

Mechanisms of sphingosine and sphingosine 1-phosphate generation in human platelets

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Abstract The bioactive molecule sphingosine 1-phosphate (S1P) is abundantly stored in platelets and can be released extracellularly. However, although they have high sphingosine (Sph) kinase activity, platelets lack the *de novo* sphingolipid biosynthesis necessary to provide the substrates. Here, we reveal a generation pathway for Sph, the precursor of S1P, in human platelets. Platelets incorporated extracellular ³H-labeled Sph much faster than human megakaryoblastic cells and rapidly converted it to S1P. Furthermore, Sph formed from plasma sphingomyelin (SM) by bacterial sphingomyelinase (SMase) and neutral ceramidase (CDase) was rapidly incorporated into platelets and converted to S1P, suggesting that platelets use extracellular Sph as a source of S1P. Platelets abundantly express SM, possibly supplied from plasma lipoproteins, at the cell surface. Treating platelets with bacterial SMase resulted in Sph generation at the cell surface, conceivably by the action of membrane-bound neutral CDase. Simultaneously, a time-dependent increase in S1P levels was observed. Finally, we demonstrated that secretory acid SMase also induces S1P increases in platelets. **In conclusion, our results suggest that in platelets, Sph is supplied from at least two sources: generation in the plasma followed by incorporation, and generation at the outer leaflet of the plasma membrane, initiated by cell surface SM degradation.**—Tani, M., T. Sano, M. Ito, and Y. Igarashi. **Mechanisms of sphingosine and sphingosine 1-phosphate generation in human platelets.** *J. Lipid Res.* 2005. 46: 2458–2467.

Supplementary key words ceramide • sphingomyelinase • neutral ceramidase

Sphingolipid metabolites have been implicated as modulators of membrane signal transduction systems and shown to be involved in diverse cellular processes (1–4). Generally, the sphingolipid sphingomyelin (SM), which is distributed mainly on the exoplasmic side of the plasma membrane, is broken down by signal-activated sphingomyelinases (SMases), leading to the formation of ceramide (Cer), an important

signaling molecule with diverse cellular functions (2). Subsequently, Cer is deacylated by ceramidase (CDase) to generate sphingosine (Sph); because Sph is not produced by *de novo* synthesis, its generation is restricted to this pathway (5), referred to here as the “SM degradation pathway.” In a final step, which occurs mainly in the cytoplasmic region of the cells, Sph is phosphorylated by Sph kinase into sphingosine 1-phosphate (S1P).

S1P has attracted a great deal of attention as a key cell signaling molecule, functioning both as an intracellular second messenger and as a ligand for five G protein-coupled receptors, the endothelial differentiation gene receptors, now named S1P₁ to S1P₅ (4). With such multiple and pleiotropic targets, S1P as an extracellular messenger influences numerous cell functions, including vascular maturation during development (6), heart rate (7), and lymphocyte recirculation (8).

S1Ps are present in plasma and serum (9). This extracellular S1P is thought to be generated through two pathways: phosphorylation of extracellular Sph by Sph kinase released from endothelial cells (10), and release of intracellular S1P from certain cell types (11, 12). At present, it is widely accepted that the most important source for extracellular S1P is platelets. Platelets abundantly store S1P intracellularly and release it extracellularly after stimulation by protein kinase C activators (13, 14). Generally, S1P levels in cells are low because of its degradation by S1P lyase (15), but platelets lack S1P lyase activity and have highly active Sph kinases, so they accumulate high concentrations of S1P (14, 16). However, just how the precursor Sph is generated in platelets remains unclear.

In the current study, we determined that Sph generated in the plasma or in the outer leaflet of the platelet plasma membrane is converted to S1P within the platelets. Al-

Abbreviations: CDase, ceramidase; Cer, ceramide; FB₁, fumonisin B₁; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; SM, sphingomyelin; SMase, sphingomyelinase; Sph, sphingosine; S1P, sphingosine 1-phosphate.

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though the de novo sphingolipid biosynthetic pathway in platelets was found to be very weak, the platelets rapidly incorporated extracellular Sph and converted it to SIP. Additionally, at their cell surface, the platelets abundantly expressed SM, possibly produced from a supply of plasma lipoproteins. Exogenously added SMase triggered Cer increase, subsequent Sph generation at the cell surface (conceivably through the action of neutral CDase), and, finally, the formation of SIP in platelets. In summary, we propose that SIP generation in platelets depends on a supply of Sph from extracellular sources, including the outer leaflet of the plasma membrane.

MATERIALS AND METHODS

Materials

CHOP cells were kindly provided by Dr. K. Nara (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan). Apyrase was purchased from Nacalai Tesque (Tokyo, Japan). BSA (essentially fatty acid-free), palmitoyl-CoA, fumonisins B₁ (FB₁), UDP-glucose, and prostaglandin E₁ were obtained from Sigma (St. Louis, MO). SMase from *Bacillus cereus* was purchased from Funakoshi (Tokyo, Japan). Precoated Silica Gel 60 TLC plates were obtained from Merck (Whitestation, NJ). [³H]Sph, [¹⁴C]serine, and [γ -³²P]ATP were purchased from Perkin-Elmer Life Sciences (Boston, MA), and [¹⁴C] palmitoyl-CoA was from American Radiolabeled Chemical, Inc. (St. Louis, MO). C6-4-nitrobenzo-2-oxa-1,3-diazole (NBD)-SM and C6-NBD-Cer were purchased from Matreya, Inc. (Pleasant Gap, PA), and C12-NBD-Cer was prepared as described elsewhere (17). All other reagents were of the highest purity available.

