

Supplemental Data for

"Fluorescence-based, rapid measurement of sphingosine-1-phosphate transport activity in erythrocytes"

Naoki Kobayashi, Masato Otsuka, Akihito Yamaguchi, and Tsuyoshi Nishi

Supplemental Table 1. Comparison of the methods for measuring S1P release from cells

Characteristics	Methods					
	TLC	HPLC	LC/MS/MS	TLC	Microplate reader	
Detected S1P species	[³ H]S1P	OPA-labeled S1P ^a	Native S1P	NBD-S1P	NBD-S1P	NBD-S1P
Lipid extraction						
Required time ^b	40 min	5 hr ^c	10 min ^d	40 min	10 min	None
Separation and detection						
Required time ^b	14 hr ^e	20 min	14 min	2 hr	0.2 sec ^f	0.2 sec ^f
Sensitivity ^g	<200 pM	20 nM	1 nM	140 pM	20 nM	ND ^h
Throughput	Low	Low	Low	Low	Medium	High
Running cost (USD) ^b	4.5	3.5	3.5	2.2	1.1	1.1
Required equipment	None/imager	HPLC	LC/MS/MS	Imager	Plate reader	Plate reader
Equipment cost (USD)	-/100,000	70,000	1,000,000	25,000	20,000	20,000
Reference ⁱ	[1]	[2, 3]	[4, 5]	Fig. 1BC	Fig. 6A	Fig. 6B

^aOPA, ortho-phthalaldehyde.

^bThe time and running cost were estimated for an application of one sample.

^cThe time includes OPA-labeling time.

^dLipid extraction means deproteinization in this case.

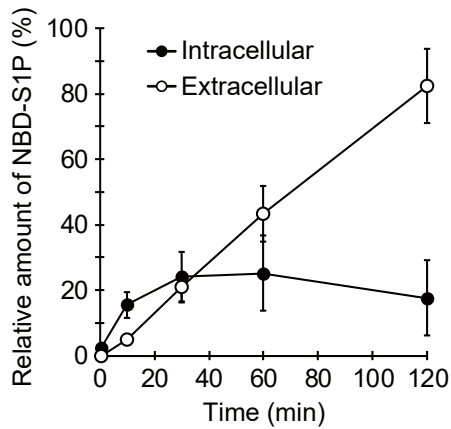
^eThe time includes exposure time.

^fSeparation is not required.

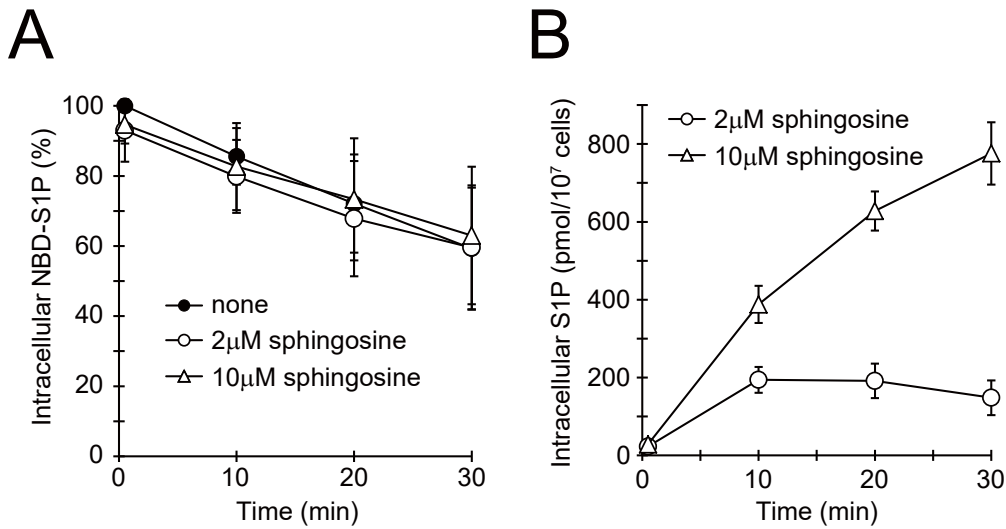
^gLimit of detectable S1P concentration in sample.

^hND, Not determined.

