Sphingosine 1-Phosphate Formation and Intracellular Ca^{2+} Mobilization in Human Platelets: Evaluation with Sphingosine Kinase Inhibitors[1]

Libo Yang,* Yutaka Yatomi,*,[2] Kaneo Satoh,* Yasuyuki Igarashi,† and Yukio Ozaki*

**Department of Laboratory Medicine, Yamanashi Medical University, Nakakoma, Yamanashi 409-3898; and †Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812*

Received March 8, 1999; accepted April 12, 1999

Sphingosine 1-phosphate (Sph-1-P) is considered to play a dual role in cellular signaling, acting intercellularly as well as intracellularly. In this study, we examined the role of Sph-1-P as a signaling molecule in human platelets, using DL-*threo*-dihydrosphingosine (DHS) and *N,N*-dimethylsphingosine (DMS), inhibitors of Sph kinase and protein kinase C. Both DMS and DL-*threo*-DHS were confirmed to be competitive inhibitors of Sph kinase obtained from platelet cytoplasmic fractions. In intact platelets labeled with [³H]Sph, stimulation with 12-*O*-tetradecanoylphorbol 13-acetate or thrombin did not affect [³H]-Sph-1-P formation. While both DMS and DL-*threo*-DHS inhibited not only [³H]Sph-1-P formation but also protein kinase C-dependent platelet aggregation, staurosporine, a potent protein kinase inhibitor, only inhibited the protein kinase C-dependent reaction. Hence, it is unlikely that Sph kinase activation and the resultant Sph-1-P formation are mediated by protein kinase C in platelets. Furthermore, Ca^{2+} mobilization induced by platelet agonists that act on G protein-coupled receptor was not affected by DMS or DL-*threo*-DHS. Our results suggest that Sph-1-P does not mediate intracellular signaling, including Ca^{2+} mobilization, in platelets.

Key words: DL-*threo*-dihydrosphingosine, *N,N*-dimethylsphingosine, platelet, sphingosine kinase, sphingosine 1-phosphate.

Sphingosine 1-phosphate (Sph-1-P), formed from sphingosine (Sph) by Sph kinase, has recently been added to the list of bioactive sphingolipids that modulate membrane signal transduction systems and are involved in diverse cellular processes (*1-3*). Originally, Sph-1-P was proposed to act as a second (intracellular) messenger. PDGF and FCS were the first extracellular stimuli reported to increase Sph-1-P formation and to act as second messengers in cell proliferation (*4*). Afterwards, regulation of Sph-1-P production and intracellular Sph-1-P targets have been clarified (*5-12*). Recently, Sph-1-P formation was reported to be involved in Ca^{2+} mobilization by high-affinity IgE receptors (FcεRI) (*5*) and by various heterotrimeric G protein-coupled receptors (*10, 11*). Sph-1-P has been shown to induce the release of Ca^{2+} from intracellular stores, and Sph kinase, which catalyzes Sph-1-P production, has been reported to play a similar role to phospholipase C; this lipase elicits inositol 1,4,5-trisphosphate formation (from phosphatidylinositol 4,5-bisphosphate) and the resultant Ca^{2+} release from internal stores in the endoplasmic reticulum (*13*).

On the other hand, many of the biological effects of Sph-1-P, including the regulation of heart rate, oxidative burst, neurite retraction, cell motility regulation, and platelet activation, have been reported to be due to binding to cell surface receptors (*14-21*). Very recently, high-affinity plasma membrane receptors for Sph-1-P have been identified; Sph-1-P has been shown to act on subfamilies of G-protein-coupled receptors, *i.e.*, the endothelial differentiation genes (Edgs) (*22-24*). Hence, Sph-1-P is now recognized as playing a dual role in cellular signaling, acting intercellularly as well as intracellularly (*14*).

To examine the signaling role of Sph-1-P, especially the intracellular role, two Sph kinase inhibitors, DL-*threo*-dihydrosphingosine (DHS) (*25*) and *N,N*-dimethylsphingosine (DMS) (*26*), have been applied. However, these agents are not specific Sph kinase inhibitors; they also inhibit protein kinase C (*27, 28*). To make the matter more complicated, it has been reported that Sph kinase activation and the resultant Sph-1-P formation depend on protein kinase C (*6-8*). Accordingly, it is important to determine whether or not the inhibitory effects of DL-*threo*-DHS and DMS are attributable specifically to Sph kinase inhibition. In this study, we compare the effects of the Sph kinase inhibitors on Sph-1-P formation and protein kinase C-dependent processes. We also examined the effects of these com-

[1] This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, The Ryoichi Naito Foundation for Medical Research, and the Clinical Pathology Research Foundation of Japan.