Platelet and plasma preparation

Platelets were isolated from the blood of healthy adult volunteers. The blood was collected in 0.2 volume of the anticoagulant ACD (0.8% citric acid, 2.2% sodium citrate, and 2.45% glucose) and centrifuged at 120 *g* for 15 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma fraction was incubated with 3 units of apyrase at 37°C for 10 min. Then, 1.0 volume of buffer A [138 mM NaCl, 3.3 mM NaH₂PO₄, 2.9 mM KCl, 1 mM MgCl₂, 20 mM HEPES-NaOH (pH 7.4), and 1 mg/ml glucose] containing 1% BSA, 1 μ M prostaglandin E₁, and 0.2 volume of ACD was added to the platelet-rich plasma fraction, followed by centrifugation at 1,107 *g* for 10 min at room temperature. The platelets were then washed with buffer A containing 1% BSA and 0.2 volume of ACD. The platelets were resuspended in buffer A containing 0.1% BSA and used as platelet suspensions. Plasma was obtained from anticoagulated blood by centrifugation at 12,000 *g* for 1 min. The plasma was buffered with HEPES-NaOH (20 mM, pH 7.4).

Cell culture and transfection

The human megakaryoblastic cell lines CMK and MEG-O1 were grown in RPMI 1640 medium supplemented with 10% FBS, 100 μ g/ml streptomycin, and 100 U/ml penicillin in a 5% CO₂ incubator. CHOP cells, which are Chinese hamster ovary cells that express the polyoma large T antigen to support the replication of eukaryotic expression vectors (18), and HEK293T human embryonic kidney cells were grown in α -MEM and DMEM, respectively, each supplemented with 10% FBS, 100 μ g/ml streptomycin, and 100 U/ml penicillin. Transfection was performed using LipofectAMINE Plus™ Reagent (Invitrogen Co., Carlsbad, CA).

Preparation of cell lysates

To prepare cell lysates, CHOP cells (1×10^6), CMK cells (2×10^6), MEG-O1 cells (1×10^6), and platelets (3.5×10^8) were washed with 1 ml of PBS, suspended in 1 ml of 10 mM Tris-HCl (pH 7.5) containing 1 \times protease inhibitor mixture (Roche Diagnostics Co., Indianapolis, IN) and 1 mM phenylmethylsulfonyl fluoride, sonicated (three times for 30 s at 4°C), and stored at -80°C before use.

Lipidase assays

The activity of neutral CDase was measured using C12-NBD-Cer as a substrate (19). CDase activity at different pH conditions was determined using GTA [3,3-dimethyl-glutaric acid, Tris(hydroxymethyl) aminomethane, and 2-amino-2-methyl-1,3-propanediol] buffer (19).

A Sph kinase assay was performed according to the method of Olivera et al. (20) with slight modifications. In brief, an appropriate amount of cell lysate was incubated with 1 μ Ci of [γ -³²P] ATP and 50 μ M Sph at 37°C for 30 min in 100 μ l of reaction mix [20 mM Tris-HCl buffer (pH 7.5) containing 0.25% Triton X-100, 5 mM MgCl₂, 12 mM β -glycerophosphate, 0.25 mM EDTA, and 1 mM sodium pyrophosphate]. The reaction was terminated by the addition of 375 μ l of chloroform-methanol-concentrated HCl (100:200:1), and lipids were extracted by the method of Bligh and Dyer (21). Portions of the lipid extract were applied to a TLC plate, which was then developed in butanol-acetic acid-water (3:1:1, v/v). Radioactive bands were visualized and quantified using an imaging analyzer (FLA5000; Fuji Film).

The activity of acid SMase was measured at pH 5.0 using C6-NBD-SM as a substrate. Briefly, C6-NBD-SM (550 pmol) was incubated at 37°C for 30 min with an appropriate amount of cell lysate in 20 μ l of reaction mix [100 mM acetate buffer (pH 5.0) containing 0.1% Triton X-100]. Where indicated, a final concentration of 0.1 mM ZnCl₂ was added to the reaction mix. The reaction was stopped by adding 100 μ l of chloroform-methanol (2:1, v/v), and the resulting lower layer was applied to a TLC plate, which was then developed with chloroform-methanol-25% ammonia (90:20:0.5, v/v). The released C6-NBD-Cer and the remaining C6-NBD-SM were quantified with an imaging analyzer.

Cer glucosyltransferase and SM synthase assays

Cer glucosyltransferase and SM synthase activities were quantified according to the method of Lipsky and Pagano (22) with slight modifications. C6-NBD-Cer (10 nmol in 10 μ l of ethanol) was mixed with 200 μ l of distilled water containing 0.01% BSA to form a BSA complex. An appropriate amount of cell lysate was incubated with 5 μ l of the BSA complex at 32°C for 1 h in 50 μ l of reaction buffer [25 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 500 μ M UDP-glucose]. The reaction was stopped by adding 150 μ l of chloroform-methanol (2:1, v/v), and the resulting lower layer was applied to a TLC plate, which was then developed with chloroform-methanol-12 mM MgCl₂ (65:25:4, v/v). C6-NBD-glucosyl-Cer and C6-NBD-SM were quantified with an imaging analyzer.

Serine palmitoyltransferase assay

Serine palmitoyltransferase activity was measured according to the method of Merrill (23) with slight modifications. Specifically, 0.5 μ Ci of [¹⁴C]serine and 50 μ M palmitoyl-CoA were incubated at 37°C for 20 min with an appropriate amount of cell lysate in 300 μ l of reaction buffer [100 mM HEPES-NaOH (pH 8.0), 5 mM EDTA, 5 mM DTT, and 50 μ M pyridoxal 5-phosphate]. The reaction was stopped by adding 600 μ l of chloroform-methanol (2:1, v/v), and the resulting lower layer was applied to a TLC plate, which was then developed with chloroform-methanol-25% ammonia (90:20:0.5, v/v). The radiolabeled 3-ketosphinganine was visualized and quantified using an imaging analyzer.

