ARTICLE
Journal of Cellular Biochemistry 109:1232–1243 (2010)

Journal of Cellular Biochemistry

Erythrocytes Serve as a Reservoir for Cellular and Extracellular Sphingosine 1-Phosphate

Constantin Bode,[1] Sven-Christian Sensken,[1] Ulrike Peest,[1] Gernot Beutel,[2] Felicitas Thol,[2] Bodo Levkau,[3] Zaiguo Li,[4] Robert Bittman,[4] Tao Huang,[5] Markus Tölle,[5] Markus van der Giet,[5] and Markus H. Gräler[1*]

[1]*Institute for Immunology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hanover, Germany*

[2]*Department of Hematology, Hemostasis, Oncology, and Stem Cell Transplantation, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hanover, Germany*

[3]*Institute of Pathophysiology, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany*

[4]*Department of Chemistry and Biochemistry, Queens College of City University of New York, Flushing, New York 11367-1597*

[5]*Department of Nephrology, Charité-Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany*

ABSTRACT

Sphingosine 1-phosphate (S1P) in blood is phosphorylated, stored, and transported by red blood cells (RBC). Release of S1P from RBC into plasma is a regulated process that does not occur in plasma- or serum-free media. Plasma fractionation and incubations with isolated and recombinant proteins identified high density lipoprotein (HDL) and serum albumin (SA) as non-redundant endogenous triggers for S1P release from RBC. S1P bound to SA and HDL was able to stimulate the $S1P_1$ receptor in calcium flux experiments. The binding capability of acceptor molecules triggers S1P release, as demonstrated with the anti-S1P antibody Sphingomab™. More S1P was extracted from RBC membranes by HDL than by SA. Blood samples from anemic patients confirmed a reduced capacity for S1P release in plasma. In co-cultures of RBC and endothelial cells (EC), we observed transcellular transportation of S1P as a second function of RBC-associated S1P in the absence of SA and HDL and during tight RBC-EC contact, mimicking conditions in tissue interstitium and capillaries. In contrast to S1P bound to SA and HDL, RBC-associated S1P was significantly incorporated by EC after S1P lyase (SGPL1) inhibition. RBC-associated S1P, therefore, has two functions: (1) It contributes to the cellular pool of SGPL1-sensitive S1P in tissues after transcellular transportation and (2) it helps maintain extracellular S1P levels via SA and HDL independently from SGPL1 activity. J. Cell. Biochem. 109: 1232–1243, 2010.

KEY WORDS: APOLIPOPROTEIN; SERUM ALBUMIN; SCRAMBLASE; S1P LYASE; PLASMA

S1P is a potent extracellular messenger with a broad spectrum of functions. Inter alia, blood-borne S1P is required for the maintenance of lymphocyte circulation. It stimulates the S1P receptor type 1 ($S1P_1$) on lymphocytes and mediates their egress from thymus and secondary lymphoid organs (SLO) [Allende et al., 2004; Matloubian et al., 2004]. RBC potently phosphorylate sphingosine (Sph) to S1P, and sphingosine kinases (SK) expressed by RBC are critical to achieve high levels of S1P in blood plasma [Hänel et al., 2007; Pappu et al., 2007]. Thrombocytes, which have been suggested as the main source for S1P in blood [Yatomi et al., 1995, 1997, 2001], do not contribute significantly in sustaining high plasma S1P levels [Hänel et al., 2007; Pappu et al., 2007]. Thus, RBC serve as the principal repository for blood-borne S1P.

The specific function of S1P in blood is only poorly understood. Mice deficient in the S1P-producing enzymes SK1 and SK2 manifest severe failure in blood vessel stability [Mizugishi et al., 2007], and deficiency of the $S1P_1$ receptor results in defective blood vessel maturation [Liu et al., 2000]. Both phenotypes are embryonic lethal. Targeted deletion of SK1 and SK2 in hematopoietic and vascular endothelial cells (EC) render mice lymphopenic because of the loss of

Grant sponsor: German Research Foundation (DFG); Grant numbers: GR-1943/1-4, GR-1943/2-1, GR-1943/3-1; Grant sponsor: National Institutes of Health (NIH); Grant number: HL-083187.

*Correspondence to: Dr. Markus H. Gräler, Institute for Immunology, Hannover Medical School, Bldg. K11, OE 9422, Carl-Neuberg-Str. 1, 30625 Hanover, Germany. E-mail: graeler.markus@mh-hannover.de

Received 19 October 2009; Accepted 29 December 2009 • DOI 10.1002/jcb.22507 • © 2010 Wiley-Liss, Inc.
Published online 23 February 2010 in Wiley InterScience (www.interscience.wiley.com).

S1P, which is a required stimulus for lymphocyte exit from thymus and SLO in blood and lymph [Pappu et al., 2007]. Targeted deletion of the $S1P_1$ receptor in lymphocytes constitutes a similar phenotype, indicative of the constitutive presence of S1P in blood and lymph, and $S1P_1$ receptor expression on lymphocytes as the driving force for lymphocyte circulation [Allende et al., 2004; Matloubian et al., 2004]. In addition, the anti-S1P antibody Sphingomab™ shows promising efficacy in preclinical animal studies for cancer treatment [Sabbadini, 2006; Visentin et al., 2006; Caballero et al., 2008; Swaney et al., 2008]. It prevents tumor growth and cancer progression by inhibiting tumor-associated angiogenesis and the release of proangiogenic cytokines. Blood-borne S1P is, therefore, an important factor for blood vessel maturation, angiogenesis, and lymphocyte circulation.

