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## **Lack of sphingosine 1-phosphate-degrading enzymes in erythrocytes**

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## **Abstract**

Platelets are known to store a large amount of the bioactive lipid molecule sphingosine 1-phosphate (S1P) and to release it into the plasma in a stimuli-dependent manner. Erythrocytes can also release S1P, independently from any stimuli. We measured the S1P and sphingosine (Sph) levels in erythrocytes by HPLC and found that the contribution of erythrocyte S1P to whole blood S1P levels is actually higher than that of platelets. *In vitro* assays demonstrated that erythrocytes possess much weaker Sph kinase activity compared to platelets but lack the S1P-degrading activities of either S1P lyase or S1P phosphohydrolase. This combination may enable erythrocytes to maintain a high S1P content relative to Sph. The absence of both S1P-degrading enzymes has not been reported for other cell types. Thus, erythrocytes may be specialized cells for storing and supplying plasma S1P.

*Keywords:* Sphingosine 1-phosphate; Erythrocyte; Platelet; Sphingolipid; Lipid

## **Introduction**

The bioactive lipid molecule sphingosine 1-phosphate (S1P) elicits a variety of cellular responses through binding to its cell surface receptors, which are members of the S1P/Edg family [1]. S1P is abundant in blood, and its actions on cells exposed to plasma, such as vascular endothelial cells and lymphocytes, are physiologically its most important.

S1P is generated by the phosphorylation of sphingosine (Sph) by Sph kinase. In most cells, generated S1P is rapidly degraded either by lyase, to hexadecenal and phosphoethanolamine, or by phosphohydrolase to Sph. These S1P-degrading enzymes are responsible for maintaining low levels of intracellular S1P.

*In vitro* results have indicated that platelets are a major source of plasma S1P [2]. Since platelets possess high Sph kinase activity [2] but lack S1P lyase activity [3], they accumulate high levels of S1P [2]. The phospholipid is stored in the inner leaflet of the platelet plasma membrane, and stimuli that activate platelets, such as thrombin and phorbol ester, trigger its release in an ATP-dependent manner [4].

Although most cell types do not readily release intracellular S1P to the extracellular space, blood cells in general can efficiently export S1P. In addition to the stimuli-dependent release by platelets [3,5], erythrocytes, neutrophils, and mononuclear cells have all been shown to release S1P, though mostly in a stimuli-independent manner [6]. Moreover, these blood cells can efficiently import exogenous Sph and convert it to S1P [6].

Considering that erythrocytes are the most abundant blood cells, it is reasonable to

speculate that these cells may be a major source for plasma S1P in addition to platelets.

In the present study, we found that about half of all blood S1P is indeed found in erythrocytes. In addition, we investigated the activities in erythrocytes of those enzymes that are important for S1P production and degradation. From these analyses, we determined that erythrocytes possess a simple Sph/S1P metabolic pathway, i.e. they contain only Sph kinase and not S1P lyase, S1P phosphohydrolase, or ceramidase. Thus, erythrocytes may import exogenous Sph, convert it to S1P, and then release the S1P, with no degradation of the phospholipid.

## **Materials and methods**

*Preparation of erythrocytes, platelets, and plasma.* Erythrocytes and platelets were prepared essentially as described elsewhere [6]. Blood cell numbers were determined using a COULTER Gen-S™ Hematology Analyzer (Beckman Coulter, Fullerton, CA).

*Quantitative analysis of Sph and S1P levels by HPLC.* Sph and S1P levels were measured by HPLC (Agilent 1100 series; Agilent Technologies, Palo Alto, CA) after derivatization with *o*-phthalaldehyde, essentially as described elsewhere [7]. In the experiment examining metabolism and transport of exogenous Sph, 1/100 volume of 100  $\mu$ M Sph (final concentration 1  $\mu$ M) in DMSO was added to the medium.