TABLE 1. Activity of sphingolipid synthesizing and metabolizing enzymes

Enzyme	CHOP	CMK	MEG-O1	Platelets
	<i>pmol/min/mg protein</i>			
Serine palmitoyltransferase	5.09 ± 0.32	6.11 ± 0.50	4.67 ± 0.33	0.48 ± 0.07
Sphinganine <i>N</i> -acyltransferase	17.86 ± 1.72	31.68 ± 1.74	28.19 ± 0.36	11.75 ± 0.26
Ceramide glucosyltransferase	53.58 ± 1.42	42.09 ± 0.98	34.83 ± 1.04	1.95 ± 0.64
Sphingomyelin synthase	7.32 ± 0.79	8.47 ± 0.04	5.58 ± 0.34	2.50 ± 0.12
Ceramidase (pH 7.5)	0.62 ± 0.02	0.61 ± 0.06	0.79 ± 0.05	1.17 ± 0.05
Sphingosine kinase	4.46 ± 0.19	0.14 ± 0.002	0.11 ± 0.007	29.06 ± 4.28

Total cell lysates prepared from each cell line were used. Results are means ± SD for at least three independent experiments.

and labeled lipids were analyzed by TLC. Human megakaryoblastic MEG-O1 and CMK cells were also labeled as controls. In the megakaryoblastic cells, radiolabeled SM and Cer, as well as glycerophospholipids, were detected (Fig. 1A). However, in platelets, despite efficient labeling of glycerophospholipids, little SM or Cer was detected.

Reportedly, the inhibition of sphinganine *N*-acyltransferase by FB₁ causes a rapid increase in sphinganine, an intermediate of de novo sphingolipid biosynthesis, and, consequently, in the levels of sphinganine 1-phosphate (28). Using this inhibitor, we examined the effect of de novo sphingolipid biosynthesis in platelets. To confirm the effectiveness of FB₁ on Cer synthesis of platelets, platelets were incubated with [³H]Sph in the presence of 100 μM FB₁ for 1, 3, and 8 h. As a result, FB₁ strongly inhibited the conversion of [³H]Sph to [³H]Cer in platelets, but no inhibitory activity was observed in the conversion of [³H]Sph to [³H]S1P (data not shown). As expected, marked increases in intracellular sphinganine and sphinganine 1-phosphate levels were observed in MEG-O1 and CMK cells after a 5 h FB₁ treatment (Fig. 1B). In contrast, no accumulation was observed in platelets even after an 18 h treatment with FB₁. Together, these results indicate that platelets synthesize very few sphingolipids de novo.

The inability of platelets to synthesize sphingolipids de novo suggested that the initial step of sphingolipid biosynthesis, the condensation reaction of serine and palmitoyl-CoA to synthesize 3-ketosphinganine, is defective. We examined the activity of enzymes involved in sphingolipid synthesis and metabolism using lysates of human platelets and CHOP, CMK, and MEG-O1 cells. As shown in Table 1, platelets exhibited little serine palmitoyltransferase activity (<10% of the activity in the control cells). Similarly, Cer glucosyltransferase, SM synthase, and sphinganine *N*-acyltransferase activities were all lower in platelets compared with the other cell lines. In contrast, the CDase activity (pH 7.5) of platelets was higher than that of the other cell lines. As expected, platelets had high Sph kinase activity. It appears that a loss in serine palmitoyltransferase activity during megakaryocyte differentiation into platelets leads to an inability to synthesize sphingolipids de novo.

Incorporation of extracellular Sph

Because platelets have little de novo sphingolipid biosynthesis, it is possible that S1P generation in platelets depends on an extracellular supply. Thus, we investigated

the ability of platelets to incorporate and phosphorylate extracellular Sph, the precursor of S1P. Exogenously added [³H]Sph was rapidly incorporated into platelets and efficiently converted to S1P (Fig. 2). The incorporation into