[2] To whom correspondence should be addressed. Tel: +81-552-73-9884, Fax: +81-552-73-6713, E-mail: yatomiy@res.yamanashi-med.ac.jp

Abbreviations: Sph-1-P, sphingosine 1-phosphate; Sph, sphingosine; DHS, dihydrosphingosine; DMS, *N,N*-dimethylsphingosine; Cer, ceramide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

© 1999 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

pounds on Ca^{2+} signaling *via* G protein-coupled receptors.

MATERIALS AND METHODS

Materials—The following materials were obtained from the indicated suppliers: DMS, DL-*threo*-DHS, and Sph-1-P (Biomol, Plymouth Meeting, PA); Sph, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and ceramide (Cer) (type III) (Sigma, St. Louis, MO); thrombin (Mochida Pharmaceutical, Tokyo); staurosporine (Kyowa Medex, Tokyo); U46619 (Cayman Chemical, Ann Arbor, MI); PAF (Avanti polar lipids, AL, USA); fura2-AM (Dojin Chemicals, Kumamoto); [3-^{3}H]Sph (22.0 Ci/mmol) (Du Pont-New England Nuclear, Boston, MA).

Preparation of Platelets—Platelets were isolated from the blood of healthy adult volunteers. The blood was anticoagulated with 3.8% sodium citrate (9 volumes of blood to 1 volume of sodium citrate), then centrifuged at $120\times g$ for 10 min to obtain platelet-rich plasma. The washed platelets were then prepared and handled as described previously (*20*). The final platelet suspensions were adjusted to 3×10^8/ml, and supplemented with EGTA or $CaCl_2$ *plus* fibrinogen when indicated. All experiments using intact platelet suspensions were performed at 37°C.

Sph Kinase Activity Assay—Sph kinase was obtained from the soluble fraction of washed platelets (2.5×10^{10} cells) as described previously (*26*).

The reaction mixture (200 μl) for the Sph kinase assay contained 16.7 μM [^{3}H]Sph (0.1 μCi), 80 mM Tris-HCl, 20 mM $MgCl_2$, 1 mM ATP, and enzyme preparation (200 μg 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), the lipids were extracted, and the phases were separated by the method of Bligh and Dyer (*29*). The resultant lower chloroform phase samples were subjected to TLC developed in butanol/acetic acid/water (3:1:1), followed by autoradiography. Silica gel areas containing [^{3}H]Sph-1-P (formed from [^{3}H]Sph) were scraped off and counted by liquid scintillation counting.

Metabolism of [^{3}H]Sph in Platelets—Platelet suspensions (0.5 ml) were incubated with 1 μM (0.2 μCi) [^{3}H]-Sph, and the reaction was terminated by the addition of 1.875 ml of ice-cold chloroform/methanol/concentrated HCl (100:200:1), and the lipids were extracted and analyzed for [^{3}H]Sph metabolism as described previously (*20*). When indicated, silica gel areas containing radiolabeled sphingolipids were scraped off and counted by liquid scintillation counting.

Platelet Aggregation—Platelet aggregation was determined turbidometrically (*30*) in an AA-100 platelet aggregation analyzer (Sysmex, Kobe). Human fibrinogen (500 μg/ml) and 1 mM $CaCl_2$ were added to platelet suspensions 1 min prior to TPA addition.

Platelet $[Ca^{2+}]_i$ Measurement—$[Ca^{2+}]_i$ was measured using a Ca^{2+}-sensitive fluorophore, fura2, as described previously (*31*), except that fluorescence measurements were made using an FS100 (Kowa, Tokyo). The $[Ca^{2+}]_i$ values were determined from the ratio of fura2 fluorescence intensity at 340 and 380 nm excitation (*32*). The data shown are those obtained using platelets supplemented with 100 μM EGTA to abolish the influx of extracellular Ca^{2+}. In separate experiments, increases in $[Ca^{2+}]_i$ induced by 0.1 U/ml thrombin were found to be 351 ± 61 nM (mean$\pm$SD, $n=6$).