S1P concentrations are high in blood and lymph, and low in tissues [Schwab et al., 2005; Peest et al., 2008]. This difference in S1P distribution is maintained by SK1/2, which catalyzes the phosphorylation of Sph in the rate-limiting step for the generation of S1P in blood, and by SGPL1, which degrades S1P irreversibly into 2-hexadecenal and phosphoethanolamine in tissues [Schwab et al., 2005; Pappu et al., 2007]. Despite the phenotype of correspondent gene deficient mice [Mizugishi et al., 2005; Vogel et al., 2009], SGPL1 deficiency had no effect on extracellular S1P degradation, indicating that clearance of extracellular S1P is predominantly a SGPL1 independent process [Peest et al., 2008]. Furthermore S1P and sphingosine accumulation in splenocytes was not maintained ex vivo [Peest et al., 2008], and SGPL1 deficient neurons required exogenous addition of Sph or S1P for massive cellular S1P accumulation [Hagen et al., 2009]. Therefore, we hypothesized that a systemic source of S1P may serve as substrate for SGPL1 in local tissues.

To this end we investigated the release of S1P from RBC. We identified two major pathways: Firstly, release of the SGPL1 inaccessible and receptor-available signaling molecule S1P into the extracellular space via binding molecules, with SA and HDL as the endogenous acceptors in plasma and RBC as donors in blood, and secondly, transcellular transportation of S1P via RBC-EC-tissue cell contact, which contributes to the cellular pool of SGPL1 susceptible S1P in tissues.

MATERIALS AND METHODS

CHEMICALS

The anti-S1P antibody Sphingomab™ was kindly provided by Lpath (San Diego, CA). S1P, 1,4-dithioerythritol (DTE), apolipoproteins ApoCI and ApoCII, sodium orthovanadate (SOV), and *N*-ethylmaleimide (NEM) were obtained from Sigma (St. Louis, MO). 1,3-Dihydroxy-2-amino-4*E*-heptadecene-1-phosphate (the C17 analog of S1P, C17-S1P) and 1,3-dihydroxy-2-amino-4*E*-heptadecene (the C17 analog of Sph, C17-Sph) were purchased from Otto Nordwald (Hamburg, Germany). Sph was purchased from Tocris (Ellisville, MO), and fluorescein-labeled S1P (S1P-FITC) was from Tebu-Bio (Offenbach, Germany). Phosphotungistic acid was obtained from Fluka (Buchs, Switzerland). S1P, Sph, C17-Sph, and C17-S1P were dissolved in methanol. BODIPY-D-*erythro*-sphingosine (Sph-BODIPY) was prepared via an *E*-selective olefin cross-metathesis reaction of (*S*)-Garner allylic alcohol ((*S*)-*tert*-butyl 4-((*R*)-1-hydroxyallyl)-2,2-methyloxazolidine-3-carboxylate) with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-undecen-3-undec-10-ene using a reported procedure [Chandrasekhar et al., 2005; Sudhakar et al., 2005; Peters et al., 2007], followed by deprotection with BF_3OEt_2 in the presence of 4 Å molecular sieves. Sph-BODIPY was dissolved in methanol.

LIPID EXTRACTION

Biological samples (1 ml of medium or 50 µl of erythrocyte cell pellet, or 100 µl of plasma) were adjusted to 1 ml sample volume with 1 M NaCl in H_2O and transferred into a glass centrifuge tube. After addition of 1 ml of methanol and 200 µl of 6 M HCl, the samples were vortexed. Chloroform (2 ml) was added, and the samples were again vigorously vortexed for 2 min. After the samples were centrifuged for 3 min at 1,900*g*, the lower chloroform phase was transferred to another glass centrifuge tube. After the lipid extraction was repeated with 2 ml of chloroform, the chloroform phases were combined and vacuum dried in a speed-vac for 45 min at 50°C.

DERIVATIZATION OF SPHINGOLIPIDS WITH 9-FLUORENYLMETHYL CHLOROFORMATE (FMOC-Cl)

Vacuum-dried samples were dissolved in 200 µl of dioxane with subsequent addition of 200 µl of 70 mM K_2HPO_4, and 200 µl of FMOC-Cl solution [Andréani and Gräler, 2006].

HPLC ANALYSIS OF FLUORESCENTLY LABELED SPHINGOLIPIDS

Chromatographic detection of sphingolipids was performed as described [Andréani and Gräler, 2006] using the Merck–Hitachi Elite LaChrom System. The injection-pump delivery rate was 1.3 ml/min. The eluent was methanol, 70 mM K_2HPO_4, and H_2O, forming a gradient over a period of 68 min for FMOC-labeled sphingolipids. A sample volume of 10 µl was injected using the cut-injection method. The sphingolipids were separated by reversed phase HPLC using a 250 mm × 4.6 mm Kromasil 100-5 C18 column and a 17 mm × 4 mm Kromasil 100-5 C18 pre-column. The column temperature was 35°C, and detection was performed with a fluorescence detector (excitation at 263 nm and emission at 316 nm). For detection of Sph-FITC and S1P-FITC a gradient of 30 min (Table I) was used, and the detection was carried out at 494 nm excitation and 518 nm emission wavelengths [Peest et al., 2008].