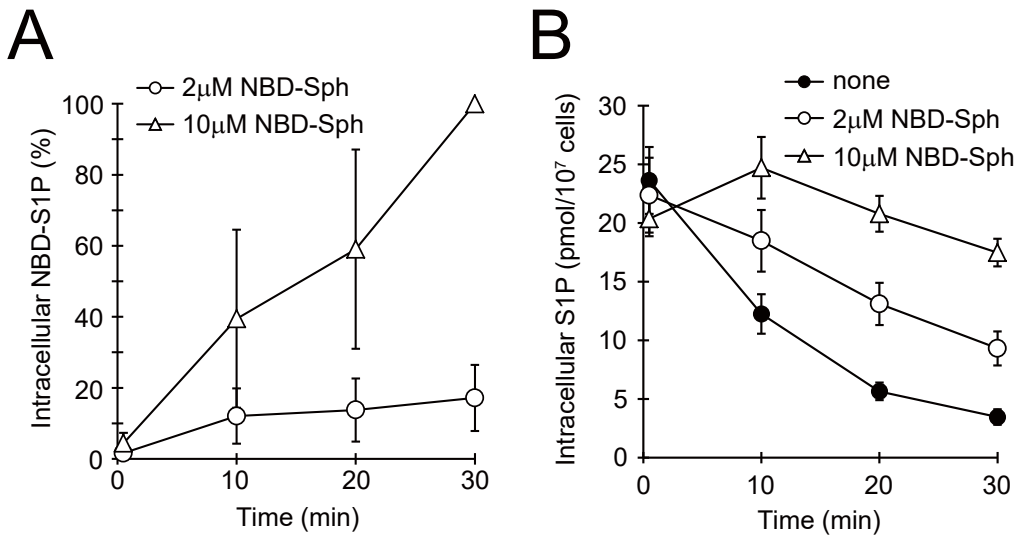
ⁱ[1] *J. Biol. Chem.* **284**, 21192-21200 (2009). [2] *J. Biol. Chem.* **286**, 1758-1766 (2011). [3] *Anal. Biochem.* **303**, 167-175 (2002). [4] *PLoS One.* **7**, e38941 (2012). [5] *Anal. Bioanal. Chem.* **403**, 1897-1905 (2012).



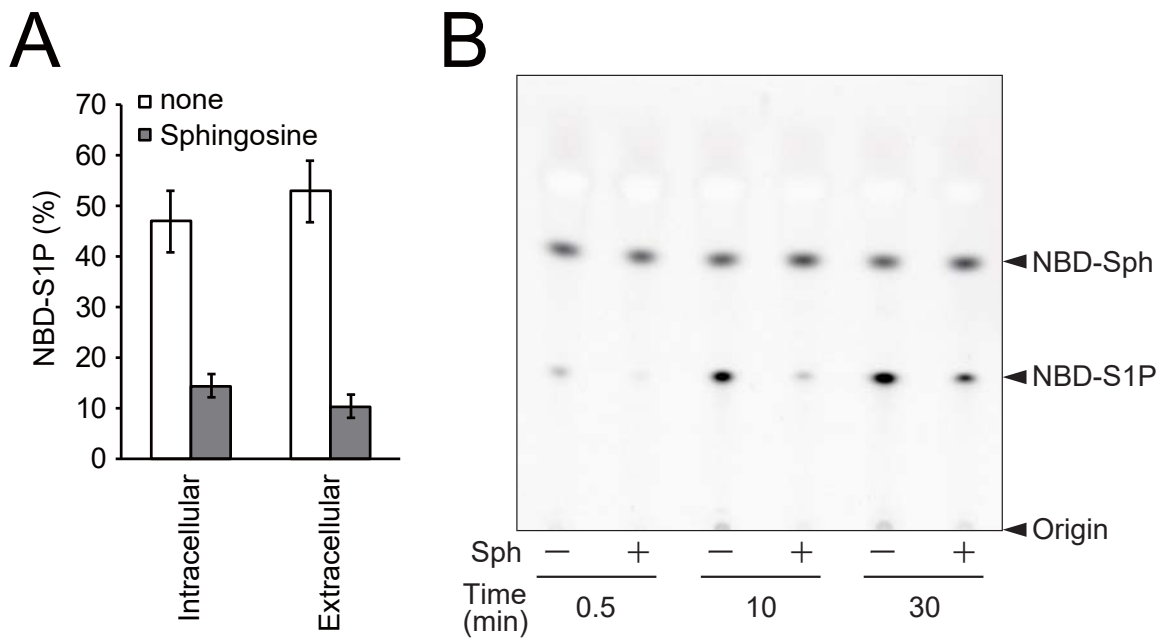
Supplemental Figure 1. NBD-S1P release from erythrocytes. The relative amounts of NBD-S1P extracted from the samples containing 1% BSA were calculated from the NBD-S1P spots on the TLC plates in Figure 1BC. The total amount of NBD-S1P at 120 min was set at 100%. The filled and open circles indicate intracellular and extracellular NBD-S1P, respectively. The experiments were repeated three times, and the error bars indicate the standard deviation (S.D.).



