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Sphingosine 1-Phosphate, a Bioactive Sphingolipid Abundantly Stored in Platelets, Is a Normal Constituent of Human Plasma and Serum[1]

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Although sphingosine 1-phosphate (Sph-1-P) is reportedly involved in diverse cellular processes and the physiological roles of this bioactive sphingolipid have been strongly suggested, few studies have revealed the presence of Sph-1-P in human samples, including body fluids and cells, under physiological conditions. In this study, we identified Sph-1-P as a normal constituent of human plasma and serum. The Sph-1-P levels in plasma and serum were 191±79 and 484±82 pmol/ml (mean±SD, $n=8$), respectively. Furthermore, when Sph-1-P was measured in paired plasma and serum samples obtained from 6 healthy adults, the serum Sph-1-P/plasma Sph-1-P ratio was found to be 2.65±1.26 (mean±SD). It is most likely that the source of discharged Sph-1-P during blood clotting is platelets, because platelets abundantly store Sph-1-P compared with other blood cells, and release part of their stored Sph-1-P extracellularly upon stimulation. We also studied Sph-1-P-related metabolism in plasma. [^{3}H]Sph was stable and not metabolized at all in plasma, but was rapidly incorporated into platelets and metabolized mainly to Sph-1-P in platelet-rich plasma. [^{3}H]Sph-1-P was found to be unchanged in plasma, revealing that plasma does not contain the enzymes needed for Sph-1-P degradation. In summary, platelets can convert Sph into Sph-1-P, and are storage sites for the latter in the blood. In view of the diverse biological effects of Sph-1-P, the release of Sph-1-P from activated platelets may be involved in a variety of physiological and pathophysiological processes, including thrombosis, hemostasis, atherosclerosis and wound healing.

Key words: plasma, platelet, sphingosine, sphingosine kinase, sphingosine 1-phosphate.

The phosphorylated sphingoid base, sphingosine 1-phosphate (Sph-1-P), is the initial product of the catabolism of sphingosine (Sph) by Sph kinase and, generally, it is then cleaved by Sph-1-P lyase to yield ethanolamine phosphate and a fatty aldehyde (*1-3*). Sph-1-P has several important physiologic functions in addition to its role as a metabolite of Sph. Sph-1-P has been shown to be involved in a variety of cellular functions, including the stimulation of fibroblast growth (*4, 5*), regulation of cell motility (*6, 7*), activation of muscarinic K^+ currents in atrial myocytes (*8, 9*), mediation of FcεRI antigen receptor signaling (*10*), neurite retraction (*11*), and suppression of ceramide-mediated apoptosis (*12*). In non-proliferative, terminally differentiated platelets, Sph-1-P induces a shape change and aggregation reactions by itself, and synergistically elicits aggregation in combination with weak platelet agonists such as epinephrine and ADP (*13*).

Although the physiological roles of Sph-1-P have been strongly suggested, the current evidence for the involvement of Sph-1-P in signal transduction or cellular function(s) consists largely of data on the cellular and biochemical effects of exogenous Sph-1-P. Few studies have revealed the presence and quantitative changes of Sph-1-P in human tissues or body fluids under physiological conditions. To obtain clear evidence implicating endogenous Sph-1-P in cellular signal transduction, and to assess its physiological and pathophysiological functions, we recently developed a method for quantifying the mass of Sph-1-P through its acylation into *N*-[^{3}H]acetylated Sph-1-P with [^{3}H]acetic anhydride, which made it possible to measure Sph-1-P in biological samples (*14*). Here we report that Sph-1-P is a normal constituent of plasma and serum, and that platelets seem to release Sph-1-P into the serum during the blood clotting process. We also studied Sph-1-P-related metabolism in plasma.

MATERIALS AND METHODS

Materials—Sph-1-P (*15*) and C_2-ceramide (C_2-Cer) (*16*) were prepared as previously described. C_2-Ceramide 1-phosphate (C_2-Cer-1-P) was formed by acylation of Sph-1-P with acetic anhydride, which was confirmed by FAB-MS analysis (*14*). [3-^{3}H]Sph-1-P was prepared by ATP-dependent phosphorylation of [3-^{3}H]Sph, which was catalyzed by

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Abbreviations: Cer, ceramide; Cer-1-P, ceramide 1-phosphate; Sph, sphingosine; Sph-1-P, sphingosine 1-phosphate.

Sph kinase obtained from BALB/c 3T3 fibroblasts (*17*).