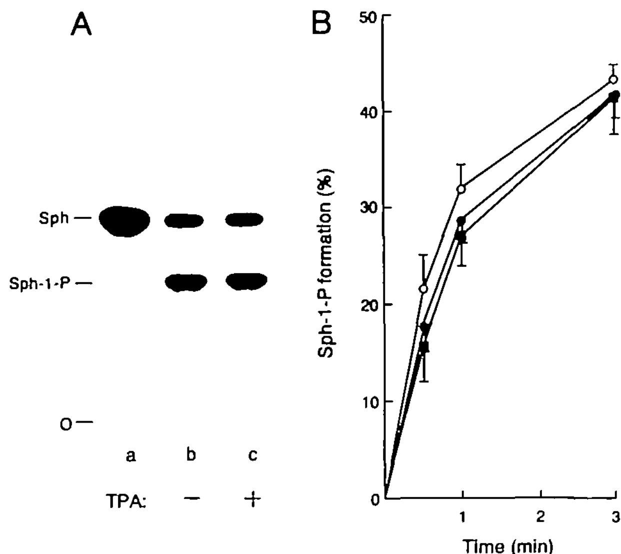

Fig. 1. **Failure of TPA or thrombin to affect [^{3}H]Sph conversion into [^{3}H]Sph-1-P in platelets.** (A) Human platelets were pretreated without (b) or with 100 nM TPA for 30 s (c) and then incubated with [^{3}H]Sph for 3 min. Lipids were extracted, and [^{3}H]-Sph-1-P formation from [^{3}H]Sph was analyzed by TLC autoradiography. O, origin. Added [^{3}H]Sph without interaction with intact platelets is in lane a. A representative autoradiogram from one of three experiments is shown. (B) Platelets were treated without (open circles) or with 100 nM TPA (closed circles) or 0.1 U/ml of thrombin (closed squares) for 30 s and then incubated with [^{3}H]Sph for various times. Percent Sph-1-P formation was calculated as $100\times$ [^{3}H]Sph-1-P formed/[^{3}H]Sph added. The results are presented as the mean$\pm$SD ($n=3$).

RESULTS

Competitive Inhibition of Platelet Sph Kinase by DMS and DL-threo-DHS—We previously reported that DMS inhibits Sph kinase obtained from platelet cytoplasmic fractions much more strongly than DL-*threo*-DHS (*26*), a widely-used, competitive inhibitor of Sph kinase (*4, 25*). Considering the structural resemblance between DMS and Sph (Sph kinase substrate), it is likely that DMS also acts as a competitive inhibitor of Sph kinase. We first confirmed this using platelet Sph kinase. As expected, both DMS and DL-*threo*-DHS were found to be potent competitive inhibitors of Sph kinase, as confirmed by their Lineweaver-Burk plots (data not shown). The K_i values for DMS and DL-*threo*-DHS were calculated to be 2.5 and 12 μM, respectively.

Sph Kinase-Mediated Sph-1-P Formation Occurs Independently of Protein Kinase C in Platelets—Although it is now clear that both DMS and DL-*threo*-DHS are potent competitive inhibitors of Sph kinase, it should be remembered that they are also protein kinase C inhibitors (*27, 28, 33*). Furthermore, it has been reported that Sph kinase activation depends on protein kinase C in some nucleate cells (*6-8*), making it important to determine whether the inhibitory effects of DMS and DL-*threo*-DHS on Sph-1-P formation in intact cells are attributable specifically to Sph kinase inhibition. Accordingly, we examined the correla-