COUPLING OF FLUORESCENCE ANALYSIS AND LC/MS/MS

For coupled analysis of BODIPY-labeled molecules via fluorescence detection and LC/MS/MS, a gradient over a period of 30 min was used (Table I). The separation was performed with a Zorbax

TABLE I. Gradient Table for Fluorescence and Mass Detection of FITC- and BODIPY-Labeled Sphingolipids

Time	MeOH (%)	1% formic acid in H_2O (%)
0	50	50
5	50	50
20	100	0
30	100	0

Extend-C18 column (2.1 mm × 50 mm, 3.5 μm; Agilent Technologies, Böblingen, Germany) at a flow rate of 300 μl/min. Detection with the fluorescence detector was performed at 495 nm excitation and 505 nm emission wavelengths.

FLUORESCENCE MICROSCOPY

RBC (50% hematocrit) were incubated for 1 h at 37°C with 60 μM Sph-BODIPY or 20 μM S1P-FITC, washed two times with phosphate-buffered saline (PBS), and then analyzed using a confocal microscope (Leica DM IRB with a TCS SP2 AOBS scan head, Leica Microsystems, Wetzlar, Germany). HUVEC were incubated and analyzed in the chambered coverglass System Lab-TEK™ (Nunc, Langenselbold, Germany). Nuclear DNA was stained with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI, 1 μg/ml, Roche Diagnostics, Mannheim, Germany), which was added to HUVEC 3 h before analysis.

SAMPLE PREPARATION FOR LC/MS/MS ANALYSIS

The extracted lipids were dissolved in methanol/chloroform (4:1, v/v) and stored at −20°C. C17-Sph (300 pmol), which was employed as the internal standard, was added prior to lipid extraction.

ESI LC/MS/MS ANALYSIS

A QTrap triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) interfaced with a Merck-Hitachi Elite LaChrom series 3.1.3 chromatograph and autosampler (VWR, Darmstadt, Germany) was used for ESI LC/MS/MS analysis. Positive ion ESI LC/MS/MS analysis was employed for detection of all analytes. The multiple reaction monitoring transitions for the detection were as follows: C17-Sph *m*/*z* 286/268, S1P *m*/*z* 380/264, Sph-BODIPY *m*/*z* 476/408, S1P-BODIPY *m*/*z* 556/408. Liquid chromatographic resolution of all analytes was achieved using a Zorbax Extend-C18 column (2.1 mm × 50 mm, 3.5 μm; Agilent Technologies). The elution protocol was composed of a 10-min column equilibration with 10% solvent A (methanol), 50% solvent D (100 mM HCl), and 40% solvent C (H_2O) followed by sample injection and a 20-min period with 100% solvent A. Samples were infused into the electrospray ion source (ESI) through an electrode tube at a rate of 300 μl/min. Standard curves, with C17-Sph as the internal standard, were constructed by adding increasing concentrations of the analytes to 300 pmol of C17-Sph (internal standard). Linearity of the standard curves and correlation coefficients were obtained by linear regression analyses. All mass spectrometry analyses were performed with Analyst 1.4 (Applied Biosystems, Darmstadt, Germany).

FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC)

All chromatographic runs were performed with an Äkta™ design system (Amersham Biosciences, Freiburg, Germany). The chromatographic material used for FPLC was purchased from GE Healthcare (Munich, Germany). Chromatographic separation with the HiTrap Blue column was achieved with a step gradient of B in A at a flow rate of 1 ml/min (HiTrap™ Blue: A = 50 mM KH_2PO_4, pH 7.0; B = Buffer C containing 1.5 M KCl). Separation for the HiTrap™ Q was achieved with a linear gradient of D in C at the same flow rate (HiTrap™ Q: C = 20 mM Tris–HCl, pH 8.0; D = Buffer A containing 1 M NaCl). Further purification was obtained by a size-exclusion column (HiPrep™ 16/60 Sephacryl; 50 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) at a flow rate of 300 μl/min. The pooled fractions were concentrated via Centricons (Millipore, Eschborn, Germany) with a nominal molecular mass cutoff of 10 kDa.

PRECIPITATION OF LIPOPROTEINS

Lipoproteins were sequentially precipitated via an increasing $Na_3P(W_3O_{10})_4$ concentration as previously described [Burstein et al., 1970]. For chylomicron and very low density lipoprotein (VLDL) precipitation, 25 μl of 1% $Na_3P(W_3O_{10})_4$ and 25 μl of a 2 M $MgCl_2$ solution were added to 500 μl plasma. After brief mixing and 15 min incubation at room temperature, the samples were centrifuged for 50 min at 16,000*g* and 4°C. Supernatants were transferred to new tubes for further precipitation. For low density lipoprotein (LDL) precipitation, 25 μl of 4% $Na_3P(W_3O_{10})_4$ and 25 μl of a 2 M $MgCl_2$ solution were added to the first supernatant. After 15 min incubation at RT, the samples were centrifuged for 10 min at 6,000*g* and 4°C. Again the supernatants were transferred to new tubes. For HDL precipitation, 25 μl of 4% $Na_3P(W_3O_{10})_4$ and 25 μl of a 2 M $MgCl_2$ solution were added to the second supernatant. After 2 h incubation at room temperature, the samples were centrifuged for 30 min at 18,000*g* and 4°C. If not used immediately, the pellets were stored at −20°C.