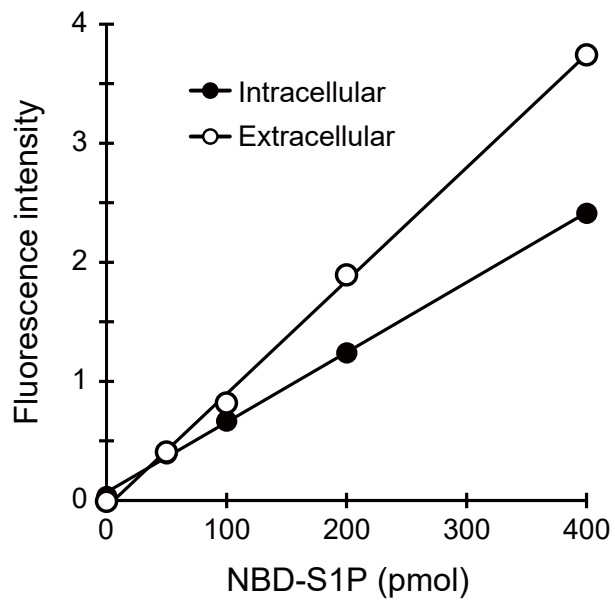
Supplemental Figure 2. Amount of intracellular NBD-S1P and S1P in competitive inhibition of NBD-S1P release by intracellular S1P. The experiments were performed as described in the legend of Fig. 3A. The lipids in erythrocytes were extracted under alkaline conditions, as described in the materials and methods section. The amounts of intracellular NBD-S1P (A) are expressed as the % of NBD-S1P at 0.5 min in the absence of sphingosine. The amounts of intracellular S1P (B) were measured by HPLC. The experiments were performed at least three times, and the error bars indicate the S.D.



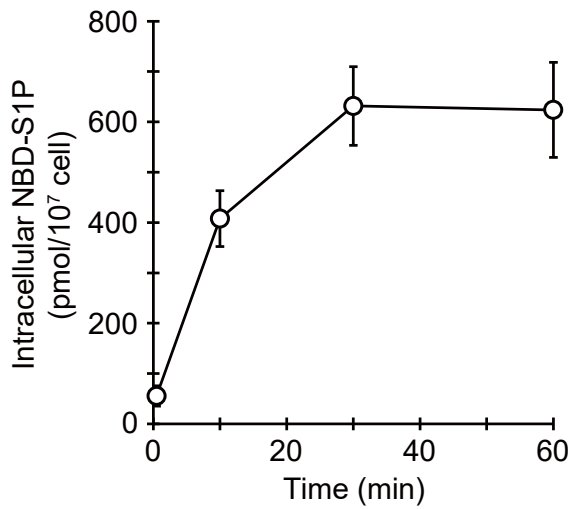
Supplemental Figure 3. Amount of intracellular NBD-S1P and S1P in competitive inhibition of S1P release by intracellular NBD-S1P. The experiments were performed as described in the legend of Fig. 3B. The lipids in the erythrocytes (intracellular) were extracted under alkaline conditions, as described in the materials and methods section. For NBD-S1P quantification, a 100- μ L aliquot of the upper phase was dried and analyzed by TLC. The amounts of intracellular NBD-S1P (A) was expressed as the % of NBD-S1P at 30 min in the presence of 10 μ M NBD-Sph. The amounts of intracellular S1P (B) were measured by HPLC. The experiments were performed at least three times, and the error bars indicate the S.D.