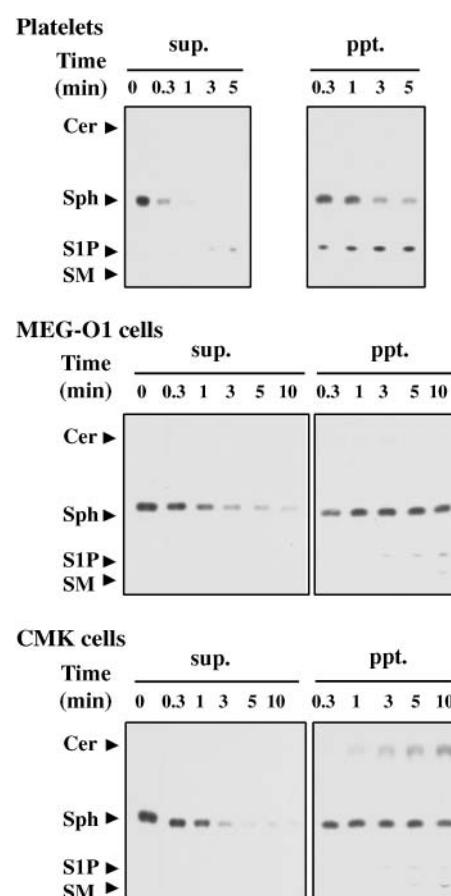


Fig. 2. Incorporation of [³H]sphingosine (Sph) into human platelets and megakaryoblastic cells. Platelets (7×10^7), MEG-O1 cells (2×10^5), and CMK cells (4×10^5), suspended in 200 μl of buffer containing 0.1% BSA, were incubated with 1 μM [³H]Sph (0.05 μCi) at 37°C. At the indicated times, the incubation was terminated by centrifugation at 12,000 *g* for 5 s at 4°C to separate cell pellets and medium supernatant. Lipids were extracted from the supernatants (sup.) and the cell pellets (ppt.) by the method described by Yatomi et al. (13) and were applied to TLC plates, which were then developed in butanol-acetic acid-water (3:1:1, v/v). The bands of radioactive sphingolipids were visualized with autoradiography using Kodak X-Omat film at -80°C for 1 day. SM, sphingomyelin; S1P, sphingosine 1-phosphate.

platelets was notably much faster than that in CMK or MEG-O1 cells, so that the [^3H]Sph had completely disappeared from the supernatant of the platelets by 1 min but was still detectable after 10 min in the supernatants of the CMK and MEG-O1 cells (Fig. 2).

In physiologic conditions, plasma would be the major extracellular source of sphingolipids available to platelets. We recently reported that neutral CDase, a rate-limiting enzyme in the generation of Sph, is present in mouse plasma (29). Using C12-NBD-Cer as a substrate, we found CDase activity in human plasma with an optimum pH near 7.0 (Fig. 3A). The pH dependence of the human plasma enzyme was similar to that of purified mouse (19) and rat neutral CDases (27). To investigate whether this CDase activity is involved in the hydrolysis of Cer formed in plasma, human plasma was treated with SMase from *B. cereus* at 37°C for 5 min. Cer in the plasma was markedly increased (Fig. 3B), and, simultaneously, Sph levels increased, presumably as a result of the hydrolysis of Cer by the CDase in plasma (Fig. 3C). Furthermore, this Sph formation was greatly enhanced upon the addition of conditioned medium containing secretory neutral CDase, indicating that neutral CDase plays a role in Sph formation in the plasma (Fig. 3C). Additionally, when plasma that had been treated with the bacterial SMase and secretory neutral CDase was added to a platelet suspension for 5 min at 37°C, a marked increase in the S1P levels in the platelets was observed (Fig. 3D). A significant increase in platelet S1P levels was also found when plasma treated with only SMase was added to platelets, whereas the addition of untreated plasma had no effect (Fig. 3D). These results indicate that platelets produce S1P from Sph that has been generated from plasma SM.

Sph and S1P generation by hydrolysis of cell surface SM in platelets

Reportedly, phospholipids, including SM, are supplied to platelets from plasma lipoproteins via an endocytosis-independent transfer reaction (30). We found that the SM content of platelets was higher than that of CMK or MEG-O1 cells despite the lack of de novo sphingolipid biosynthesis (Fig. 4A). Furthermore, the majority of the SM appears to be at the outer leaflet of the plasma membrane, because exogenous treatment with bacterial SMase resulted in a marked decrease in SM levels in platelets (Fig. 4B).

Because SM is the initial compound in the pathway generating Sph and S1P, we examined the possibility that in platelets Sph and S1P are generated as a consequence of the hydrolysis of SM at the cell surface. As expected, treating platelets with bacterial SMase for 5 min induced a remarkable increase in Cer levels (Fig. 4C). Sph levels were also increased rapidly, reaching a plateau at 10 min (Fig. 4D, panel a), possibly as a result of CDase activity within the platelets. A concomitant increase in S1P levels was observed, to levels ~20-fold higher than in controls (Fig. 4D, panel b). In contrast, in CHOP cells treated with the SMase, both the Sph and S1P levels transiently increased over control values (75.2 and 2.3 pmol/mg protein, respectively), peaking 30 min after treatment (131.5 and 8.0 pmol/mg