LIPOPROTEIN ANALYSES

HDL (d = 1.125-1.210 g/ml) from healthy controls was isolated from human plasma via ultracentrifugation using a SW40 Ti rotor [Havel et al., 1955]. Levels of total cholesterol were determined using chromogenic assays (Diasys, Holzheim, Germany). Separation of HDL in lipid and protein fraction was done by using a 2-propanol-hexane-protocol as previously described [Hara and Radin, 1978]. Both fractions were dried with a speed-vac (Thermo Fisher Scientific, Dreieich, Germany). Protein fractions were resolved in guanidinium hydrochloride (3 M) and separated by HPLC using a Grace Vydac C18 column (Alltech, Rottenburg, Germany). Fractions were collected as indicated for the experiments. All fractions were dried with a speed-vac and resolved in the indicated buffers.

S1P RELEASE TEST PROCEDURE

RBC (8×10^8 cells) were incubated with 0.2, 0.4, 1, or 2 ml of the sample for 2 h in a heat block at 37°C and 900 rpm. The incubation medium (pH 7.4) contained the following (mM): NaCl (150), KCl (5), Tris–HCl (21), and glucose (5) (RBC buffer). After centrifugation for 2 min at 3,000*g*, the amount of S1P in the supernatant was analyzed by HPLC or LC/MS/MS analysis. For higher test sensitivity, RBC (8×10^8 cells) were loaded first with 4 nmol Sph for 1 h at 37°C, washed two times, and stored at 4°C.

PRETREATMENT OF RBC WITH SH REAGENTS

Modifications were performed in RBC buffer (10% hematocrit, 37°C) as described [Kamp et al., 2001]. Incubations were carried out with 0.8 mM NEM for 20 min and with 10 mM DTE for 45 min, followed by two washing steps with PBS.

TREATMENT OF RBC WITH SOV

RBC (8×10^7 cells) were preincubated with 1 mM SOV for 5 min at 37°C. After centrifugation for 2 min at 3,000*g*, the supernatant was removed and 1 ml RBC-lysis buffer (138 mM NH_4CL, 1 mM $KHCO_3$, and 0.1 mM EDTA) was added. The sample was vortexed shortly and centrifuged again. The absorbance of the supernatant was measured at 405 nm with the Infinite M-200 plate reader (Tecan, Crailsheim, Germany) to detect RBC lysis.

CELL CULTURE

Wild type (wt) rat hepatoma cells (HTC_4) and HTC_4 expressing human $S1P_1$ were cultured in Eagle's minimum essential medium (MEM) with Earle's salts supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 × non-essential amino acids, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. HTC_4 cells carrying the human $S1P_1$ construct were cultured in the presence of G418 sulfate (0.4 mg/ml). Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Verviers, Belgium) and cultured in EGM-2 medium with supplements according to the supplier's protocol. Cells were grown at 37°C in a humidified incubator with 5% CO_2.

DOP TREATMENT

HUVEC were preincubated with 0.5 mM 4-deoxypyridoxine (DOP) overnight at 37°C and 5% CO_2 in 6-wells. Cells were incubated with or without 2 × 10E8 RBC (loaded with 2 nmol C17-S1P) or 50 μl BSA (preloaded via 2 h incubation with C17-S1P loaded RBC) in the presence or absence of 0.5 mM DOP for 2 h at 37°C. Cells were vigorously washed on ice at least three times and scraped from the plate for measurement of C17-S1P concentrations.

CALCIUM FLUX

HTC_4 cells were harvested with trypsin and washed three times with Ca^{2+}/Mg^{2+}-free Hank's BSS supplemented with 10% charcoal-adsorbed FBS. Cells (1×10^7 cells/ml) were loaded for 30 min at 37°C with 2.5μM FURA-2/AM (Cabiochem–Novabiochem, Bad Soden, Germany) in the same buffer. After three additional washing steps, 2×10^6 cells/ml were resuspended in Ca^{2+}-containing Hank's BSS supplemented with 10% charcoal-adsorbed FBS. Calcium release was determined at 37°C in a 3 ml quartz cuvette using the spectrofluorophotometer Hyper RF-5301PC (Shimadzu, Duisburg, Germany) with 335 nm excitation wavelength and 505 nm emission wavelength. Relative calcium responses were normalized to the corresponding signal with 10 μM ATP in each single experiment, which was set to 100%.

CLINICAL DATA

Analysis of blood samples from anemic patients and healthy volunteers were performed according to the June 1964 Declaration of Helsinki entitled "Ethical Principles for Medical Research Involving Human Subjects."

