N,N-Dimethylsphingosine 1-phosphate activates human platelets

Yutaka Yatomi[a,*], Soichiro Yamamura[1,b], Fuqiang Ruan[2,b], Shoji Kume[a], Yukio Ozaki[a], Yasuyuki Igarashi[3,b]

[a]*Department of Laboratory Medicine, Yamanashi Medical University, Tamaho-cho, Nakakoma-gun, Yamanashi 409-38, Japan*
[b]*The Biomembrane Institute, Seattle, WA 98195, USA*

Received 30 September 1997

Abstract We recently reported that *N,N*-dimethylsphingosine 1-phosphate (DMS-1-P) can be formed from *N,N*-dimethylsphingosine (DMS) in activated platelets [Y. Yatomi et al., Biochem. Biophys. Res. Commun. 231 (1997) 848–851]. In this study, we synthesized, for the first time, DMS-1-P and examined the functional effects of DMS-1-P and its related sphingolipids on platelets. Although exogenous DMS was inactive, its phosphorylated derivative, DMS-1-P, induced platelet intracellular Ca^{2+} mobilization and shape change, but not aggregation or release reactions. Since sphingosine 1-phosphate (Sph-1-P) is structurally related to DMS-1-P and activates platelets more strongly than DMS-1-P, a competitive binding experiment for [^{3}H]Sph-1-P was performed using DMS-1-P. DMS-1-P reduced the binding of [^{3}H]Sph-1-P to platelets almost as much as unlabeled Sph-1-P did. These results suggest that DMS-1-P activates platelets via an interaction with a platelet surface receptor for Sph-1-P.

Key words: *N,N*-Dimethylsphingosine 1-phosphate; *N,N*-Dimethylsphingosine; Sphingosine 1-phosphate; Sphingolipid; Platelet activation

1. Introduction

The sphingolipids, whose fundamental backbone structure is sphingosine (Sph), have been shown to be signal-transducing lipids [1–5]. The distinguishing characteristic of the sphingolipids is their participation in the pro- or anti-proliferative pathways of cell regulation. Ceramide (Cer) [1–3] and Sph [6,7] are claimed to be important in the regulation of programmed cell death (apoptosis), while sphingosine 1-phosphate (Sph-1-P) induces mitogenesis and has been implicated as a second messenger in the cellular proliferation induced by platelet-derived growth factor and serum [3,5].

Anucleate blood platelets are considered to be a model system for working out stimulus-response coupling pathways, which have general implications for other cell types. Evidence has accumulated on the involvement of glycerophospholipid metabolites in platelet signaling pathways [8]. However, less is known of the signaling pathways and the functional effects of sphingolipids in these non-proliferative, terminally differentiated cells. We previously reported that Sph-1-P, which is the initial product of catabolism of Sph [3,5], induces platelet shape change and aggregation reactions by itself and synergistically elicits aggregation in combination with weak platelet agonists such as epinephrine and ADP [9,10]. These functional responses, induced by Sph-1-P, were accompanied by intracellular Ca^{2+} mobilization [9,10] and activation of the non-receptor tyrosine kinase Syk [11]. These findings suggest that Sph derivatives can be signaling molecules not only in nucleated cells but also in anucleate platelets. Furthermore, we recently investigated the metabolism of various Sph derivatives in human platelets and reported that *N,N*-dimethylsphingosine 1-phosphate (DMS-1-P) can be generated from *N,N*-dimethylsphingosine (DMS) upon agonist stimulation, the first report to describe the formation of DMS-1-P in a biological system [12]. Although the physiological significance of this phosphorylated and methylated Sph DMS-1-P is unknown, it is important that the action of this novel sphingolipid be elucidated considering that both Sph-1-P [3–5,10] and DMS [4,13,14] have important and distinct functions in the signal transduction pathways and that DMS-1-P could be an inhibitor of Sph-1-P lyase [15]. In this study, we describe a method for DMS-1-P preparation and show that exogenous DMS-1-P activates platelets, through its action on a postulated Sph-1-P receptor.

2. Materials and methods

2.1. Materials

Sph-1-P was prepared from sphingosylphosphorylcholine (SPC) with bacterial phospholipase D [16]. DMS [13] and *N*-acetylsphingosine (C_2-Cer) [17] were synthesized as described previously. Ceramide 1-phosphate (Cer-1-P) was prepared by diacylglycerol kinase-catalyzed phosphorylation of Cer. Lactosylceramide was prepared from dog erythrocytes [18]. [3-^{3}H]Sph-1-P was prepared by ATP-dependent phosphorylation of [3-^{3}H]Sph catalyzed by Sph kinase obtained from BALB/c 3T3 fibroblasts [19].