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

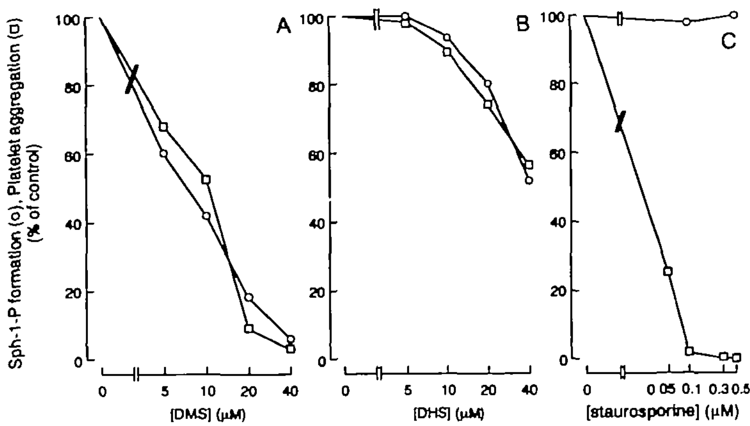

Fig. 2. **Effects of DMS, DL-*threo*-DHS, and staurosporine on platelet [³H]Sph-1-P formation (from [³H]-Sph) and TPA-induced aggregation.** Platelets were incubated with various concentrations of DMS (A), DL-*threo*-DHS (B), or staurosporine (C) for 5 min, and then [³H]Sph-1-P formation from [³H]Sph (circles) and platelet aggregation elicited by 100 nM TPA (squares) were examined as described in "MATERIALS AND METHODS." The results are expressed as percentage of control (without pretreatment). The results are from a single experiment representative of at least three separate determinations.

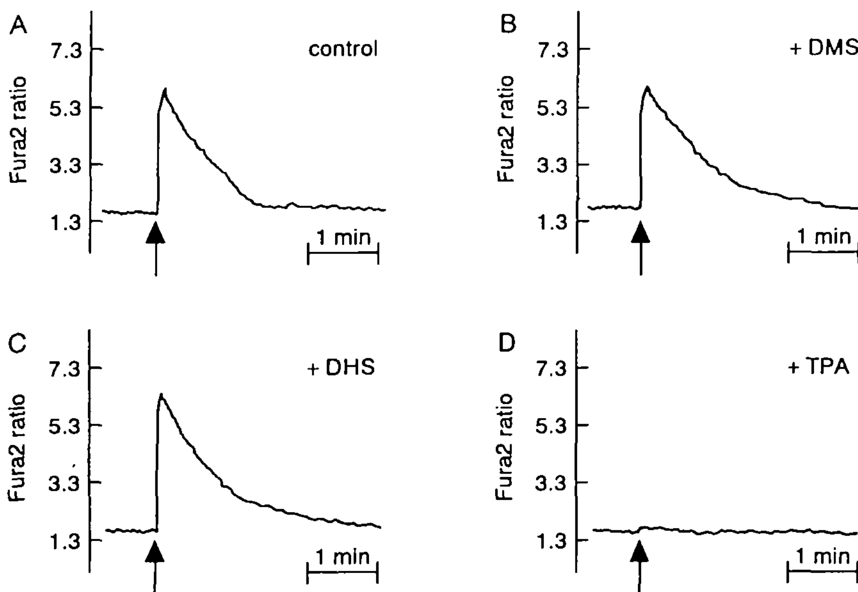

Fig. 3. **Effects of DMS, DL-*threo*-DHS, and TPA on platelet intracellular Ca^{2+} mobilization triggered by thrombin.** Fura2-loaded platelets were incubated without (A) or with 20 μM DMS (B), 20 μM DL-*threo*-DHS (C), or 1 μM TPA (D) for 5 min. The cells were then stimulated with 0.1 U/ml of thrombin, and the $[Ca^{2+}]_i$ changes were monitored by the ratio of fura2 fluorescence. Similar results were obtained in two independent experiments.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

tion between Sph kinase and protein kinase C in platelets.

When [³H]Sph was added exogenously to platelet suspensions, the label was efficiently removed from the medium, and the [³H]Sph incorporated into platelets was rapidly converted to [³H]Sph-1-P (Fig. 1A). This conversion, which reflects Sph kinase activation in intact platelets, was not enhanced by treatment with TPA or thrombin (Fig. 1, A and B). Sph kinase activity was rather inhibited by these agents, although the inhibitory effects were not statistically significant (Fig. 1B). Under our conditions, a marked increase in the phosphorylation of pleckstrin, an established protein kinase C substrate in platelets (*34*), was induced by TPA or thrombin (data not shown). TPA is a direct protein kinase C activator, while thrombin activates protein kinase C by inducing phosphoinositide turnover with resultant diacylglycerol formation (*35*).

We next compared platelet [³H]Sph-1-P formation (from [³H]Sph) with TPA-induced platelet aggregation, which is dependent on protein kinase C activation (*31*). Both DMS and DL-*threo*-DHS caused a dose-dependent inhibition of Sph-1-P formation and TPA-induced aggregation in a similar fashion (Fig. 2, A and B). In contrast, staurosporine, a potent protein kinase inhibitor (*36*), strongly inhibited protein kinase C-dependent, TPA-induced aggregation, but had no effect at all on Sph kinase (Fig. 2C). This suggests that the inhibitory effects of DMS and DL-*threo*-DHS on Sph kinase are not due to the inhibition of protein kinase C, although they inhibit both.