RESULTS

HDL AND SA INDUCE S1P RELEASE FROM RBC

We have recently shown that S1P release from RBC occurs in plasma and serum but not in serum-free media, suggesting that an unknown plasma factor accounts for its secretion [Hänel et al., 2007]. In order to isolate relevant components for S1P release, plasma was fractionated by FPLC, and S1P release was tested by incubating the resulting FPLC fractions with RBC. After three subsequent separations using affinity chromatography, anion exchange chromatography, and gel filtration, the protein peak with the main S1P-releasing activity was eluted at a similar retention time as SA (Fig. 1A). To test whether or not S1P release from RBC was triggered by SA, recombinant human SA expressed in the yeast strain *Pichia pastoris* was tested for its activity and induced massive S1P release from RBC, whereas the control medium was ineffective (Fig. 1B). All subsequent experiments were performed with bovine SA (BSA) up to the physiological plasma concentration of 50 mg/ml. Incubation of RBC with different low amounts of SA revealed a ratio of 2 S1P molecules that are extracted by 1,000 SA molecules (Fig. 1C). We next addressed the S1P-releasing activity of lipoproteins, which were not eluted during the initial affinity chromatography and were therefore excluded from all activity tests. Chylomicrons/VLDL, LDL, HDL, and lipoprotein-free plasma were isolated by sequential lipoprotein precipitation [Burstein et al., 1970]. Subsequent incubation of each plasma fraction revealed HDL being highly active in inducing S1P release from RBC, which was only slightly reduced compared to the release induced by lipoprotein-free plasma containing serum albumin (SA) (Fig. 1D). This result was confirmed by incubation of RBC with HDL isolated from plasma using density gradient centrifugation (Fig. 1E). Fractionation of HDL into four main protein fractions after delipidation elicited the major releasing activity in fraction 2 (Fig. 1F), and further subfractionation identified fraction 2.2 as the active S1P-releasing protein fraction (Fig. 1G) with ApoCI and ApoCII as major components (Fig. 1H). However, commercially available preparations of ApoCI and ApoCII were not able to induce S1P-release from RBC (data not shown). Incubation of RBC with HDL demonstrated a faster and more efficient extraction compared to SA (Fig. 1I).

SPECIFICITY, REGULATION, AND MOLECULAR REQUIREMENTS OF S1P RELEASE FROM RBC

Since SA is the most abundant plasma protein, the specificity of its interaction with RBC was investigated. SA induced S1P release and reached a plateau at 10 mg/ml or 150 μM with an EC_{50} of 30 μM, whereas ovalbumin was inactive (Fig. 2A). Incubation of RBC enriched with S1P yielded more S1P in SA-containing medium than control RBC. Recurrent 1 h incubations of control and S1P-loaded RBC preparations with the same SA-containing medium showed maximal release of S1P after the third round of 1 h incubations in both RBC preparations (Fig. 2B). This result led us to the assumption that the ratio of RBC-bound cellular and extracellularly released S1P is balanced, and that incorporation and release of S1P is initiated in case of an imbalance. In fact, when the synthetic S1P analog C17-S1P was added to RBC incubated with SA, it was partially taken up by cells (Fig. 2C). In the absence of SA all exogenously added S1P and