The following materials were obtained from the indicated suppliers: Sph, Cer (type III), and DL-*threo*-dihydrosphingosine (Sigma, St. Louis, MO); fura2-AM (Molecular Probes, Eugene, OR); [3-^{3}H]Sph (22.0 Ci/mmol) (Du Pont-New England Nuclear, Boston, MA).

2.2. DMS-1-P preparation

Attempts to prepare DMS-1-P from Sph-1-P by reductive methylation were not successful, presumably due to the insolubility of Sph-1-P under such reaction conditions. Therefore, we synthesized D-*erythro*-DMS-1-P by a semi-enzymatic approach (Scheme 1), using SPC as a starting material. Since SPC purchased from Sigma was a mixture of the D-*erythro*- and L-*threo* forms [16,20], reductive methylation of SPC afforded dimethyl-SPC (DMSPC) as a mixture of both stereoisomers, which were readily separated by chromatography on silica gel. Treat-

*Corresponding author. Fax: (81) (552) 73-6713.

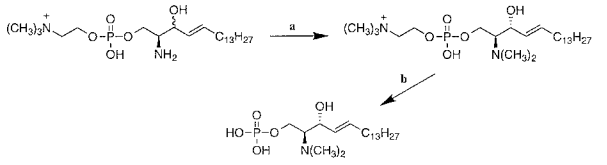
[1]Present address: Pacific Northwest Research Foundation, Division of Biomembrane Research, Seattle, WA 98122, USA.

[2]Present address: Molecumetics, Bellevue, WA 98005, USA.

[3]Present address: Fred Hutchinson Cancer Research Center, Seattle, WA 98104, USA.

Abbreviations: DMS-1-P, *N,N*-dimethylsphingosine 1-phosphate; DMS, *N,N*-dimethylsphingosine; Sph-1-P, sphingosine 1-phosphate; Sph, sphingosine; Cer, ceramide; Cer-1-P, ceramide 1-phosphate; SPC, sphingosylphosphorylcholine

PII S0014-5793(97)01321-5



(a) 37% CH_2O, $NaCNBH_3$, NaOAc - HOAc; (b) Phospholipase D, 50 mM NH_4OAc buffer (pH = 8)

Scheme 1.

ment of D-*erythro*-DMSPC with phospholipase D (*Streptomyces chromofuscus*) gave D-*erythro*-DMS-1-P. The configuration of DMS-1-P was confirmed by NMR analysis (data not shown). Unlike Sph-1-P, D-*erythro*-DMS-1-P was quite water-soluble.

2.3. Platelet preparation

Washed platelets (3×10^8/ml) were prepared as described [9], and supplemented with 1 mM $CaCl_2$ unless stated otherwise. For the shape change studies, the platelets were left for at least 60 min at 37°C after their final centrifugation, because this resulted in a greater response. All of the experiments using intact platelet suspensions were performed at 37°C.

2.4. Platelet shape change and aggregation

Platelet shape change and aggregation were determined turbidimetrically in a platelet ionized calcium aggregometer (PICA) (Chrono-Log, Havertown, PA), while being stirred at 1000 rpm. The instrument was calibrated with a platelet suspension for zero light transmission and the buffer for 100% transmission. When aggregation was measured, human fibrinogen (500 μg/ml) was added to platelet suspensions shortly before the addition of stimuli.

The shape change was observed by adding 5 mM EDTA (instead of Ca^{2+}) before stimulation to prevent aggregation and indicated by a decrease in light transmission. For the quantitative evaluation of both processes, the results are expressed as the maximum change in the transmission of light within 5 min after stimulation.