*Biochemical assays.* Sph kinase assays were performed essentially as described elsewhere [8]. S1P lyase assays were performed using [4,5-<sup>3</sup>H]dihydrosphingosine 1-phosphate (dihydro-S1P) essentially as described previously [9]. S1P phosphohydrolase assay using [3-<sup>3</sup>H]S1P was performed essentially as described previously [10]. Ceramidase activities were measured using 4-nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled ceramide (C12:0, d18:1) (C<sub>12</sub>-NBD-ceramide) as described previously [11].

## Results

### *Significant contribution of erythrocytes to blood S1P*

Until now, only platelets have been considered to be a major source of plasma S1P [2]. However, the most abundant blood cells, erythrocytes, can also release S1P extracellularly [6], and their role as a major source of plasma S1P has never been excluded. Therefore, quantitative and comparative analyses of S1P from erythrocytes were performed. Using HPLC we measured S1P levels, as well as those of its precursor Sph, in human whole blood, plasma, platelets, and erythrocytes. In whole blood, S1P and Sph were present at levels of 1155 pmol/ml and 79 pmol/ml, respectively (Fig. 1). The plasma and platelets tested contained very low levels of Sph, whereas the erythrocytes had levels similar to those found in the whole blood, indicating that most of the Sph found in the whole blood was derived from erythrocytes. In plasma, platelets, and erythrocytes we found 153, 341, and 589 pmol S1P per ml blood, respectively. Thus, erythrocytes contain more S1P than platelets do. Calculations based on cell numbers in the whole blood used ( $4.2 \times 10^9$  erythrocytes per ml,  $2.1 \times 10^8$  platelets per ml, and  $5.2 \times 10^6$  leukocytes per ml) indicated that one platelet contained an approximately 9 fold higher amount of S1P than did one erythrocyte (Fig. 1B).

We also counted the platelets in the erythrocyte preparation and found only negligible platelet contamination (0.37% of erythrocytes). On the other hand, a significant number of erythrocytes (7.6% of platelets) were included in the platelet fraction. However, we estimated that this contamination affected the platelet S1P levels by less than 1%, considering the S1P amount in erythrocytes. Thus, the S1P amount in

platelets or erythrocytes was essentially derived from a relatively pure source. In conclusion, one platelet stores a much higher amount of S1P than one erythrocyte. However, the cell numbers found in the whole blood above correspond to 95.3% of the total cells being erythrocytes, and 4.6% being platelets. So, despite the higher amount of S1P in platelets, the overall contribution of total S1P in whole blood from erythrocytes is considerably higher than that from platelets.

#### *Quantitative analysis of S1P release from erythrocytes*

An earlier study demonstrated that erythrocytes can efficiently import exogenous [<sup>3</sup>H]Sph, convert it to S1P, and release the S1P into medium [6]. In the present study, we performed a quantitative analysis of S1P release from erythrocytes using HPLC. Erythrocytes were treated with 1  $\mu$ M (100 pmol) Sph and incubated at 37°C for appropriate time periods, then separated from the medium by centrifugation. Sph and S1P levels were measured in both the pellet and supernatant. Within the first 5 min, most of the added Sph had disappeared from the medium (Fig. 2A), indicating that the exogenous Sph was rapidly imported into the erythrocytes. The Sph levels in the cell fraction reached a maximum (22 pmol) at 5 min and gradually decreased over 30 min (Fig. 2A). The S1P levels were already higher than the Sph levels at 5 min (Fig. 2B), indicating that Sph was rapidly converted to S1P. Cellular S1P reached a maximal level (44 pmol) at 15 min then gradually decreased, due to its release into the medium (Fig. 2B). Release of S1P into the medium was delayed by its production, so those totals increased significantly from 5 to 30 min, then more slowly from 30 min to 90 min. By

90 min almost all of the added Sph had been converted to S1P, of which nearly 60% was released into the medium. The sum of the Sph and S1P levels remained nearly constant during the incubation period, suggesting that further metabolism of Sph beyond S1P does not occur in erythrocytes. These results indicate that erythrocytes possess a high capacity to produce and release S1P in the absence of any stimuli.