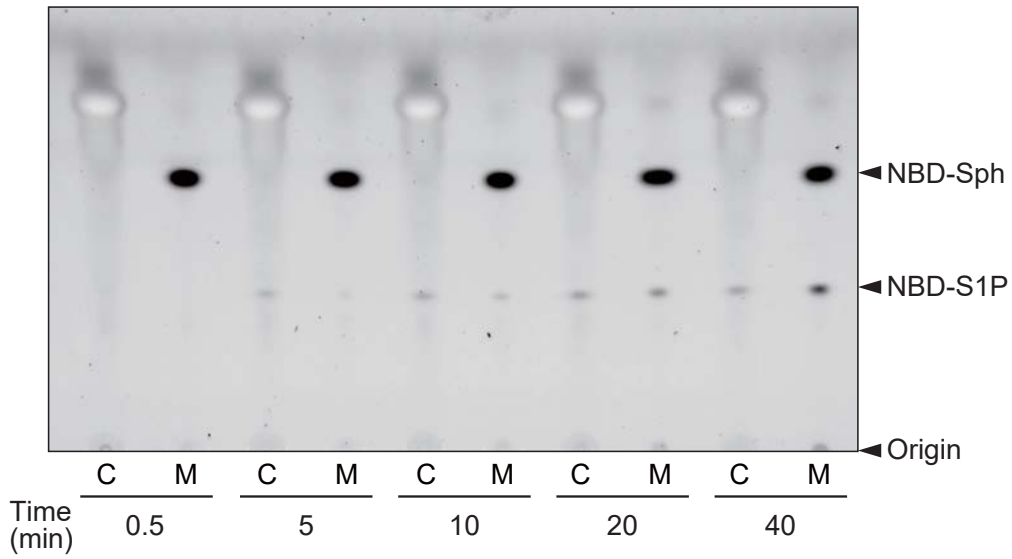
Supplemental Figure 4. Effect of sphingosine on NBD-Sph phosphorylation. Erythrocytes were incubated (5×10^7 cells/mL) in a buffer containing 1% BSA and $5 \mu\text{M}$ NBD-Sph in the presence or absence of sphingosine (Sph, $5 \mu\text{M}$) at 37°C . The erythrocytes and assay buffer were separated by brief centrifugation. Then, the lipids were extracted from the erythrocytes (intracellular) and the assay buffer (extracellular) under acidic conditions and analyzed by TLC. (A) Erythrocytes were incubated for 30 min. The sum of the fluorescence intensity of the intracellular and extracellular NBD-S1P in the sphingosine-free sample was used as 100%. The experiments were repeated three times, and the error bars indicate the S.D. (B) Erythrocytes were incubated for the indicated times. The positions where samples were spotted on the TLC plates are denoted as the origins.



Supplemental Figure 5. Standard curves for quantification of NBD-S1P by using a fluorescence microplate reader. Known concentrations of NBD-S1P were added to the assay buffer or erythrocyte lysates and extracted, and the fluorescence measurements of the NBD-S1P were collected as described in the materials and methods section.



Supplemental Figure 6. Detection of NBD-S1P production in rat erythrocytes by using a microplate reader. The experiments were performed as described in the legend of Fig. 6A. The lipids in the erythrocytes (intracellular) were extracted under alkaline conditions. NBD-S1P standards were prepared to quantify intracellular NBD-S1P (Supplemental Fig. 5). Duplicate experiments were performed four times, and the error bars indicate the S.D.



Supplemental Figure 7. Incorporation of NBD-sphingosine into rat erythrocytes. Rat erythrocytes (1.8×10^6 cells) were incubated with a buffer containing 1% BSA and $0.01 \mu\text{M}$ NBD-sphingosine at 37°C for the indicated times. The erythrocytes and assay buffer were separated by brief centrifugation. Then, the lipids were extracted from the erythrocytes (C) and the assay buffer (M) and analyzed by TLC. The positions where samples were spotted on the TLC plates are indicated as the origins.