Failure of DMS and DL-threo-DHS to Affect Ca^{2+} Signaling by G Protein-Coupled Receptors in Platelets—The involvement of Sph kinase activation, leading to Sph-1-P production, in Ca^{2+} signaling was proposed for Fc receptors (*5, 9*). Very recently, this idea has been extended to Ca^{2+} signaling mediated by G protein-coupled receptors (*10, 11*), based on the fact that Ca^{2+} signaling by various G protein-coupled receptors in different cell types is attenuated by the Sph kinase inhibitors DMS and DL-*threo*-DHS (*10, 11*). We examined whether this is the case for platelets, since most platelet agonists act on G protein-coupled receptors and one of the major early events triggered by these receptors is the increase in $[Ca^{2+}]_i$ (*35*).

Thrombin, the stable thromboxane A_2 analogue U46619, and PAF are well-established platelet agonists that act on their own G protein-coupled receptors (*35*). When platelets were stimulated with various concentrations of thrombin, the stimulated intracellular Ca^{2+} mobilization was not

affected by pretreatment with DMS or DL-*threo*-DHS (Figs. 3, 4, and 5). Similar results were obtained using platelets challenged with U46619 or PAF (Fig. 5). In contrast, Ca^{2+} mobilization was abolished by TPA pretreatment (Figs. 3 and 5), because the activation of protein kinase C attenuates the phospholipase C signaling pathway (*37*).

DISCUSSION

As expected from the structural resemblance between DMS and the Sph kinase substrate Sph, we found that DMS, as well as DL-*threo*-DHS, acts as a competitive inhibitor of Sph kinase. Our observation is consistent with a recent report on the purification and characterization of rat kidney Sph kinase, which is competitively inhibited by both DMS and DL-*threo*-DHS (*38*). However, whether this kinase is the same as the platelet enzyme remains to be determined. Although DMS and DL-*threo*-DHS act not only as Sph kinase inhibitors but also as protein kinase C inhibitors (*27, 28, 33*), the Sph kinase inhibition most likely occurs independently of the effect on protein kinase C. Sph kinase is present as an active enzyme in resting platelets, whereas protein kinase C is inactive (*35, 39*). Activation of protein kinase C by TPA or thrombin does not affect Sph-1-P formation in platelets. Furthermore, staurosporine does not inhibit Sph kinase at all, whereas it inhibits protein kinase C-dependent platelet aggregation.

The importance of stimulation-dependent Sph kinase activation (with the resultant Sph-1-P formation) and its dependence on protein kinase C activation have been reported in various cellular responses, including stimulation of the proliferation and inhibition of apoptosis (*6-8*). However, neither stimulation-dependent Sph-1-P formation nor protein kinase C-dependent Sph kinase activation was observed in platelets. Presumably, the functional role(s) and hence metabolism of sphingolipids in anucleate and highly-differentiated platelets are unique, compared with nucleate cells.

Although DL-*threo*-DHS is a useful tool, it is not a physiological compound; the D-*erythro* form (sphinganine) is the natural form. On the other hand, (D-*erythro*-)DMS can be detected biologically at least in some systems. *N*-Methylation of Sph has been observed in brain homogenates (*40, 41*), and labeling corresponding to DMS has been observed in A431 (human epidermoid carcinoma) (*42*) and CTLL (mouse T cell line) cells (*43*) metabolically labeled with [^{3}H]serine. However, [^{3}H]DMS was not detected in [^{3}H]Sph-labeled platelets under any conditions (data not shown), as we reported (*26*) previously. It is very important to clarify the presence of and quantitative changes in DMS in various cells or tissues under physiological conditions.