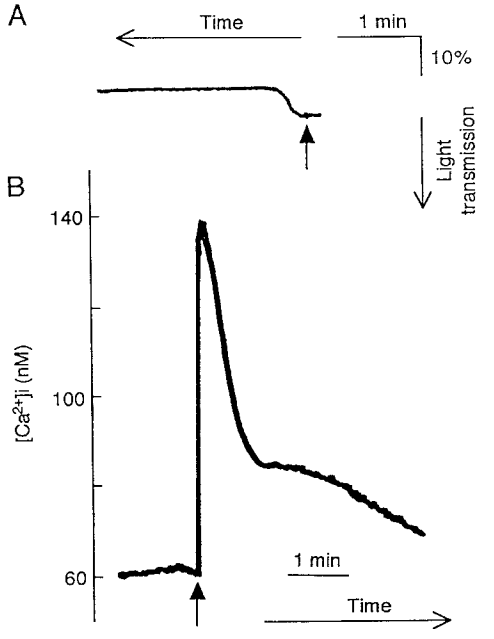
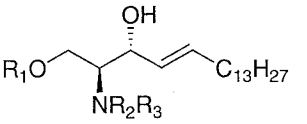


Fig. 1. DMS-1-P-induced platelet activation. A: Platelets were challenged with 20 μM DMS-1-P as indicated by the arrow. The shape change was observed turbidimetrically. The decrease in light transmission of a platelet suspension monitors the change from the discoid to the spheroid shape [24]. B: Platelets were stimulated with 40 μM DMS-1-P as indicated by the arrow. The intracellular Ca^{2+} concentration was monitored using the Ca^{2+}-sensitive fluorophore fura2.



1. $R_1 = PO_3H_2$, $R_2 = H$, $R_3 = H$,
2. $R_1 = PO_3H_2$, $R_2 = CH_3$, $R_3 = CH_3$,
3. $R_1 = PO_3H_2$, $R_2 = H$, $R_3 = C(O)C_{17}H_{25}$,
4. $R_1 = H$, $R_2 = H$, $R_3 = H$,
5. $R_1 = H$, $R_2 = CH_3$, $R_3 = CH_3$,
6. $R_1 = H$, $R_2 = H$, $R_3 = C(O)C_{17}H_{25}$

Fig. 2. Chemical structure of sphingolipids used in this study. 1: Sph-1-P; 2: DMS-1-P; 3: Cer-1-P; 4: Sph; 5: DMS; 6: Cer

2.5. Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was measured using the Ca^{2+}-sensitive fluorophore fura2 as previously described [9]. The values of the calcium peak increases after the addition of each agent were quantified by fluorometry.

2.6. [^{3}H]Sph-1-P binding assays

The binding assays were performed by incubating intact platelets (1×10^8 cells), which were suspended in phosphate-buffered saline, with 2.1 nM [^{3}H]Sph-1-P. The reactions were initiated by the addition of a ligand and were incubated at 4°C for 1 h. The platelets were then washed three times with a solution of 1 mg/ml of bovine serum albumin, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5). The radioactivity of the platelets was counted by liquid scintillation counting. Under the experimental conditions, the added [^{3}H]Sph-1-P remained intact, as confirmed by separation by thin layer chromatography.

Total binding was defined as the amount of radioactivity bound to platelets in the absence of a competing ligand. Non-specific binding was defined as the amount of [^{3}H]Sph-1-P binding that occurred in the presence of excess amount of (50 μM) Sph-1-P. Specific binding was defined as the difference between the total and the non-specific binding.

3. Results and discussion

3.1. Effects of various Sph derivatives, including DMS-1-P, on platelet functional responses

Platelets show several responses that can be studied separately in vitro but are closely linked during hemostasis in vivo: adhesion, shape change, aggregation, and secretion [8]. If platelets are in a suspension, the first response to stimulation is shape change, followed by aggregation and secretion. Metabolic energy is required at all stages of platelet activation, and the various responses have different energy requirements increasing in the order: shape change < aggregation < granule secretion [21,22]. When human platelets were stimulated with DMS-1-P, a marked shape change reaction was observed by the turbidimetric method (Fig. 1A). However, DMS-1-P did not elicit platelet aggregation or secretion of 5-hydroxytryptamine (data not shown). DMS-1-P, which was only able to induce the shape change reaction, may be classified as a weak platelet agonist.

It is acknowledged that intracellular Ca^{2+} mobilization plays a central role in eliciting platelet functional responses [8]. Hence, we studied the effects of DMS-1-P on Ca^{2+} levels in human platelets. As expected, DMS-1-P induced a prompt increase in $[Ca^{2+}]i$, peaking within 20 s of addition (Fig. 1B). The intracellular Ca^{2+} mobilization seems important for platelet activation induced by DMS-1-P, as is the case with Sph-1-P [9,10].