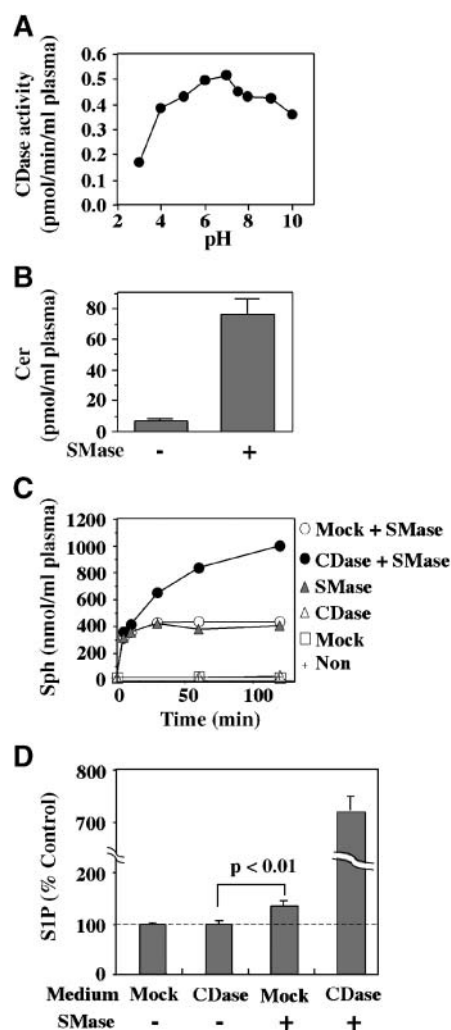


Fig. 3. Generation of Sph in human plasma by bacterial sphingomyelinase (SMase) and neutral ceramidase (CDase) and its conversion in platelets to S1P. **A:** CDase activity in human plasma. C12-4-nitrobenzo-2-oxa-1,3-diazole (NBD)-Cer (550 pmol) was incubated with 10 μl of human plasma at 37°C for 3 h under different pH conditions. Data were averaged from two independent experiments. **B:** Cer generation in human plasma after the addition of bacterial SMase. Human plasma (100 μl) was treated with 1 milliunit of SMase from *B. cereus* at 37°C for 5 min. After incubation, lipids were extracted and total Cer content was measured using *sn*-1,2-diacylglycerol kinase (26). Data represent means \pm SD from three independent experiments. **C:** Time course for Sph generation in human plasma by bacterial SMase and neutral CDase. For each time point, 100 μl of human plasma was incubated at 37°C with 1 milliunit of bacterial SMase and 50 μl of conditioned medium from HEK293T cells transfected with either secretory neutral CDase or mock plasmids. At the indicated times, the sample was subjected to HPLC analysis to measure the total amount of Sph present. Data were averaged from two independent experiments. **D:** S1P generation in platelets from plasma-derived Sph. Human plasma was treated for 2 h with the two conditioned media and bacterial SMase as described for C. Treated plasma (500 μl) was then added to a 1 ml suspension of platelets (3.5×10^8) and incubated at 37°C for 5 min. The samples were centrifuged, and the cells were washed. The total amount of S1P in each sample was measured by HPLC and is presented as a percentage relative to that in control platelets incubated with plasma that had been treated with mock-conditioned medium in the absence of bacterial SMase. Data represent means \pm SD from three independent experiments. Statistical analysis was done using Student's *t*-test.

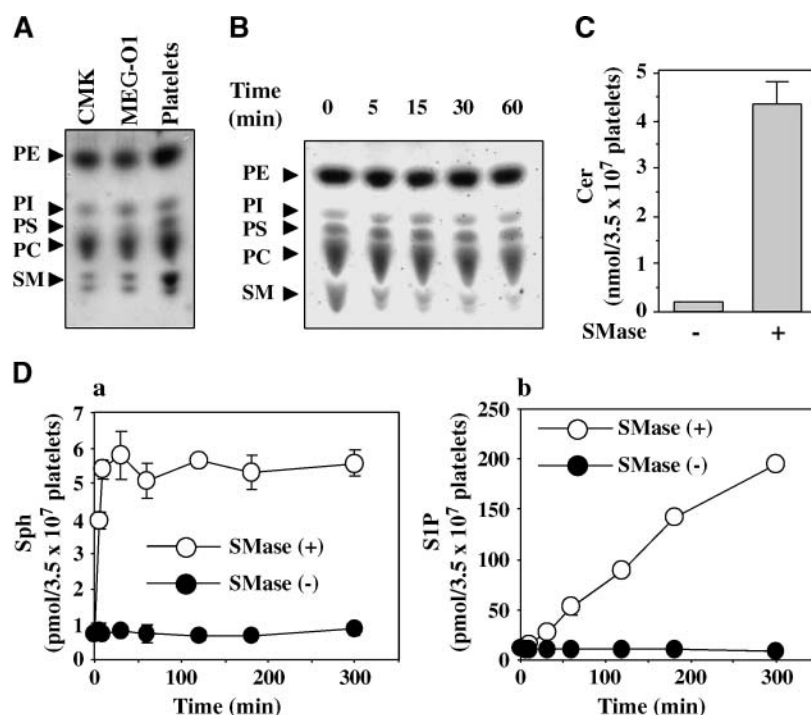


Fig. 4. Hydrolysis of cell-surface SM triggers Sph and SIP generation in platelets. **A:** Distribution of SM in platelets, CMK cells, and MEG-O1 cells. Suspensions of each cell type, corresponding to 270 μ g of cellular protein, were centrifuged, and the total lipids were extracted from the cells by the method of Bligh and Dyer (21). Lipids were applied to TLC plates, resolved in chloroform-methanol-15 mM CaCl_2 (5:4:1, v/v), and visualized by Dittmer reagent. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. **B:** Platelet suspensions (1.1×10^8 cells in 300 μ l) were treated with 3 milliunits of bacterial SMase at 37°C for the indicated times. Lipids were extracted and analyzed as described for **A**. **C:** Cer generation in platelets by exogenously added bacterial SMase. Platelet suspensions (3.5×10^8 cells in 1 ml) were treated without (–) or with (+) 10 milliunits of bacterial SMase at 37°C for 5 min. Lipids were extracted, and the Cer content was determined using *sn*-1,2-diacylglycerol kinase (26). Data represent means \pm SD from three independent experiments. **D:** Sph and SIP generation in platelets after SMase treatment. After treatment with SMase for the indicated times, platelet suspensions were subjected to HPLC analysis to measure the total amount of Sph (**a**) and SIP (**b**). Data represent means \pm SD from three independent experiments.

protein, respectively) and returning to basal levels by 3 h. The difference in increases between platelets and CHOP cells is probably attributable to the presence of SIP lyase in the CHOP cells. Nevertheless, these results indicate that the hydrolysis of cell surface SM in platelets induces increases in Cer and Sph levels and a subsequent accumulation of SIP.

Involvement of neutral CDase in Sph generation at the platelet cell surface

To investigate where the generation of Sph occurs after bacterial SMase treatment, platelets were treated with SMase at 37°C for 5 min, then washed in buffer with or without 1% BSA at 4°C. After a 1% BSA wash, the cell-bound Sph was reduced in the SMase-treated platelets and detected in the supernatant, whereas in the absence of BSA, little Sph was detected in the supernatant (**Fig. 5A**). This result suggests that the generation of Sph in the SMase-treated platelets occurred at the outer leaflet of the plasma membrane.