Although Sph kinase activation and the resultant Sph-1-P formation reportedly mediate Ca^{2+} signaling (*5, 9-11*),

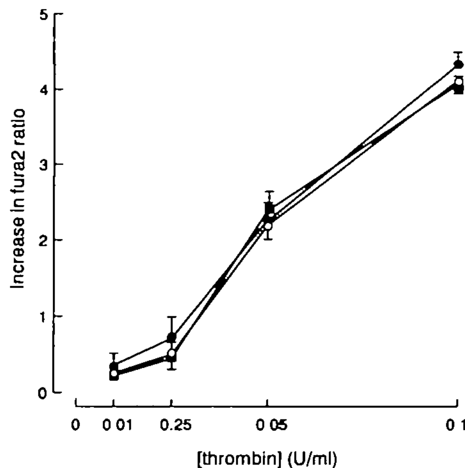

Fig. 4. **Effects of DMS and DL-*threo*-DHS on platelet intracellular Ca^{2+} mobilization induced by various concentrations of thrombin.** Fura2-loaded platelets were incubated without (open circles) or with 20 μM DMS (closed circles) or DL-*threo*-DHS (closed squares) for 5 min. The cells were then stimulated with the indicated concentrations of thrombin, and the increases in $[Ca^{2+}]_i$ (the ratio of fura2 fluorescence) were measured. The results are presented as the mean±SD (n=3).

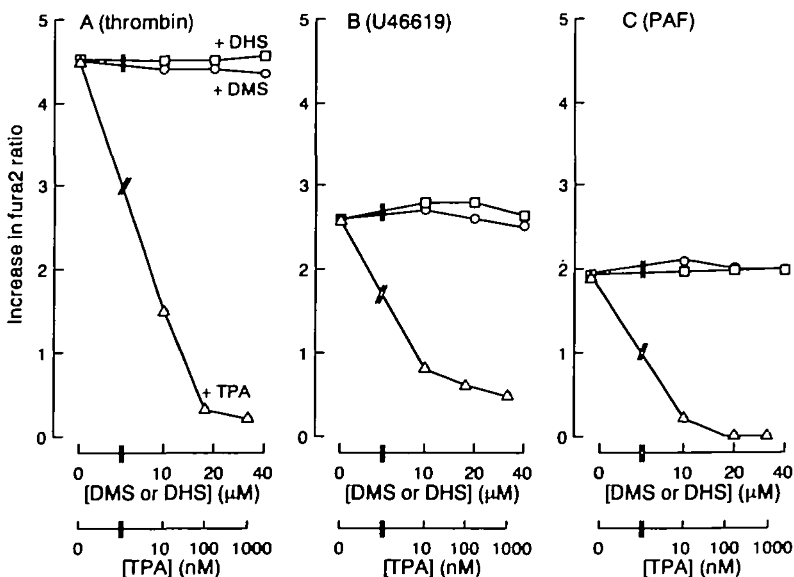

Fig. 5. **Effects of DMS, DL-*threo*-DHS, and TPA on platelet intracellular Ca^{2+} mobilization induced by thrombin, U46619, and PAF.** Fura2-loaded platelets were incubated without or with various concentrations of DMS (circles), DL-*threo*-DHS (squares), or TPA (triangles) for 5 min. The cells were then stimulated with 0.1 U/ml of thrombin (A), 1 μM U46619 (B), or 1 μM PAF (C), and peak $[Ca^{2+}]_i$ increases were measured. The results are from a single experiment representative of at least three separate determinations.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

including that originating from G protein-coupled receptors (*10, 11*), this does not seem to be true for platelet agonists. In platelets, no stimulation-dependent Sph kinase activation was observed; thrombin, which induces strong intracellular Ca^{2+} mobilization through its G protein-coupled receptor, failed to enhance Sph-1-P formation. Consistently, Ca^{2+} mobilization induced by three major agonists (which act on G protein-coupled receptor) was not affected by the Sph kinase inhibitors DMS and DL-*threo*-DHS.

In summary, in platelets, both DMS and DL-*threo*-DHS inhibit Sph kinase competitively, independently of protein kinase C inhibition. However, the findings obtained using these inhibitors are different in platelets from those in nucleate cells where Sph-1-P acts as an intracellular messenger. It is unlikely that Sph-1-P mediates intracellular signaling, including Ca^{2+} mobilization, in highly-differentiated, anucleate platelets.