We next investigated whether erythrocytes indeed release endogenous S1P (i.e. stored) in the absence of an exogenous supply of Sph. To minimize the S1P release during the preparation, we isolated erythrocytes at 4°C then started the reaction by shifting the cells to 37°C. Subsequently, the S1P levels in the media increased in a time-dependent manner (Fig. 2B). S1P associated with the erythrocytes decreased concurrently, reaching nearly half the original level by 4 h. This S1P release was not caused by membrane damage, since almost no leakage of lactose dehydrogenase was observed during the incubation periods (data not shown). In addition, when erythrocytes were incubated at 4°C, no release was stimulated (data not shown). Thus, erythrocytes do possess the capacity to release stored S1P into plasma.

#### *Activities of S1P-synthesizing and -degrading enzymes*

S1P is produced from Sph by Sph kinase and degraded either by S1P lyase to hexadecenal and phosphoethanolamine or by phosphohydrolase to Sph. We investigated the activities of these enzymes in erythrocytes and platelets. Platelets exhibited high Sph kinase activity (69 pmol/min/mg; Fig. 3A), while Sph kinase activity in erythrocytes was extremely limited (3 pmol/min/mg; Fig. 3A). Thus, Sph kinase activity was much

lower (~25-fold per protein and ~3 fold per cell) in erythrocytes than in platelets.

Next, we investigated *in vitro* S1P lyase activity using [<sup>3</sup>H]dihydro-S1P as a substrate. Platelets are known to lack S1P lyase activity [3]. Consistent with this, platelet lysates could not produce hexadecanal from dihydro-S1P, in contrast to the positive control cells (Fig. 3B). Erythrocytes also generated no hexadecanal (Fig. 3B), indicating that erythrocytes also lack S1P lyase activity. The lack of the S1P lyase SPL in platelets and erythrocytes was confirmed by immunoblotting (data not shown).

We also performed an *in vitro* S1P phosphohydrolase assay using [<sup>3</sup>H]S1P in the presence of 100 nM unlabeled S1P. Platelets exhibited an approximately 2-fold lower S1P phosphohydrolase activity than did the positive control cells (Fig. 3C). In erythrocytes, however, we could not detect any S1P phosphohydrolase activity (Fig. 3C). In summary, erythrocytes contain Sph kinase activity but lack both S1P lyase and S1P phosphohydrolase activities. The absence of these degrading enzymes has not been reported in other cell types.

#### *Lack of ceramidase activity in erythrocytes*

The S1P precursor Sph is synthesized from ceramide by ceramidase. Three types of ceramidases are known, acid, neutral, and alkaline ceramidases, which differ in their optimum pH for activity. To investigate a possible source of erythrocyte S1P, we measured ceramidase activity in these cells. Erythrocyte lysates and, for comparison, platelet lysates were incubated with fluorescence-labeled ceramide (C<sub>12</sub>-NBD-ceramide) at pH 4.5, pH 7.5, or pH 9.4. As previously described [12], platelet cell lysates produced

NBD-fatty acid (NBD-dodecanoic acid) at each of the 3 pH, with the highest activity observed at pH 7.5 (Fig. 4). In contrast, NBD-dodecanoic acid was not produced by erythrocyte lysates at any pH tested (Fig. 4). This indicates that erythrocytes contain no ceramidase and, thus, lack an ability to produce Sph.

## Discussion

In the present study, we have demonstrated that erythrocyte S1P accounts for about half of the total S1P levels in whole blood. Since erythrocytes efficiently release S1P into the surrounding fluid in a stimuli-independent manner (Fig. 2) [6], these cells may supply a significant amount of S1P to the plasma. We speculate that platelets and erythrocytes are two major sources of plasma S1P.

Plasma must exhibit a basal level of S1P to enable a S1P gradient between plasma and the secondary lymphoid organs or thymus. This S1P gradient is important in inducing lymphocyte egress from these organs [13,14], and its disruption causes lymphopenia [15]. Moreover, high basal levels of S1P in plasma may be important for constant activation of the S1P receptor on vascular endothelial cells and the subsequent stimulation of adherens junction assembly, vasorelaxation (via the production of nitric oxide), and cell survival [1].