The following materials were obtained from the indicated suppliers: [^{3}H]acetic anhydride (50 mCi/mmol) and [3-^{3}H]Sph (22.0 Ci/mmol) (Du Pont-New England Nuclear, Boston, MA); and Sph, Cer (type III), and palmitic acid (Sigma, St. Louis, MO).

Preparation of Platelets, Neutrophils, Erythrocytes, Plasma, Serum, and Other Samples—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) and 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 (*13*). Neutrophils were isolated by dextran sedimentation and centrifugation on a Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) cushion, followed by hypotonic lysis to remove contaminating erythrocytes (*18*). Erythrocytes were washed in normal saline and the buffy coat was carefully removed. The washed blood cells were resuspended in a buffer comprising 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1.0 mM $MgCl_2$, 1 mg/ml of glucose, and 20 mM Hepes (pH 7.4). NaH_2PO_4 was omitted when indicated.

To obtain plasma, venous blood was mixed with 15% volume of ACD anticoagulant (*19*), and then centrifuged at $2,000\times g$ for 15 min. The resultant plasma supernatant was carefully collected. To obtain serum, venous blood was directly put into glass tubes and left for 60 min at room temperature to allow blood clots to form. Then, 15% volume of ACD was added to the samples and the serum was separated by centrifugation at $2,000\times g$ for 15 min. The urine, ascites, pleural effusion, and cerebrospinal fluid samples used in this study were the rest of those obtained for routine laboratory analyses at Yamanashi Medical University Hospital.

Quantitative Measurement of Sph-1-P—Sph-1-P was extracted from 0.5-1.0 ml of body fluids, $1\text{-}2\times10^9$ platelets, $5\text{-}10\times10^7$ neutrophils, and $1\text{-}5\times10^9$ erythrocytes, and quantitatively measured by *N*-acylation with [^{3}H]-acetic anhydride into [^{3}H]C_2-Cer-1-P (*N*-[^{3}H]acetylated Sph-1-P) as described previously (*14*). This assay allows quantification of Sph-1-P in the range of about 30 pmol to 10 nmol (*14*). The amounts of Sph-1-P extracted were calculated by extrapolation of those of Sph-1-P standards subjected to the same procedures.

Phospholipid Quantitation—Blood cells were suspended in 0.8 ml of the above buffer without NaH_2PO_4. The lipids were extracted and assayed for phosphorus as described (*20*).

[^{3}H]Sph Metabolism in Plasma and Platelet-Rich Plasma—When plasma [^{3}H]Sph metabolism was studied (Fig. 2A), plasma (0.5 ml) was incubated with 5 μM [^{3}H]Sph at 37°C and, after the reaction, lipids were extracted as described previously (*13*). Lipids obtained from the lower chloroform phase were separated by reversed phase HPLC on a TSK gel ODS-80Ts column (TOSOH, Tokyo) developed with 85% methanol plus 1% phosphoric acid at 1 ml/min. Radioactivity in the eluate was monitored on-line with a 171 radioisotope detector (Beckman, Fullerton, CA).

When [^{3}H]Sph metabolism in platelet-rich plasma was studied (Fig. 2B), platelet-rich plasma (0.5 ml) incubated with 5 μM [^{3}H]Sph at 37°C was centrifuged for 15 s at $12,000\times g$ after the reaction. Lipids were then extracted from the resultant platelet pellet and analyzed for [^{3}H]Sph metabolism as described previously (*13*).

[^{3}H]Sph-1-P Metabolism in Plasma—Plasma (0.5 ml) was incubated with [^{3}H]Sph-1-P (5 μM) at 37°C, and the metabolic fate of the label was determined as described previously for the Sph-1-P lyase assay (*3*) except that [3-^{3}H]Sph-1-P instead of [4,5-^{3}H]dihydrosphingosine-1-phosphate was used as the substrate. The reaction products were applied to silica gel 60 HPTLC plates and the plates were developed with chloroform/methanol/acetic acid (50 : 50 : 1) or butanol/acetic acid/water (3 : 1 : 1).