We next compared the effects of various sphingolipids, in-

Table 1
Effects of various Sph derivatives on platelet shape change, aggregation, and $[Ca^{2+}]_i$ increase

Sph derivative	Shape change (%)	Aggregation (%)	$[Ca^{2+}]_i$ increase (nM)
Sph	ND	ND	ND
Sph-1-P	4.8 ± 0.2	14.7 ± 3.0	54.7 ± 8.9
DMS	ND	ND	ND
DMS-1-P	4.4 ± 0.5	ND	40.3 ± 10.7
Cer (type III)	ND	ND	ND
C_2-Cer	ND	ND	ND
Cer-1-P	4.5 ± 0.1	ND	NP
DL-*threo*-Dihydrosphingosine	ND	ND	ND
Lactosylceramide	ND	ND	ND

Platelets were stimulated with various Sph derivatives, and shape change, aggregation, and $[Ca^{2+}]_i$ increase were examined. The concentrations of Sph derivatives used were 10 μM for shape change and $[Ca^{2+}]_i$ increase, and 40 μM for aggregation. The values represent the mean ± S.D. ($n = 3$). ND, not detectable. NP, not performed.

cluding DMS-1-P, on platelet shape change, aggregation, and $[Ca^{2+}]i$ increase under the same conditions (Fig. 2 and Table 1). In addition to Sph-1-P, which had been found to be a platelet activator [9,10], both DMS-1-P (see above) and Cer-1-P induced a marked shape change. When platelet aggregation, a more energy-requiring process than shape change [21,22], was examined, only Sph-1-P was capable of inducing the response. Sph-1-P and DMS-1-P were confirmed to induce intracellular Ca^{2+} mobilization, which is related to platelet activation, especially shape change [8]. Although phosphorylated sphingolipids such as Sph-1-P, DMS-1-P, and Cer-1-P elicited platelet functional responses, their unphosphorylated compounds, i.e. Sph, DMS, and Cer, did not. It should be noted that DMS and DMS-1-P exert contrasting effects on platelets; DMS inhibits protein kinase C and Sph kinase and hence suppresses platelet functional responses [13,14,23], while DMS-1-P is a platelet activator (see above).

3.2. *Inhibition by DMS-1-P of specific [^{3}H]Sph-1-P binding to platelets*

As described above, phosphorylated sphingolipids specifically elicit platelet activation, and among them Sph-1-P is the strongest platelet activator in that it was the only one capable of inducing platelet aggregation, a more energy-requiring process. We are now postulating that Sph-1-P acts on platelets via an interaction with a plasma membrane receptor. Platelets were found to possess two binding sites for Sph-1-P; the K_d values were estimated to be 110 nM and 2.6 μM, and the numbers of binding sites approximately 200/cell and 2400/cell, respectively [10]. Since the existence of a mono-ester phosphate moiety seems important in the ligand-receptor interaction of the sphingolipids, we hypothesized that the mono-ester phosphate moiety of Sph-1-P is important for its interaction with the platelet surface receptor and that DMS-1-P, through the moiety including mono-ester phosphate, cross-reacts with the putative Sph-1-P receptor. Accordingly, competition binding experiments of [^{3}H]Sph-1-P were performed using DMS-1-P (Fig. 3). DMS-1-P reduced the binding of [^{3}H]Sph-1-P to platelets almost as much as unlabeled Sph-1-P did, while Sph was unable to compete for the [^{3}H]Sph-1-P binding sites on platelets under the same conditions. Although DMS-1-P effectively displaces [^{3}H]Sph-1-P binding to platelets, it is unlikely that DMS-1-P fully activates the pathways derived from the Sph-1-P receptor. Rather, DMS-1-P may be a partial agonist for this receptor, which may be related to the facts that only Sph-1-P (but not DMS-1-P) induces platelet aggregation, and that Sph-1-P induces intracellular Ca^{2+} mobilization more strongly than DMS-1-P.

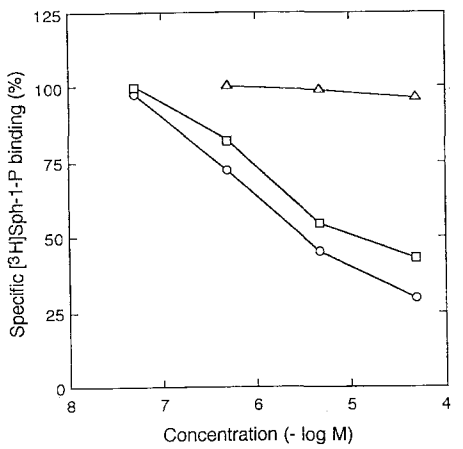


Fig. 3. Inhibition of the [^{3}H]Sph-1-P binding to platelets by DMS-1-P. Platelets were incubated with [^{3}H]Sph-1-P alone or in the additional presence of various concentrations of unlabeled Sph-1-P (○), DMS-1-P (□), and Sph (△). The specific [^{3}H]Sph-1-P binding to platelets is expressed as a percentage of the control (without the unlabeled ligand).