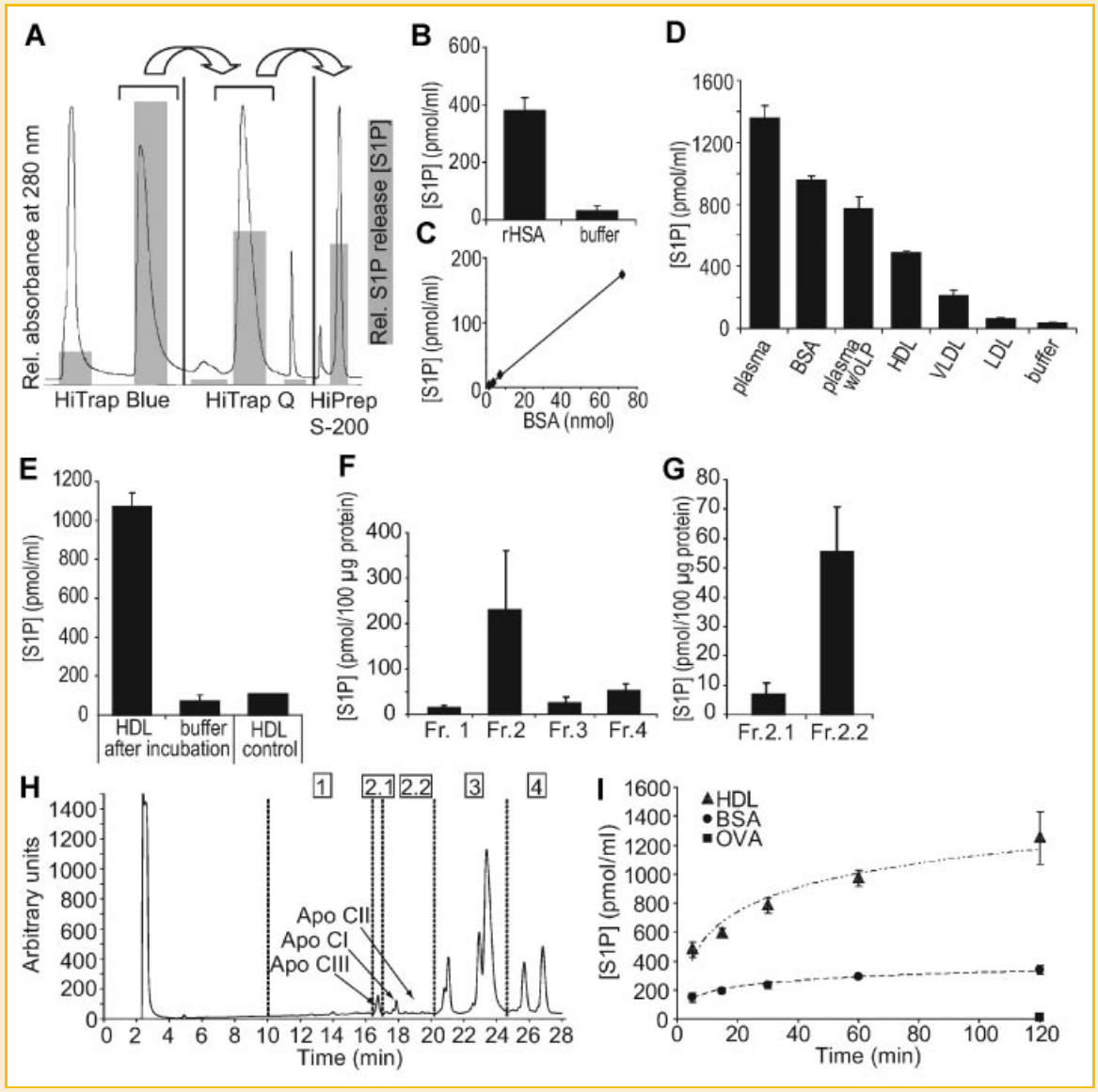

Fig. 1. Identification of plasma proteins inducing S1P release from RBC. A: FPLC chromatograms with activity profiles for S1P release of correspondent fractions from human plasma. From left: HiTrap™ Blue column loaded with 250 µl of human plasma; the next columns (HiTrap™ Q and HiPrep™ Sephacryl S-200) were subsequently loaded with the protein fraction of the previous separation showing the highest activity in the S1P release test (grey bars). B–G: Incubations and subsequent LC/MS/MS analysis of S1P-preloaded RBC (8×10^8 cells) at 37°C for 2 h with (B) 10 mg recombinant human SA (rHSA), (C) increasing amounts of BSA, (D) 200 µl of plasma, 50 mg/ml BSA, lipoprotein-free plasma (plasma w/o LP), high density lipoproteins (HDL), very low density lipoproteins (VLDL, low density lipoproteins (LDL), and buffer, (E) HDL purified from 200 µl plasma, and (F,G). HDL protein fractions. H: Representative analytical HPLC chromatogram of HDL protein fractions isolated from healthy patients (N = 6). I: S1P release after incubation of RBC (8×10^8 cells) with 1 mg HDL and 1 mg BSA. Shown are means ± SD of at least two independent experiments.

C17-S1P was taken up by RBC (data not shown). This result points to an equilibrium between RBC-associated S1P and extracellular S1P. Both SA and HDL are known to be highly associated with S1P [Murata et al., 2000], suggesting that their binding capacity for S1P may be required for inducing S1P release from RBC.

To test this hypothesis, RBC were incubated with Sphingomab™, a high affinity monoclonal antibody for S1P [Visentin et al., 2006]. Sphingomab™ was indeed able to extract S1P from RBC at a 1:1 molar ratio, which was not detected in control incubations without added antibody (Fig. 2D). The capacity to bind S1P is, therefore, not only required, but also sufficient to induce S1P release from RBC. To allow antibody binding, S1P must be accessible to the antibody, which requires its localization in the outer RBC membrane. Confocal microscopy of RBC incubated with 60 µM Sph-BODIPY demonstrated its presence in RBC membranes (Fig. 2E). The BODIPY moiety was introduced at position 13 of the alkenyl chain (Fig. 2F). LC/MS/MS analysis of RBC incubated for 20 h with Sph-BODIPY proved that most of the added fluorescently labeled Sph was phosphorylated to S1P-BODIPY (Fig. 2G). Release of S1P was not affected by the phosphate analog and inhibitor of ABC transporters vanadate (Fig. 2H). Vanadate was shown before to enter RBC [Cantley et al., 1978], and RBC lysis in hypotonic buffer was significantly less