Erythrocytes and platelets exhibit distinct characteristic S1P metabolism. Platelets contain high Sph kinase activity (Fig. 3A) [2] and no S1P lyase activity (Fig. 3B) [3]. The high synthesizing activity and low degrading activity correspond well to the high levels of stored S1P found in platelets [2]. On the other hand, the Sph kinase activity of erythrocytes is much lower (~25-fold) than that of platelets (Fig. 3A), which may correlate with the lower S1P amount found in erythrocytes (~9 fold lower in one erythrocyte compared to one platelet) (Fig. 1B). However, erythrocytes convert exogenous Sph to S1P efficiently (Fig. 2), considering such low Sph kinase activity.

In addition, we found here that erythrocytes contain no degrading activity by

either phosphohydrolase (Fig. 3C) or S1P lyase (Fig. 3B), for which the gene responsible has been identified as SPL [16]. S1P can be dephosphorylated either by members of the S1P-specific SPP family or by enzymes in a different phosphate phosphohydrolase family, LPP, which has broad substrate specificity. Two SPP members (SPP1 and SPP2) [10,17] and three LPP members (LPP1, LPP2, and LPP3) have been identified [18]. SPP1, LPP1, and LPP3 are expressed almost ubiquitously among tissues [10,17,19], as is SPL [20]. Interestingly, until now only platelets have been known to lack S1P lyase [3], and no cell types have been reported to lack S1P phosphohydrolase activity. Thus, erythrocytes possess extremely unique S1P metabolism. The absence of the S1P-degrading enzymes may enable erythrocytes to generate and store S1P efficiently.

[<sup>3</sup>H]Sph labeling assays have demonstrated that platelets convert Sph to ceramide and further to sphingomyelin, albeit weakly [2,6], yet such conversion could not be detected in erythrocytes [6]. Thus, the Sph/S1P metabolism in erythrocytes is quite simple: Sph is metabolized only to S1P. Ceramide synthase, S1P lyase, and S1P phosphohydrolase SPP members are all localized in the ER of cells [10,21,22]. On the other hand, LPP family members are localized in the plasma membrane and also in the internal membranes [23,24], and Sph kinase is localized in the cytosol (primarily) and plasma membrane [25]. Thus the lack of ceramide synthase and S1P lyase activities in erythrocytes would seem reasonable, since erythrocytes have no ER. The absence of S1P phosphohydrolase activity suggests, though, that erythrocytes contain neither SPP nor LPP members, which contrasts with platelets having at least LPP1 [26].

Here, we report the amount of Sph in erythrocytes. The Sph levels were significantly lower than S1P levels both in platelets and erythrocytes (Fig. 1). The observed Sph/S1P balance is contrary to that in other cell types, in which the Sph level is much higher than the S1P level. Thus, erythrocytes and platelets are cells specialized for accumulating S1P. Our *in vitro* ceramidase assay revealed that ceramidase was detected in platelets but not in erythrocytes (Fig. 4), consistent with a previous *in vivo* [<sup>3</sup>H]C<sub>6</sub>-ceramide labeling assay [6]. These results indicate that erythrocytes cannot synthesize Sph. However, erythrocytes contain a significant amount of Sph (Fig. 1), suggesting that they import plasma Sph continuously. The release of endogenous S1P from erythrocytes into culture medium gradually decreased after initiating incubation (Fig. 2B). Under physiological conditions, though, it is possible that the Sph supply enables erythrocytes to produce and release S1P constantly. In fact, a very recent report demonstrated that erythrocytes can store S1P, protecting it from cellular degradation, and release it into plasma, which the authors propose to be a buffer system [27]. However, the authors did not consider the possibility that erythrocytes can recycle the S1P degradation product Sph as suggested in our study.

Recently, the existence in platelets of two S1P transporters, an ATP-dependent and a Ca<sup>2+</sup>-dependent transporter, was reported [4]. However, future studies identifying the S1P transporters both in platelets and erythrocytes will be necessary to elucidate the molecular mechanism of S1P release.

