Biochimica et Biophysica Acta 1841 (2014) 1581–1589

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Granule-mediated release of sphingosine-1-phosphate by activated platelets

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ARTICLE INFO

Article history:
Received 20 June 2014
Received in revised form 13 August 2014
Accepted 19 August 2014
Available online 23 August 2014

Keywords:
Platelet
Sphingosine
Secretion
Munc13-4
Platelet activation

ABSTRACT

Sphingosine-1-phosphate (S1P) is an intracellularly generated bioactive lipid essential for development, vascular integrity, and immunity. These functions are mediated by S1P-selective cell surface G-protein coupled receptors. S1P signaling therefore requires extracellular release of this lipid. Several cell types release S1P and evidence for both plasma membrane transporter-mediated and vesicle-dependent secretion has been presented. Platelets are an important source of S1P and can release it in response to agonists generated at sites of vascular injury. S1P release from agonist-stimulated platelets was measured in the presence of a carrier molecule (albumin) using HPLC-MS/MS. The kinetics and agonist-dependence of S1P release were similar to that of other granule cargo *e.g.* platelet factor IV (PF4). Agonist-stimulated S1P release was defective in platelets from *Unc13d*^{jinx} (Munc13-4 null) mice demonstrating a critical role for regulated membrane fusion in this process. Consistent with this observation, platelets efficiently converted fluorescent NBD-sphingosine to its phosphorylated derivative which accumulated in granules. Fractionation of platelet organelles revealed the presence of S1P in both the plasma membrane and in α-granules. Resting platelets contained a second pool of constitutively releasable S1P that was more rapidly labeled by exogenously added sphingosine. Our studies indicate that platelets contain two pools of S1P that are released extracellularly: a readily-exchangeable, metabolically active pool of S1P, perhaps in the plasma membrane, and a granular pool that requires platelet activation and regulated exocytosis for release.

1. Introduction

Bioactive lipids contribute to many cellular signaling processes [1,2]. In most cases, the biological actions of these lipids are mediated by cell surface receptors. These lipids therefore must be generated and/or released extracellularly. The focus of the present study is to examine the route by which a bioactive lipid, sphingosine-1-phosphate (S1P), is released from activated platelets. S1P regulates many physiological processes, *e.g.* cell migration, maturation, differentiation, vascular integrity, and angiogenesis [3]. Its effects are facilitated by a family of receptors called S1PR1-5 (previously called Endothelial Differentiation Gene receptors) [4]. A reciprocal axis of VEGF/S1P signaling is key to normal angiogenesis and absence of S1P results in hypersprouting of blood vessels leading to vascular leakage [5]. Localized availability of S1P is necessary to maintain the junctional integrity of vascular endothelial cells. S1P also regulates lymphocyte trafficking between the lymphatic system and peripheral circulation [6]. Therefore, regulating the availability of extracellular S1P is critical for several aspects of vascular function.

Blood contains both cellular and plasma associated pools of S1P. Plasma S1P is almost exclusively associated with carriers: the apolipoprotein M component of HDL or albumin [7,8], and is predominantly produced by erythrocytes [9], and vascular endothelial cells [11]. Indeed, erythrocyte transfusion reduces the vascular leakiness seen in genetically-modified mice, deficient in plasma S1P [12] and human plasma S1P levels correlate with hematocrit [25]. Release of S1P from erythrocytes is passive, requiring only the presence of a carrier molecule to adsorb S1P [13]. Although not a primary determinant of circulating S1P levels, platelets contain a regulated pool of S1P that is mobilized in response to extracellular signals resulting in localized increases in extracellular S1P. The relevance of this pool is demonstrated by the role that platelet-released S1P plays to maintain the integrity of high endothelial venules (HEVs) [14]. Platelet production of S1P is robust given their high levels of sphingosine kinase (SphK) and lack of S1P phosphatase and S1P lyase [10,15,16]. How platelets release S1P has been the subject of several studies, yet the mechanisms are unclear [17]. Platelets passively release S1P and also exhibit an activation-dependent release, which requires stimulation with agonists such as thrombin [18]. Initial theories held that S1P, made on the cytoplasmic