RESULTS

Sph-1-P Levels in Human Body Fluids—We recently developed a method for quantitatively measuring Sph-1-P by means of its acylation with radioactive acetic anhydride into C_2-Cer-1-P (*14*). When plasma or serum extracts were *N*-acylated with [^{3}H]acetic anhydride for Sph-1-P measurements, one clear band was detected, with a high degree of resolution, on TLC (Fig. 1A). The band (acylation product in plasma and serum) coincided with FAB-MS-identified C_2-Cer-1-P in TLC mobility with three different solvent systems, *i.e.*, butanol/acetic acid/water (3 : 1 : 1) (Fig. 1A), chloroform/methanol/7 N NH_4OH/water (80 : 20 : 0.5 : 0.5) (data not shown), and chloroform/methanol/acetic acid/water (65 : 43 : 1 : 3) (data not shown). These results indicate that Sph-1-P was clearly detected and

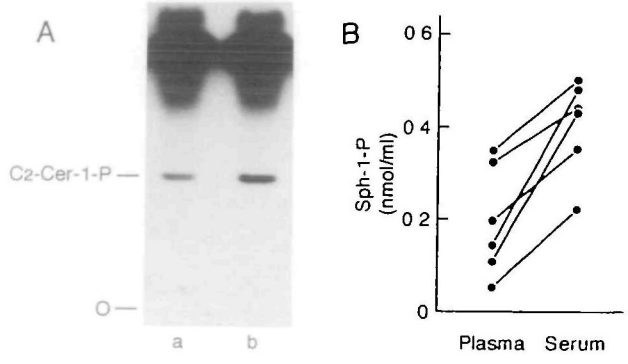

Fig. 1. **Determination of the Sph-1-P levels in human plasma and serum.** (A) Sph-1-P extracted from human plasma (lane a) and serum (lane b) was *N*-acylated with [^{3}H]acetic anhydride into [^{3}H]-C_2-Cer-1-P. Radioactive spots corresponding to [^{3}H]C_2-Cer-1-P on the TLC plate shown were scraped off and counted for quantification of Sph-1-P. A representative autoradiogram is shown. (B) Determination of the Sph-1-P levels in paired plasma and serum samples obtained from 6 healthy adults. The serum Sph-1-P/plasma Sph-1-P ratio was calculated to be 2.65 ± 1.26 (mean$\pm$SD, $n=6$).

TABLE I. **Sph-1-P levels in human body fluids.** Various body fluids were assayed for Sph-1-P. Values are means$\pm$SD, and the numbers of experiments are shown in parentheses. ND, not detectable (<30 pmol/ml).

	Sph-1-P concentration (pmol/ml)
Plasma	191 ± 79 ($n=8$)
Serum	484 ± 82 ($n=8$)
Urine	ND ($n=10$)
Ascites	ND ($n=6$)
Pleural effusion	ND ($n=2$)
Cerebrospinal fluid	ND ($n=6$)

identified as a normal constituent of plasma and serum with our procedures. Human plasma, which does not include platelet discharge, contained about 190 pmol/ml of Sph-1-P, while clotted blood serum, into which the contents of platelets should be released, contained about 480 pmol/ml of Sph-1-P (Table I). When the Sph-1-P levels in paired plasma and serum samples obtained from 6 healthy adults were measured, the serum Sph-1-P/plasma Sph-1-P ratio was found to range from 1.36 to 4.05, the average being 2.65±1.26 (mean±SD) (Fig. 1B). We also analyzed other body fluids, but Sph-1-P was not detectable in urine, ascites, pleural effusion or cerebrospinal fluid (Table I).

Determination of Sph-1-P Level in Blood Cells—Next, we analyzed blood cells for Sph-1-P. Platelets possess a very active Sph kinase but lack lyase activity for the degradation of Sph-1-P into a fatty aldehyde and ethanolamine phosphate (*21-24*). In agreement with this, we previously reported that platelets contain as much as 1.4 nmol of Sph-1-P/10^9 platelets and that the amount of Sph-1-P present in platelets is much higher than that of Sph (*14*). When a comparison was made using the mol% Sph-1-P/phospholipid value, the Sph-1-P level in platelets was found to be over 10 times higher than that in neutrophils or erythrocytes (Table II). Since Sph-1-P is most abundantly stored in platelets, compared with in other blood cells, and can be released into the medium upon the stimulation of platelets (*13, 14*), it is most likely that the source of discharged Sph-1-P during the clotting process is platelets.

TABLE II. **Sph-1-P levels in human platelets, neutrophils, and erythrocytes.** Sph-1-P extracted from blood platelets, neutrophils and erythrocytes was measured and adjusted as to the phospholipid level. Values are means±SD (n=3).

	Cell count	Sph-1-P (pmol)	mol% Sph-1-P/phospholipid
Platelets	10^8	141±4	0.211±0.014
Neutrophils	10^7	15.2±2.8	0.018±0.003
Erythrocytes	10^8	7.17±1.66	0.019±0.002

Metabolism of [^{3}H]Sph in Plasma and Platelet-Rich Plasma—We previously reported [^{3}H]Sph metabolism in washed platelet suspensions (*13*). Since Sph-1-P was found to be a normal constituent of plasma, the metabolism of Sph, the substrate of Sph kinase for the production of Sph-1-P, was studied in the presence of plasma. [^{3}H]Sph was metabolically stable in plasma and remained totally unchanged for at least 2 h (Fig. 2A). This indicates that plasma does not contain the enzymes for Sph metabolism and that Sph-1-P cannot be formed from Sph in the plasma.