REFERENCES

1. Igarashi, Y. (1997) Functional roles of sphingosine, sphingosine 1-phosphate, and methylsphingosines: In regard to membrane sphingolipid signaling pathways. *J. Biochem.* **122**, 1080-1087
2. Spiegel, S., Foster, D., and Kolesnick, R. (1996) Signal transduction through lipid second messengers. *Curr. Opin. Cell Biol.* **8**, 159-167
3. Hannun, Y.A. (1994) The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* **269**, 3125-3128
4. Olivera, A. and Spiegel, S. (1993) Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* **365**, 557-560
5. Choi, O.H., Kim, J.-H., and Kinet, J.-P. (1996) Calcium mobilization via sphingosine kinase in signalling by the FceRI antigen receptor. *Nature* **380**, 634-636
6. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P.G., Coso, O.A., Gutkind, J.S., and Spiegel, S. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* **381**, 800-803
7. Cuvillier, O., Rosenthal, D.S., Smulson, M.E., and Spiegel, S. (1998) Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. *J. Biol. Chem.* **273**, 2910-2916
8. Kleuser, B., Cuvillier, O., and Spiegel, S. (1998) 1α,25-Dihydroxyvitamin D_3 inhibits programmed cell death in HL-60 cells by activation of sphingosine kinase. *Cancer Res.* **58**, 1817-1824
9. Melendez, A., Floto, R.A., Gillooly, D.J., Harnett, M.M., and Allen, J.M. (1998) FcgRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. *J. Biol. Chem.* **273**, 9393-9402
10. Meyer zu Heringdorf, D., Lass, H., Alemany, R., Laser, K.T., Neumann, E., Zhang, C., Schmidt, M., Rauen, U., Jakobs, K.H., and van Koppen, C.J. (1998) Sphingosine kinase-mediated Ca^{2+} signalling by G-protein-coupled receptors. *EMBO J.* **17**, 2830-2837
11. Alemany, R., Meyer zu Heringdorf, D., van Koppen, C.J., and Jakobs, K.H. (1999) Formyl peptide receptor signaling in HL-60 cells through sphingosine kinase. *J. Biol. Chem.* **274**, 3994-3999
12. Ghosh, T.K., Bian, J., and Gill, D.L. (1994) Sphingosine 1-phosphate generated in the endoplasmic reticulum membrane activates release of stored calcium. *J. Biol. Chem.* **269**, 22628-22635
13. Beaven, M.A. (1996) Calcium signalling: sphingosine kinase *versus* phospholipase C? *Curr. Biol.* **6**, 798-801
14. Meyer zu Heringdorf, D., van Koppen, C.J., and Jakobs, K.H. (1997) Molecular diversity of sphingolipid signalling. *FEBS Lett.* **410**, 34-38
15. van Koppen, C.J., Meyer zu Heringdorf, D., Laser, K.T., Zhang, C., Jakobs, K.H., Bunemann, M., and Pott, L. (1996) Activation of a high affinity G_i protein-coupled plasma membrane receptor by sphingosine-1-phosphate. *J. Biol. Chem.* **271**, 2082-2087
16. Bunemann, M., Brandts, B., Meyer zu Heringdorf, D., van Koppen, C.J., Jakobs, K.H., and Pott, L. (1995) Activation of muscarinic K^+ current in guinea-pig atrial myocytes by sphingosine-1-phosphate. *J. Physiol.* **489**, 701-707
17. Okajima, F., Tomura, H., Sho, K., Kimura, T., Sato, K., Im, D.S., Akbar, M., and Kondo, Y. (1997) Sphingosine 1-phosphate stimulates hydrogen peroxide generation through activation of phospholipase C-Ca^{2+} system in FRTL-5 thyroid cells: possible involvement of guanosine triphosphate-binding proteins in the lipid signaling. *Endocrinology* **138**, 220-229
18. Postma, F.R., Jalink, K., Hengeveld, T., and Moolenaar, W.H. (1996) Sphingosine-1-phosphate rapidly induces Rho-dependent neurite retraction: action through a specific cell surface receptor. *EMBO J.* **15**, 2388-2395
19. Yamamura, S., Yatomi, Y., Ruan, F., Sweeney, E.A., Hakomori, S., and Igarashi, Y. (1997) Sphingosine 1-phosphate regulates melanoma cell motility through a receptor-coupled extracellular action and in a pertussis toxin-insensitive manner. *Biochemistry* **36**, 10751-10759
20. Yatomi, Y., Ruan, F., Hakomori, S., and Igarashi, Y. (1995) Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood* **86**, 193-202
21. Yatomi, Y., Yamamura, S., Ruan, F., and Igarashi, Y. (1997) Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.* **272**, 5291-5297
22. Lee, M.-J., Van Brocklyn, J.R., Thangada, S., Liu, C.H., Hand, A.R., Menzeleev, R., Spiegel, S., and Hla, T. (1998) Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* **279**, 1552-1555
23. Zondag, G.C.M., Postma, F.R., van Etten, I., Verlaan, I., and Moolenaar, W.H. (1998) Sphingosine 1-phosphate signalling through the G-protein-coupled receptor Edg-1. *Biochem. J.* **330**, 605-609
24. Goetzl, E.J. and An, S. (1998) Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12**, 1589-1598
25. Buehrer, B.M. and Bell, R.M. (1992) Inhibition of sphingosine kinase *in vitro* and in platelets. *J. Biol. Chem.* **267**, 3154-3159
26. Yatomi, Y., Ruan, F., Megidish, T., Toyokuni, T., Hakomori, S., and Igarashi, Y. (1996) *N,N*-Dimethylsphingosine inhibition of sphingosine kinase and sphingosine 1-phosphate activity in human platelets. *Biochemistry* **35**, 626-633
27. Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., and Racker, E. (1989) Effect of chemically well-defined sphingosine and its *N*-methyl derivatives on protein kinase C and src kinase activities. *Biochemistry* **28**, 6796-6800
28. Schwartz, G.K., Jiang, J., Kelsen, D., and Albino, A.P. (1993) Protein kinase C: a novel target for inhibiting gastric cancer cell invasion. *J. Natl. Cancer Inst.* **85**, 402-407
29. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917
30. Zucker, M.B. (1989) Platelet aggregation measured by the photometric method. *Methods Enzymol.* **169**, 117-133
31. Yatomi, Y., Hazeki, O., Kume, S., and Ui, M. (1992) Suppression by wortmannin of platelet responses to stimuli due to inhibition of pleckstrin phosphorylation. *Biochem. J.* **285**, 745-751
32. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450
33. Khan, W.A., Dobrowsky, R., Touny, S.E., and Hannun, Y.A. (1990) Protein kinase C and platelet inhibition by D-*erythro*-sphingosine: comparison with *N,N*-dimethylsphingosine and commercial preparation. *Biochem. Biophys. Res. Commun.* **172**, 683-691
34. Tyers, M., Rachubinski, R.A., Stewart, M.I., Varrichio, A.M.,