A previous study demonstrated that in HEK293 and CHOP cells, overexpressed neutral CDase is not only re-

leased into the medium in a soluble form but is also distributed at the plasma membrane as a type II integral membrane protein, the catalytic domain of which is located on the extracellular side (29). In CHOP cells treated with bacterial SMase, overexpression of neutral CDase resulted in accelerated Sph and SIP production, indicating that plasma membrane-bound neutral CDase is involved in Cer hydrolysis at the cell surface (unpublished observation). In platelets, we detected CDase activity with an optimum pH near 7.5 (**Fig. 5B**), which corresponds to the pH dependence of neutral CDase (19, 27). Furthermore, nearly all of the activity at pH 7.5 was found in the membrane pellets of cell lysates that had been centrifuged at 105,000 *g* for 90 min (7.04 pmol/min/mg membrane protein vs. 0.25 pmol/min/mg cytosolic protein). Together, these data support the hypothesis that in platelets, neutral CDase is involved in the generation of Sph at the cell surface.

Secretory acid SMase-mediated SIP generation in platelets

Acid SMase is not just localized in lysosomes and endosomes but is also reportedly secreted to the extracellular

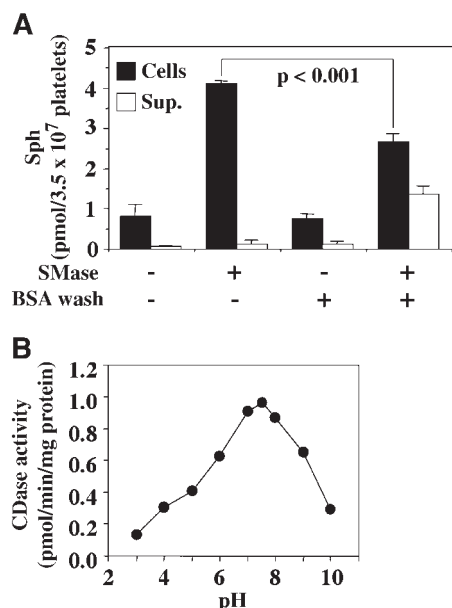


Fig. 5. Cell surface generation of Sph in platelets after bacterial SMase treatment. A: Removal of cell surface Sph by BSA treatment. Each sample (3.5×10^8) was suspended in 1 ml of buffer without BSA and then treated with or without 10 milliunits of bacterial SMase at 37°C for 5 min, washed twice with buffer A containing 0% or 1% BSA at 4°C for 5 min, and centrifuged. The total amount of Sph in each cell pellet or supernatant was measured by HPLC. Data represent means \pm SD from three independent experiments. Statistical analysis was done using Student's *t*-test, and *P* values are indicated. B: CDase activity in platelet cell lysates. C12-NBD-Cer (550 pmol) was incubated with 10 μ l of platelet cell lysate at 37°C for 3 h at the indicated pH. Data were averaged from two independent experiments.

space by macrophages and endothelial cells (31), and detectable levels of activity are present in human plasma (32). We investigated whether secretory acid SMase is involved in the hydrolysis of SM at the cell surface of platelets and in S1P generation after Cer and Sph generation. Consistent with a previous result using CHO cells (31), a massive amount of acid SMase activity was found in the conditioned medium from HEK293T cells that were transfected with a plasmid vector containing mouse acid SMase cDNA. The secreted enzyme was highly Zn^{2+} -dependent, as the activity was activated to ~ 30 -fold greater in the presence of 0.1 mM ZnCl_2 (Fig. 6A). When this acid SMase-conditioned medium was added to platelets in the presence of 0.1 mM ZnCl_2 , a significant increase in Cer levels was observed after 30 min, yet no increment was observed in the absence of ZnCl_2 or with conditioned medium from mock-transfected cells (Fig. 6B). We also analyzed the Sph and S1P levels in platelets in the presence of the acid SMase and ZnCl_2 . As shown in Fig. 6C, treating platelets with the secretory acid SMase-conditioned medium for 30 min at 37°C leads to increased Sph levels. Additionally, S1P levels were subsequently increased after a 3 h incubation. These data demonstrate that secretory acid SMase can hydrolyze SM at the cell surface of platelets in a Zn^{2+} -dependent manner, triggering a cascade that results in significant S1P generation.

DISCUSSION

Yatomi et al. (13) demonstrated that in platelets, highly active Sph kinases rapidly convert Sph to S1P, which is then abundantly stored. This conclusion was mainly derived from an experiment using exogenously added ^3H -labeled Sph, so the mechanism behind the generation of Sph itself was not addressed. In the present study in platelets, we found that the precursor Sph is supplied from at least two sources: incorporation of plasma Sph, which at least in part is generated in the plasma by secreted enzymes; and generation at the outer leaflet of the plasma membrane, triggered by cell surface SM degradation. This scenario is summarized in Fig. 7. We found that platelets have little de novo sphingolipid biosynthesis activity, mainly because of very weak serine palmitoyltransferase activity (Fig. 1, Table 1). Thus, it is likely that S1P generation in platelets depends on an extracellular supply of sphingolipids. Reportedly, phospholipids including SM, phosphatidylcholine, and phosphatidylethanolamine are converted from plasma lipoproteins supplied by selective transfer, not by endocytosis (30). Indeed, despite their defective de novo sphingolipid biosynthesis, platelets are rich in SM, which represents 21% of their total phospholipids and 13% of their total lipids (33). The proportion of SM is similar to that found in erythrocytes and considerably higher than that in normal lymphocytes or polymorphonuclear leukocytes (34). We confirmed here that the SM content in platelets is also higher than that in megakaryoblastic cells (Fig. 4A). Additionally, we found the majority of the SM to be located at the outer leaflet of the plasma membrane of the platelets (Fig. 4B), in agreement with another report (35). Sph is generally thought to be produced not by de novo synthesis but rather by the SM degradation pathway (5). In this context, it is likely that the high content of SM and its localization in platelets are related to the high concentration of S1P, a contention supported by the observation that the hydrolysis of cell surface SM induced increases in intracellular S1P levels (Figs. 4D, 6C).