Next we studied the metabolic fate of [^{3}H]Sph in platelet-rich plasma (Fig. 2B). [^{3}H]Sph was quickly incorporated into platelets; the uptake of 5 μM [^{3}H]Sph was always over 80% at 5 min after the label addition. The incorporated [^{3}H]Sph was rapidly metabolized. Within 5 min, about 40% of the [^{3}H]Sph added was converted to [^{3}H]Sph-1-P. [^{3}H]Sph was also converted through N-acylation into Cer (*25*) at later times. The [^{3}H]Sph metabolism

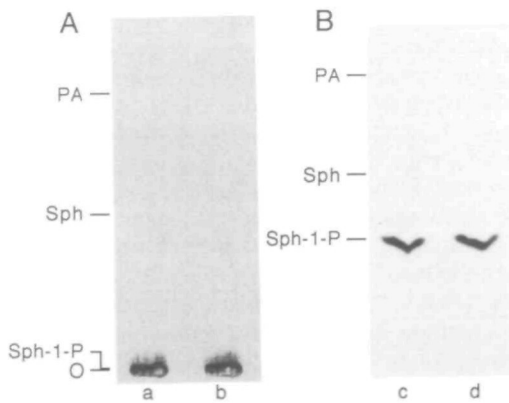

Fig. 3. **[^{3}H]Sph-1-P metabolism in plasma.** Plasma was incubated with [^{3}H]Sph-1-P (5 μM) for 0 (a and c) and 2 (b and d) h, and then lipids were extracted and analyzed by TLC autoradiography. TLC was developed with chloroform/methanol/acetic acid (50 : 50 : 1) (A) or butanol/acetic acid/water (3 : 1 : 1) (B). PA, palmitic acid.

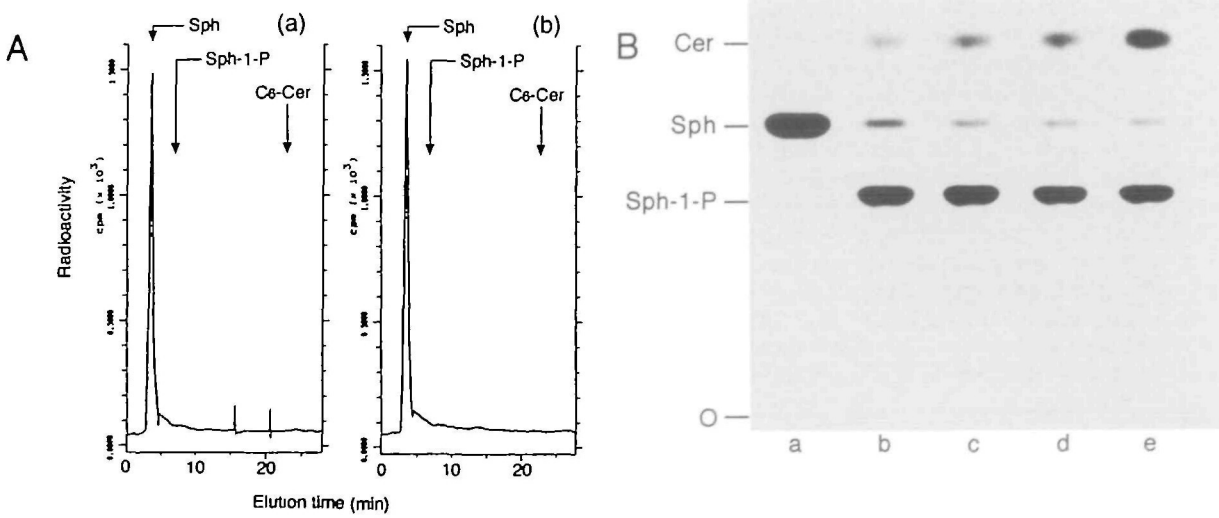

Fig. 2. **[^{3}H]Sph metabolism in plasma (A) and platelet-rich plasma (B).** (A) Plasma was incubated with [^{3}H]Sph (5 μM) for 0 (a) and 2 (b) h, and then lipids were extracted and analyzed by HPLC, followed by radioactivity monitoring. The locations of the standard lipids are indicated by arrows. (B) Platelet-rich plasma was incubated with [^{3}H]Sph (5 μM) for 0 (a), 5 (b), 20 (c), 60 (d), and 120 (e) min, and then lipids were extracted and analyzed by TLC autoradiography.

observed here is basically similar to that observed in washed platelet suspensions (in the absence of plasma) (*13*).