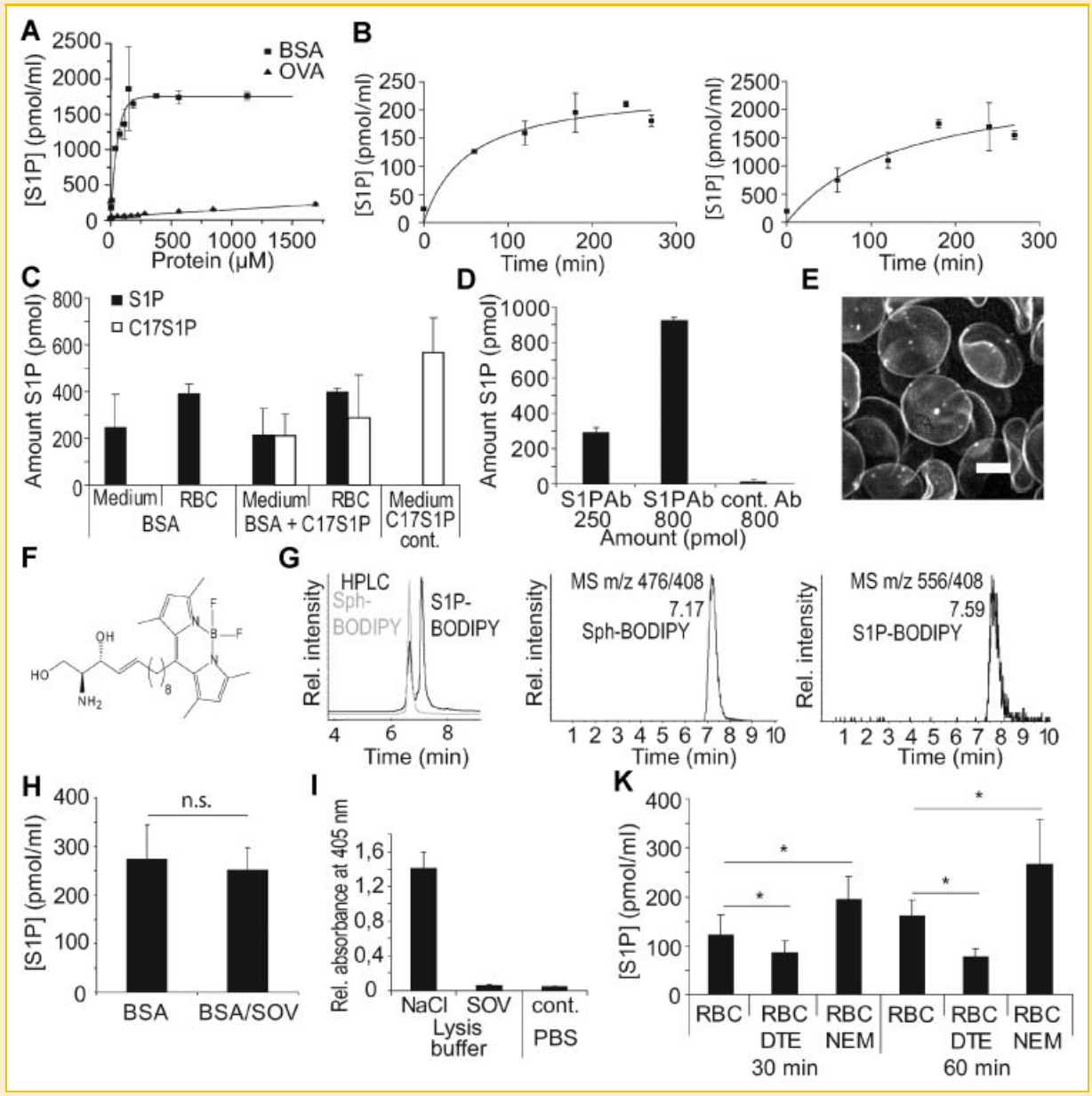

Fig. 2. Kinetics of S1P release, anti-S1P-antibody-induced S1P release, and cellular localization of S1P-BODIPY in RBC. A: Incubation of S1P-preloaded RBC (8×10^8 cells) with increasing concentrations of BSA and ovalbumin (OVA) at 37°C for 2 h. B: RBC (8×10^8 cells) unloaded (left) and S1P-preloaded (right) were incubated with 200 μl of 50 mg/ml BSA for 4.5 h at 37°C. RBC were replaced every hour with fresh RBC. C: RBC (8×10^8 cells) were incubated with 200 μl of 25 mg/ml BSA for 2 h at 37°C with and without addition of 600 pmol C17-S1P. D: Incubation of S1P-preloaded RBC (8×10^8 cells) with 250 and 800 pmol of the anti-S1P antibody Sphingomab™ and 800 pmol of isotype control antibody at 37°C for 2 h. E: RBC were analyzed by confocal laser scanning microscopy after incubation with 60 μM sphingosine-BODIPY (scale bar = 4 μm). F: Chemical structure of Sph-BODIPY. G: Phosphorylation of Sph-BODIPY demonstrated by HPLC and LC/MS/MS, monitoring the Sph-BODIPY specific mass transfer of 476.0/408.2, and the S1P-BODIPY specific mass transfer of 556.0/242.2. H: Incubation of RBC (8×10^8 cells, not preloaded with S1P) with 10 mg BSA in the presence or absence of 1 mM sodium orthovanadate (SOV) for 2 h at 37°C. I: Incubation of RBC (8×10^7 cells) with RBC lysis buffer in presence or absence of 1 mM SOV. K: Inhibitory effect of DTE and enhancing effect of NEM on S1P release from RBC. Shown are means ± SD of at least two independent experiments.

efficient in the presence of 1 mM vanadate compared to 1 mM NaCl as control, demonstrating potent inhibition of ATP-dependent ion pumps in RBC (Fig. 2I). Incubation of RBC with the scramblase inhibitor DTE, however, significantly decreased S1P release during 30 and 60 min incubations. The scramblase enhancer NEM equivalently enhanced S1P release from RBC (Fig. 2K). Therefore, the scramblase-dependent transbilayer movement of S1P in RBC membranes was important for S1P release rather than ATP-dependent ABC transporters.

ANALYSIS OF S1P IN BLOOD OF ANEMIC PATIENTS AND SIGNALING CAPACITY OF S1P IN PLASMA

In order to evaluate the significance of RBC for S1P release in plasma, we investigated blood samples from anemic patients for