In conclusion, our data demonstrate that DMS-1-P, as well as Sph-1-P, activates platelets, and that these phosphorylated sphingolipids may bind to the same cell surface receptor. In view of our previous report that DMS-1-P can be formed from DMS in activated platelets [12], the fact that phosphorylation of the anti-platelet agent DMS results in a platelet activator may be of physiological importance. We believe that the functional effects of DMS-1-P in various cell systems are an important subject for future study.

Acknowledgements: This work was supported by funds from The Biomembrane Institute, in part under research contracts with Otsuka Pharmaceutical Company and Seikagaku Corporation and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

References

[1] Hannun, Y.A. (1994) J. Biol. Chem. 269, 3125–3128.
[2] Kolesnick, R. and Golde, D.W. (1994) Cell 77, 325–328.
[3] Spiegel, S., Foster, D. and Kolesnick, R. (1996) Curr. Opin. Cell Biol. 8, 159–167.
[4] Hakomori, S. and Igarashi, Y. (1995) J. Biochem. 118, 1091–1103.
[5] Spiegel, S. and Merrill Jr., A.H. (1996) FASEB J. 10, 1388–1397.

[6] Ohta, H., Sweeney, E.A., Masamune, A., Yatomi, Y., Hakomori, S. and Igarashi, Y. (1995) Cancer Res. 55, 691–697.
[7] Nakamura, S., Kozutsumi, Y., Sun, Y., Miyake, Y., Fujita, T. and Kawasaki, T. (1996) J. Biol. Chem. 271, 1255–1257.
[8] Siess, W. (1989) Physiol. Rev. 69, 58–178.
[9] Yatomi, Y., Ruan, F., Hakomori, S. and Igarashi, Y. (1995) Blood 86, 193–202.
[10] Yatomi, Y., Yamamura, S., Ruan, F. and Igarashi, Y. (1997) J. Biol. Chem. 272, 5291–5297.
[11] Yang, L., Yatomi, Y., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Igarashi, Y., Ozaki, Y. and Kume, S. (1996) Biochem. Biophys. Res. Commun. 229, 440–444.
[12] Yatomi, Y., Ozaki, Y., Satoh, K., Kume, S., Ruan, F. and Igarashi, Y. (1997) Biochem. Biophys. Res. Commun. 231, 848–851.
[13] 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.
[14] Yatomi, Y., Ruan, F., Megidish, T., Toyokuni, T., Hakomori, S. and Igarashi, Y. (1996) Biochemistry 35, 626–633.
[15] Van Veldhoven, P.P. and Mannaerts, G.P. (1993) Adv. Lipid Res. 26, 69–98.
[16] Van Veldhoven, P.P., Foglesong, R.J. and Bell, R.M. (1989) J. Lipid Res. 30, 611–616.
[17] Vunnam, R.R. and Radin, N.S. (1979) Biochim. Biophys. Acta 573, 73–82.
[18] Hakomori, S. (1983) in: Sphingolipid Biochemistry (Kanfer, J.N. and Hakomori, S., Eds.), pp. 1–165, Plenum Press, New York.
[19] Mazurek, N., Megidish, T., Hakomori, S. and Igarashi, Y. (1994) Biochem. Biophys. Res. Commun. 198, 1–9.
[20] Ruan, F., Sadahira, Y., Hakomori, S. and Igarashi, Y. (1992) Biomed. Chem. Lett. 2, 973–978.
[21] Holmsen, H., Kaplan, K.L. and Dangelmaier, C.A. (1982) Biochem. J. 208, 9–18.
[22] Verhoeven, A.J.M., Mommersteeg, M.E. and Akkerman, J.W.N. (1984) Biochem. J. 221, 777–787.
[23] Handa, K., Igarashi, Y., Nisar, M. and Hakomori, S. (1991) Biochemistry 30, 11682–11686.
[24] Zucker, M.B. (1989) Methods Enzymol. 169, 117–133.