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http://dx.doi.org/10.1016/j.bbalip.2014.08.013
1388-1981/

leaflet of platelet plasma membranes, is translocated to the extracellular space by a transporter, such as Spinster 2 (SPNS2), and/or an ABC transporter. Though SPNS2 is important for S1P efflux from endothelial cells, it is not required in platelets [19]. Studies with the ABC transporter inhibitor, glyburide (also known as glibenclamide), suggest that an ABCA-like transporter could be involved; but, the inhibitor's effects on other platelet processes were not tested [17]. Other ABC transporter inhibitors (*i.e.* verapamil, cyclosporine and MK571) were without effect [17,20].

In this study, we sought to clarify the mechanisms by which platelets release S1P to determine if this bioactive lipid is stored in cargo-containing granules and is secreted upon stimulation. We used mass spectrometry to monitor both platelet-associated and released S1P. We examined S1P release from platelets obtained from a mouse strain with a genetic defect in the SNARE-based machinery that is required for platelet exocytosis [21]. Our results suggest a scheme to explain how platelet-derived S1P could be used to acutely and locally influence S1PR signaling in endothelial cells at vascular lesions.

2. Materials and methods

2.1. Platelet preparation and treatments

Human and mouse platelets were prepared as described [21–23]. The platelet preparations had no overt erythrocyte or nucleated cell contamination as assessed microscopically. Platelet concentrations were adjusted with Hepes Tyrode's (HT) buffer (10 mM HEPES/KOH, pH 7.4, 137 mM NaCl, 12 mM $NaHCO_3$, 5.56 mM glucose, 2.7 mM KCl, 1 mM $MgCl_2$ $6H_2O$, 0.36 mM NaH_2PO_4 H_2O) containing 1% (w/v) Fraction V, fatty acid-free, bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO), unless otherwise indicated. When stimulated with thrombin (Chrono–Log, Haverton, PA), the secretion reactions were stopped with a two-fold excess of hirudin (Sigma Aldrich). Other agonists: convulxin (Centerchem, Norwalk, CT) and ADP (Chrono–Log), were used at the indicated concentrations. Agonist concentrations were adjusted for optimal S1P release from the platelets obtained from different donors. All solvents were of reagent grade.

2.2. Lipid extraction

Lipids were extracted in acidified organic solvents as described [24]. Platelets (2.5×10^7 in 50 μL) were added to a mixture of 2 mL CH_3OH and 1 mL $CHCl_3$ in 8 mL borosilicate glass tubes. Fifty microliters of 1 μM C_{17}-S1P (Avanti Polar Lipids, Alabaster, AL) was added as an internal standard. The mixture was acidified with 0.45 mL of 0.1 M HCl, mixed for 5 min, and placed at 4 °C for 1 h. The extraction volume was increased with 1 mL $CHCl_3$ and 1.3 mL 0.1 M HCl. After mixing for 5 min, the samples were centrifuged for 10 min at 3500 ×*g*. The lower phase was transferred to a glass vial avoiding the protein interface. Samples were dried under N_2 stream and resuspended in 100 μL CH_3OH for analysis.

2.3. Quantitation of S1P by high performance liquid chromatography-electron spray ionization-mass spectrometry/mass spectrometry (HPLC-ESI-MS/MS)