Together with the finding that platelets abundantly store Sph-1-P, compared with other blood cells (Table II), it is likely that these anucleate cells act as Sph-1-P generators (from Sph) in the blood.

Metabolism of [^{3}H]Sph-1-P in Plasma—Finally, Sph-1-P metabolism in plasma was examined (Fig. 3). Generally, Sph-1-P can be converted to Sph by phosphatase (*26*) or degraded to palmitaldehyde and ethanolamine phosphate by lyase (3). However, [^{3}H]Sph-1-P, added to plasma, was metabolically stable and remained unchanged for at least 2 h. Therefore, the Sph-1-P released from activated platelets is likely to circulate in the body, at least for a certain period.

DISCUSSION

In this study, we, for the first time, identified Sph-1-P, and determined its levels in human plasma and serum; the Sph-1-P level was much higher in serum than in plasma. Since platelets, among blood cells, possess a high level of Sph-1-P and contain a number of organelles, the contents of which can be released during the blood clotting process (*27, 28*), we propose that Sph-1-P is released from platelets into the serum during blood clotting. This is consistent with the fact that part of the Sph-1-P stored in platelets is released into the medium upon stimulation, such as with thrombin, an end product of the coagulation cascade (*13, 14*) or with a protein kinase C-activating phorbol ester (*29*).

Sph was stable and its phosphorylation product, Sph-1-P, was not formed in plasma. Platelets effectively incorporated Sph and converted it into Sph-1-P not only in the absence of plasma (*13*) but also in its presence (this study). In view of the finding that platelets specifically and abundantly contain Sph-1-P, which can be released upon stimulation (*13, 14*), platelets may act as producers (from Sph) and releasers of Sph-1-P *in vivo* (in the blood).

It has already been established that platelets store a variety of biologically active molecules, and their contents are secreted upon stimulation (*27, 28*). The secreted molecules may interact with other platelets and vessel walls. In this context, the important findings reported for Sph-1-P are the activation of platelets themselves (*13*), modulation of endothelial cell signal transduction (*30*), regulation of PDGF-induced chemotaxis of human arterial smooth muscle cells (*7*), and stimulation of fibroblast proliferation (*4, 5*). Thus, Sph-1-P, released from activated platelets, may play important roles in thrombosis, hemostasis, atherosclerosis and wound healing.

We previously examined Sph-1-P release from active phorbol ester-stimulated platelets in the absence and presence of albumin, and suggested that albumin may prevent released Sph-1-P, a lipophilic molecule, from becoming nonspecifically attached to the plasma membrane surface (*29*). Furthermore, when a mixture of albumin and Sph-1-P was applied to a gel-filtration column, Sph-1-P was found to be co-eluted with albumin (data not shown). Accordingly, Sph-1-P is thought to bind tightly to albumin. Sph-1-P, most probably released from platelets and bound to albumin, was metabolically stable in plasma. We previously injected (i.v.) radio-labeled Sph-1-P and examined its bio-distribution, a large portion being found in the liver (*31*). Hence, Sph-1-P, being metabolically stable in plasma, may be finally metabolized in the liver. The molecular mechanism of Sph-1-P metabolism in the liver remains to be elucidated, but possible explanations are degradation by its lyase, which is abundant in the liver (*3*), and excretion into the bile, as seen with other phospholipids (*32*).

Since Sph-1-P is highly abundant in platelets and is released from platelets upon stimulation, the plasma Sph-1-P level may be elevated as a result of platelet activation or thrombus formation. Accordingly, the plasma level of Sph-1-P may be an index of *in vivo* platelet activation in patients with thrombotic disorders, as is the case with β-thromboglobulin (*33, 34*) and platelet factor 4 (*34*). Furthermore, some of the important bioactivities reported for Sph-1-P have been observed at concentrations of it below 100 nM (*6-9, 11*). Since the plasma concentration of Sph-1-P ranges from 50 pmol/ml (50 nM) to 400 pmol/ml (400 nM), it is possible that variations in the plasma level of Sph-1-P may be related to some clinical disorders, and measurement of the plasma Sph-1-P level may provide information on the pathophysiological implications of Sph-1-P.

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