We showed that platelets incorporated extracellular [^3H]Sph much faster than CMK and MEG-O1 cells and rapidly converted it to S1P (Fig. 2). It is still unclear, though, just how the extracellular Sph is rapidly incorporated across the plasma membrane of platelets. We predict the presence of a novel transporter that transports extracellular Sph to the inside of the cells. Rsb1p is known play a role in the translocation of sphingoid long-chain bases outward across the plasma membrane in *Saccharomyces cerevisiae* (36). We demonstrated here that Sph formed from plasma SM is rapidly incorporated into platelets and converted to S1P (Fig. 3D). To our knowledge, the present study is the first to demonstrate that Sph generated from plasma SM can be used for the generation of S1P in platelets.

We further provide here experimental evidence that secretory acid SMase is involved in S1P generation in platelets. Acid SMase is continuously secreted from macrophages and vascular endothelial cells as a Zn^{2+} -dependent enzyme and is constitutively present in plasma (31, 32). Thus, it is likely that in blood, acid SMase secreted

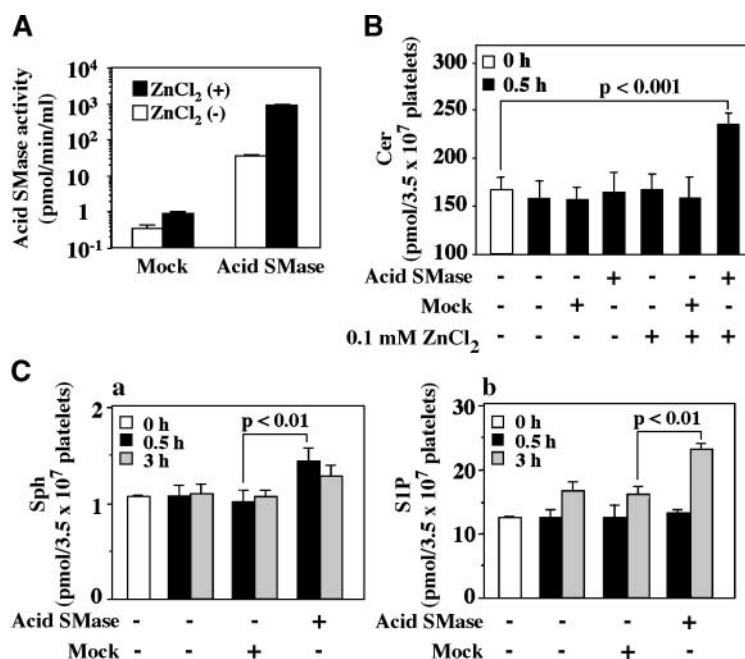


Fig. 6. Involvement of secretory acid SMase in S1P generation in platelets. **A:** Secretion of acid SMase activity from overexpressing HEK293T cells. HEK293T cells were transfected with vector alone (mock) or with an expression vector containing mouse acid SMase cDNA. Conditioned medium from each transfectant was analyzed for acid SMase activity using C6-NBD-SM in the presence or absence of 0.1 mM ZnCl₂. **B:** Cer generation in platelets by exogenously added secretory acid SMase. Platelet suspensions (3.5×10^8 in 1 ml) were treated for 30 min at 37°C with 500 μ l of secretory acid SMase- or mock-conditioned medium in the presence or absence of 0.1 mM ZnCl₂. The total Cer content in the platelet suspensions was determined using *sn*-1,2-diacylglycerol kinase (26). **C:** Platelet suspensions were treated with secretory acid SMase- or mock-conditioned medium in the presence of 0.1 mM ZnCl₂ for 30 min or 3 h. The total amount of Sph (a) and S1P (b) in each sample was measured by HPLC. All data represent means \pm SD from at least three independent experiments. Statistical analysis was done using Student's *t*-test.

from these cells constitutively hydrolyzes cell surface SM in platelets and contributes to S1P production. On the other hand, it was reported that platelets contain acid SMase but do not possess enzymatically detectable neutral or alkaline SMases (37). We also detected acid SMase activity in platelets, whereas this activity (0.86 pmol/min/mg protein) was quite low compared with that in CHOP and MEG-O1 cells (13.03 and 9.88 pmol/min/mg protein, respectively). Activation of platelets by thrombin causes the rapid release of acid SMase into the extracellular space, whereas resting platelets hardly secrete acid SMase (37). Thus, there is a possibility that acid SMase secreted from activated platelets stimulates the production of S1P in an autocrine/paracrine manner. It remains to be elucidated which is the major origin of secretory acid SMase involved in S1P production in platelets *in vivo*.