Extracted lipids were used for the detection of S1P as described in [25]. Analysis of S1P was carried out using a Shimadzu Ultra-Fast Liquid Chromatograph coupled with an ABSciex 4000-Qtrap hybrid linear ion trap triple quadrapole mass spectrometer operated in multiple reaction monitoring (MRM) mode. C_{17}-S1P or hepta-deuterated S1P were used as internal standards. Lipids were separated using an Agilent Zorbax Eclipse XDB C8 column (5 μm, 4.6 × 150 mm). The mobile phase consisted of 75/25 of CH_3OH/H_2O with formic acid (0.5%) and 5 mM $HCOONH_4$ (0.1%) as solvent A and 99/1 of CH_3OH/H_2O with formic acid (0.5%) and 5 mM $HCOONH_4$ (0.1%) as solvent B. For the analysis of S1P, the separation was achieved using a gradient of 0% B for 1 min, 0% B to 100% B in the next minute, maintained at 100% B for the next 10 min and equilibrated back to the initial conditions in 3 min. The flow rate was 0.5 mL/min with a column temperature of 30 °C. The sample injection volume was 10 μL. The mass spectrometer was operated in positive electro-spray ionization (ESI) mode with optimal ion source settings determined by synthetic standards of S1P and C_{17}-S1P with a declustering potential of 61 V, entrance potential of 10 V, collision energy of 23 V, collision cell exit potential of 16 V, curtain gas of 20 psi, ion spray voltage of 5500 V, ion source gas1/gas2 of 40 psi and temperature of 550 °C. MRM transitions monitored were as follows: 366.141/250 for C_{17}-S1P; and 380.124/264.1 for S1P.

2.4. Mouse models

C57BL/6 (wild type), and *Unc13d*^{jinx} mice were as described [21]. All experiments with animals were approved by the University of Kentucky Institutional Animal Care and Use Committee.

2.5. Fluorescence microscopy

Washed platelets (5×10^8/mL) were incubated with 1 μM mepacrine dihydrochloride (Sigma Aldrich) or 1 μM omega (7-nitro-2-1, 3-benzoxadiazol-4-yl)(2S,3R,4E)-2-amino octadec-4-ene-1,3-diol (NBD–sphingosine; Avanti Polar Lipids) for 30 min at 37 °C. The platelets were pelleted at 550 ×*g* for 8 min and resuspended in fresh HT buffer. Approximately 10 μL of the samples was placed onto a microscope slide and overlaid with a cover glass. The platelets were then allowed to settle at RT for 30 min and were viewed using a Nikon Eclipse 600 epifluorescence microscope equipped with a 100× oil objective (Melville, NY). Digital images were captured using Axiocam MRm (Carl Zeiss Microscopy, Thornwood, NY) and were processed using Axiovision software.

2.6. Thin layer chromatography

After liquid-phase lipid extraction, samples were separated by thin layer chromatography using Silica Gel 60 TLC plates (Merck KGaA, Darmstadt, Germany) with a mobile phase of $CHCl_3/CH_3OH/H_2O$ (60/35/8). After development and drying, the separated, NBD–sphingolipids were visualized using a Typhoon 9500 scanner (GE Healthcare Life Sciences, Piscataway, NJ) at excitation wavelength 457 nm and emission wavelength 532 nm. A NBD-S1P standard was a generous gift from Dr. Brian W. Wattenberg (University of Louisville).

2.7. Sucrose gradient sub-cellular fractionation

The procedure was modified from [26]. A unit of platelet rich plasma (PRP) was subjected to centrifugation at 137 ×*g* for 15 min. Platelets, in the supernatant, were pelleted at 695 ×*g* for 15 min at RT and washed twice with 50 mL Tris–citrate buffer (63 mM Tris/HCl, pH 6.5, 95 mM NaCl, 12 mM citric acid, 5 mM KCl). The platelet pellet was finally resuspended in 12 mL Tris–citrate buffer and was transferred to the cell-disruption bomb (Parr 4639, Parr Instrument Co., Moline, IL). The Parr bomb was pressurized with N_2 to 1200 psi on ice for 15 min and the pressure was rapidly released. This cycle was repeated thrice and the platelet homogenates were cleared by centrifugation at 610 ×*g* for 10 min, prior to loading the supernatant onto sucrose gradients. Linear sucrose gradients were generated by layering 1.5 mL of the sucrose solutions (containing 5 mM EDTA) of decreasing concentrations (from 60 to 30%, w/v) into an ultracentrifuge tube (Beckman Instruments, Inc., Fullerton, CA). The tubes were