complex and subtle regulation of Sph conversion into Sph-1-P by another Sph catabolite, DMS, should exist in certain cells.

Inhibition by DMS of Sph-1-P Actions as an Autocrine Stimulator of Platelets. Platelets, which lack Sph-1-P lyase activity and possess active Sph kinase, abundantly stores Sph-1-P (Figure 5). The stored Sph-1-P is released from platelets into the extracellular environment (Figure 6), possibly through mediation by protein kinase C.[3] Exogenously added Sph-1-P induces platelet functional responses such as shape change (Yatomi et al., 1995a) and aggregation (Figure 7A). These facts suggest that Sph-1-P may act intercellularly as an autocrine stimulator of platelets (Figure 8). We have shown that DMS, but not Cer, inhibits these biological actions of Sph-1-P at multiple steps. First, DMS, but not C_2-Cer, acts as a potent Sph kinase inhibitor, and administering DMS to intact platelets resulted in a decrease in Sph-1-P mass (Figure 5), as discussed above. Next, Sph-1-P release, which is most likely mediated by protein kinase C, was significantly inhibited by DMS, but not C_2-Cer (Figure 6). Finally, Sph-1-P-induced platelet aggregation was blocked by DMS, but not C_2-Cer (Figure 7). These results show that biological effects of phosphorylated Sph (Sph-1-P) are inhibited by *N*-methylated Sph (DMS), but not *N*-acylated Sph (Cer) (Figure 8). It is most likely that DMS inhibition of TPA-induced Sph-1-P release and Sph-1-P-induced platelet aggregation is related to DMS suppression of protein kinase C. This is based on the facts that both platelet aggregation (Shattil & Brass, 1987; Siess, 1989) and Sph-1-P release are protein kinase C activation-requiring processes and that DMS, as well as Sph, is a protein kinase C inhibitor (Igarashi et al., 1989; Khan et al., 1990).

Recent evidence has suggested that branching pathways of Sph metabolism may mediate contrasting or opposing effects; Cer has been reported to induce apoptosis in several types of cells, whereas Sph-1-P is mitogenic (Obeid et al., 1993; Jarvis et al., 1994; Spiegel et al., 1993). In this study, we have shown that DMS is a potent inhibitor of Sph kinase and that actions of Sph-1-P are inhibited by this methyl derivative of Sph. The biologic sequelae of Sph metabolism in different cell types must be variable depending upon the expression levels of enzymes involved in Sph catabolism.

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