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### Figure legends

Fig. 1. S1P and Sph levels in erythrocytes. Plasma, platelets, and erythrocytes were prepared from the blood of volunteers. Blood cell numbers were determined using a COULTER Gen·S™ Hematology Analyzer. After total lipids were prepared from whole blood and the isolated plasma, platelets, and erythrocytes, S1P and Sph were separated by organic phase separation. Sph was modified with *o*-phthalaldehyde and quantified by HPLC. S1P was first converted to Sph by phosphatase treatment, then quantified by HPLC. Values per ml blood (A) or per 10<sup>7</sup> cells (B) represent the mean ± SD of four samples of whole blood from separate volunteers.

Fig. 2. Constitutive release of S1P from erythrocytes. (A) Erythrocytes (10<sup>8</sup> cells in 100 μl) were treated with 1 μM (100 pmol) Sph and incubated at 37°C. At 0, 5, 15, 30, 60, or 90 min, erythrocytes were separated from the medium by centrifugation. Total lipids were prepared from both medium and erythrocytes, and Sph (upper panel) and S1P (lower panel) amounts were quantified by HPLC. Values shown represent the mean ± SD from four independent experiments. (B) Erythrocytes (10<sup>8</sup> cells in 100 μl) were prepared at 4°C and incubated at 37°C for the indicated time periods. Erythrocytes were separated from the medium by centrifugation, and total lipids were prepared from both medium and erythrocytes. S1P levels were quantified by HPLC. Values shown represent the mean ± SD from three independent experiments.

Fig. 3. Low Sph kinase activity and lack of S1P-degrading enzymes in erythrocytes.

Total lysates were prepared from platelets or erythrocytes isolated from samples of three separate donations of whole blood. The total isolated protein was determined to be  $1236 \pm 60 \mu\text{g}$  and  $136 \pm 12 \mu\text{g}$  for  $10^8$  erythrocytes and  $10^8$  platelets, respectively. (A) Sph kinase activity. Total lysates ( $25 \mu\text{g}$  protein) prepared from platelets or erythrocytes were incubated for 15 min at  $37^\circ\text{C}$  with  $20 \mu\text{M}$  Sph,  $2 \text{ mM}$  ATP, and  $5.4 \mu\text{Ci}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Lipids were extracted and separated by TLC. Radioactivities associated with S1P were quantified using a bioimaging analyzer BAS-2500. Values shown represent the mean  $\pm$  SD from three independent experiments. (B) S1P lyase activity. Total lysates ( $100 \mu\text{g}$  protein) prepared from platelets or erythrocytes and from F9 cells as a positive control were incubated with  $1 \mu\text{Ci}$   $[\text{}^3\text{H}]\text{dihydro-S1P}$  for 1 h at  $37^\circ\text{C}$ . Lipids were extracted, separated by TLC, and detected by autoradiography. (C) S1P phosphohydrolase activity. Total lysates ( $50 \mu\text{g}$  protein) were prepared from the platelets and erythrocytes, and from HEK 293T cells as a positive control. Lysates were incubated with  $100 \text{ nM}$  unlabeled S1P and  $0.1 \mu\text{Ci}$   $[\text{}^3\text{H}]\text{S1P}$  for 30 min at  $37^\circ\text{C}$ . Lipids were extracted, separated by TLC, and detected by autoradiography.

Fig. 4. Absence of ceramidase in erythrocytes. Total cell lysates ( $50 \mu\text{g}$  protein) prepared from platelets or erythrocytes were incubated with  $27.5 \mu\text{M}$   $\text{C}_{12}\text{-NBD-ceramide}$  for 3 h at  $37^\circ\text{C}$  at pH 4.5 (acid), pH 7.5 (neutral), or pH 9.4 (alkaline). Lipids were extracted and separated by TLC. The amount of NBD-dodecanoic acid was quantified using a fluoroimaging analyzer FLA-2000. Values shown represent the mean  $\pm$  SD from three independent experiments.