Because secretory acid SMase activity has a pH optimum of ~ 5.5 in *in vitro* enzyme assays and is extremely reduced at pH 7.4, which is the pH of ordinary plasma (32), the question arises of how the enzyme acts on the SM at the platelet surface (i.e., at neutral pH). One possible explanation is that glycoproteins of the platelet surface influence the near-membrane pH. In most epithelial cells, the extracellular surface of the apical plasma membrane has a large number of glycoproteins containing sialic acid, and the near-membrane pH, possibly influenced by the

negatively charged sialic acids, is markedly acidic compared with the luminal solution (38). The platelet plasma membrane contains a large number of sialoglycoproteins, GPIb, GPIIb, GPIIIa, and GPIV, which constitute the ma-

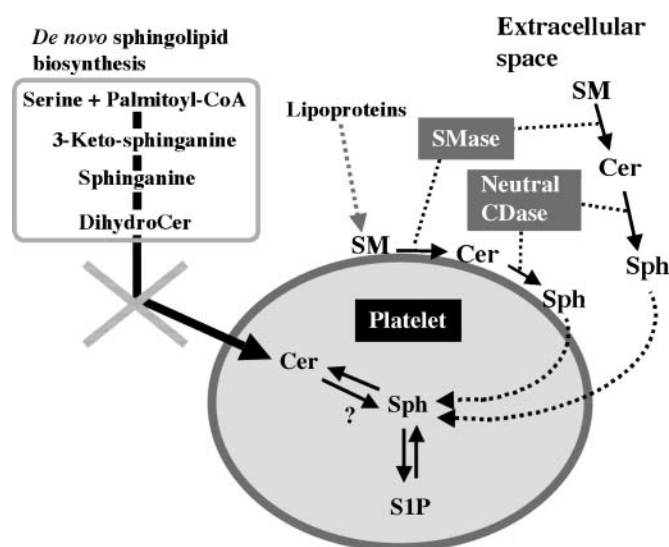



Fig. 7. Scheme for the mechanism of Sph and S1P generation in human platelets.

major portion of the total sialic acid content of platelets and provide the bulk of the negative charge at the platelet surface (39, 40). Thus, it is possible that these sialoglycoproteins alter the near-membrane pH of the platelets and affect the action of secretory acid SMase. Likewise, LDL that carries an oxidized modification is more negatively charged than unmodified LDL (41), and SM in modified LDL is reportedly hydrolyzed by secretory acid SMase much faster at neutral pH than that in the unmodified LDL (42), supporting the notion that some microenvironment changes affect the activity of secretory acid SMase toward cell surface SM.

Reportedly, Cer and Sph levels in plasma are affected by some pathological conditions. For instance, LDL extracted from human atherosclerotic lesions is enriched in Cer compared with plasma LDL, a phenomenon attributed to secretory acid SMase (42). Acute systemic inflammation after lipopolysaccharide injection induces increases in plasma lipoprotein Cer levels through two pathways, upregulation of acid SMase secretion from endothelial cells (43) and de novo sphingolipid biosynthesis in the liver (44). Because SIP generation in platelets was induced by Sph generation after Cer increase in plasma (Fig. 3) or hydrolysis of the cell surface SM by secretory acid SMase (Fig. 6), it is possible that SIP levels in platelets are also affected by these pathological conditions. Additionally, in rat congestive heart failure, serum Sph levels are increased, possibly released from cardiomyocytes, and the increase is responsible for skeletal muscle apoptosis (45). Interestingly, serum SIP levels increase with the severity of coronary artery disease, possibly because of increased release from platelets, and the SIP increases can be correlated with serum Sph levels (46). Because they can rapidly incorporate plasma Sph, platelets may be an important regulator of extracellular Sph and SIP levels, subsequently determining cell death or survival.

The finding that platelets can use Sph at the outer leaflet of the plasma membrane and in the extracellular space implies an importance for neutral CDase. Neutral CDase was shown to be localized at the plasma membrane as a type II integral membrane protein and to be secreted into the extracellular space after N-terminal anchor processing (29). It was also found to be released from mouse endothelial cells (47) and to be present in mouse serum (29). We confirmed that its activity is also detectable in human plasma and that it is involved in extracellular Sph formation (Fig. 3A). Additionally, we recently determined that plasma membrane-bound neutral CDase is involved in the hydrolysis of Cer generated at the outer leaflet of the plasma membrane (unpublished observation). Moreover, we found that after treatment with exogenously added SMase, Sph generation occurred at the cell surface of platelets (Fig. 5A) and that the platelets had membrane-bound neutral CDase activity (Fig. 5B). Interestingly, Yoshimura et al. (48) recently demonstrated that knocking down the neutral CDase gene in zebrafish during embryogenesis results in a defect in blood cell circulation, although it is still unclear why decreased neutral CDase expression would cause this phenomenon. It is possible

that the phenotype may stem from a decrease in plasma SIP levels, because plasma SIP and its receptors are important for several vascular functions, including vascular maturation (6), coronary artery blood flow (49), and lymphocyte recirculation (8). In this context, we believe that the zebrafish result reflects the significant contribution of neutral CDase to SIP production in platelets, arguably the most important source for plasma SIP.

Besides neutral CDase, there are other possible mechanisms for supplying Sph to platelets. CDases are now classified into three groups, acid, neutral, and alkaline, based on optimal pH and primary structure (50). Lysosomal acid CDase is known to be released from mouse endothelial cells, as is neutral CDase (47); thus, the possibility that acid CDase is also involved in extracellular Sph generation cannot be ruled out at present. Alkaline CDase is an endoplasmic reticulum-Golgi resident enzyme and may be involved in the regulation of Cer in intracellular compartments (51). Although platelets are defective in de novo sphingolipid biosynthesis, conversion of incorporated [^3H]Sph to Cer occurred, possibly via intracellular Cer synthase (13). Alkaline CDase may play a role in platelets in Sph generation from intracellular Cer pools and contribute to SIP production. This model is also represented in Fig. 7. 

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