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

Shorr, R.G.L., Haslam, R.J., and Harley, C.B. (1988) Molecular cloning and expression of the major protein kinase C substrate of platelets. *Nature* **333**, 470-473
35. Siess, W. (1989) Molecular mechanisms of platelet activation. *Physiol. Rev.* **69**, 58-178
36. Tamaoki, T. (1991) Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. *Methods Enzymol.* **201**, 340-347
37. Rhee, S.G. and Bae, Y.S. (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* **272**, 15045-15048
38. Olivera, A., Kohama, T., Tu, Z., Milstien, S., and Spiegel, S. (1998) Purification and characterization of rat kidney sphingosine kinase. *J. Biol. Chem.* **273**, 12576-12583
39. Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**, 693-698
40. Igarashi, Y. and Hakomori, S. (1989) Enzymatic synthesis of *N,N*-dimethyl-sphingosine: demonstration of the sphingosine: *N*-methyltransferase in mouse brain. *Biochem. Biophys. Res. Commun.* **164**, 1411-1416
41. Nudelman, E.D., Levery, S.B., Igarashi, Y., and Hakomori, S. (1992) Plasmalopsychosine, a novel plasmal (fatty aldehyde) conjugate of psychosine with cyclic acetal linkage. Isolation and characterization from human brain white matter. *J. Biol. Chem.* **267**, 11007-11016
42. Igarashi, Y., Kitamura, K., Toyokuni, T., Dean, B., Fenderson, B., Ogawa, T., and Hakomori, S. (1990) A specific enhancing effect of *N,N*-dimethylsphingosine on epidermal growth factor receptor autophosphorylation. *J. Biol. Chem.* **265**, 5385-5389
43. Felding-Habermann, B., Igarashi, Y., Fenderson, B.A., Park, L. S., Radin, N.S., Inokuchi, J., Strassmann, G., Handa, K., and Hakomori, S. (1990) A ceramide analogue inhibits T cell proliferative response through inhibition of glycosphingolipid synthesis and enhancement of *N,N*-dimethylsphingosine synthesis. *Biochemistry* **29**, 6314-6322

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012