their S1P content in blood cells and plasma. The control group consisted of six male and five female volunteers with hemoglobin (HGB) values of 16.1 ± 1 and 13.8 ± 1 g/dl, respectively. No significant gender-specific difference in S1P levels of plasma and RBC was observed. Anemic blood samples were drawn from eight male and 10 female acute myeloid leukemia patients with hemoglobin (HGB) values of 8.7 ± 1.9 and 9.4 ± 2.2 g/dl, respectively. Again, there were no significant gender-specific differences in S1P levels of plasma and RBC. Comparison of blood cells from these 11 control volunteers and 18 anemic patients yielded no significant differences in S1P (Fig. 3A). S1P levels were $2{,}313 \pm 522$ and $2{,}528 \pm 1{,}815$ pmol/10^{10} cells from control volunteers and anemic patients, respectively. Significantly different, however, were the plasma S1P levels, with 243 ± 68 and 173 ± 57 pmol/ml from control volunteers and anemic patients, respectively (Fig. 3B). The signaling capacity of S1P in plasma and S1P bound to SA was tested in calcium flux experiments using $S1P_1$ receptor expressing rat hepatoma HTC_4 cells. SA-bound S1P was equally potent as unbound S1P in $S1P_1$ receptor activation (Fig. 3C), and plasma stimulated the $S1P_1$ receptor to a comparable extent to its endogenous amount of S1P (Fig. 3D). The slightly higher signals observed in the presence of SA and plasma resulted from higher background signals in the presence of these substances (Fig. 3C,D). These results suggest that SA and HDL have no significant influence on the potency of extracellular S1P to stimulate S1P receptors.

CELLULAR EXCHANGE OF S1P

Besides the role of RBC for the maintenance of plasma S1P concentrations, we also tested their potential contribution to cellular S1P in tissue cells. When RBC were loaded with fluorescently labeled S1P-FITC and incubated with unlabeled RBC, S1P-FITC was transferred to unlabeled RBC within minutes (Fig. 4A). The FITC moiety was introduced at position 18 of the alkenyl chain of Ω-amino sphingosine (Fig. 4B). The presence of SA prevented this intercellular exchange, although S1P-FITC still faded from loaded RBC, most likely by binding to the added SA (Fig. 4C). Ten mg/ml BSA was sufficient to completely block intercellular exchange of S1P-FITC, and 1 mg/ml BSA had an intermediate effect (Fig. 4D). Similar effects were observed in co-culture experiments with HUVEC. S1P-FITC was transferred from RBC to HUVEC, whereas SA and HDL completely prevented this process (Fig. 4E,F). No dephosphorylation of S1P-FITC was observed in HUVEC cultures after 2 h, and very little conversion to Sph-FITC was seen after overnight incubation, as determined by HPLC (Fig. 4G). The same

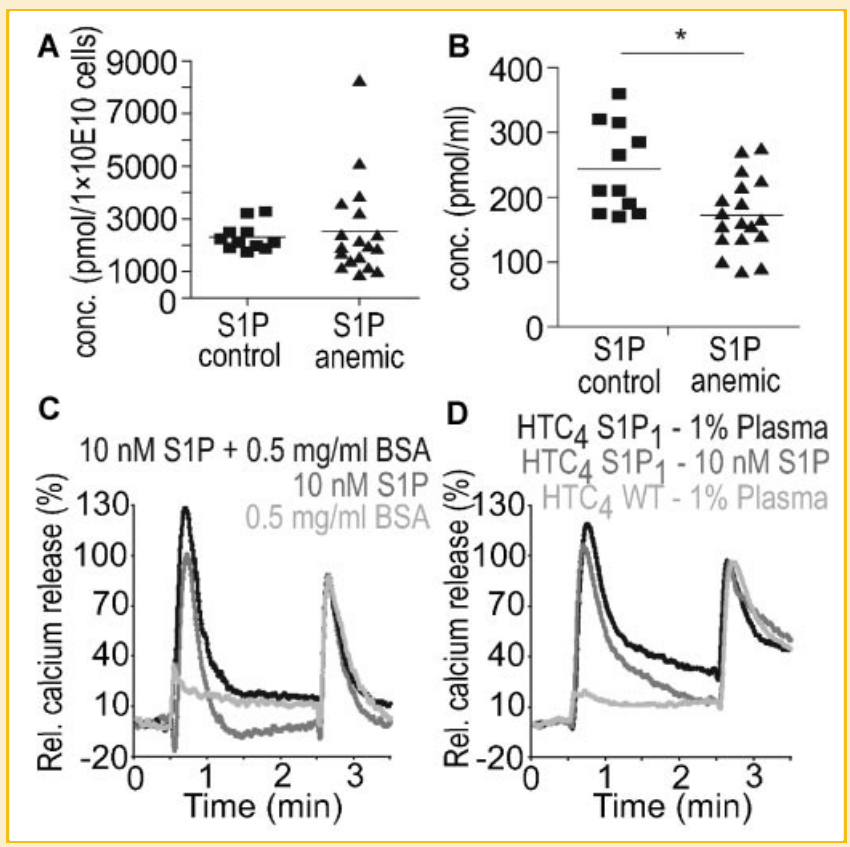

Fig. 3. S1P levels of anemic patients and S1P-receptor activation in vitro. A,B: S1P concentrations of (A) blood cells and (B) plasma from anemic patients and healthy volunteers. C: Intracellular calcium release of HTC_4 cells expressing $S1P_1$. Cells were stimulated after 0.5 min with 0.5 mg/ml BSA (light grey), 10 nM S1P (grey) or a combination of both (black). Stimulation with 10 μM ATP after 2.5 min was used for normalization (=100%). D: Intracellular calcium flux of HTC_4 control cells (HTC_4 WT) stimulated after 0.5 min with 1% C57BL/6 mouse plasma (light grey), and HTC_4 cells expressing $S1P_1$ (HTC_4 $S1P_1$) stimulated after 0.5 min with 10 nM S1P (grey), or 1% C57BL/6 mouse plasma (black). Cells were stimulated after 2.5 min with 10 μM ATP, and signals were normalized to 100%. Shown is one of three similar experiments.

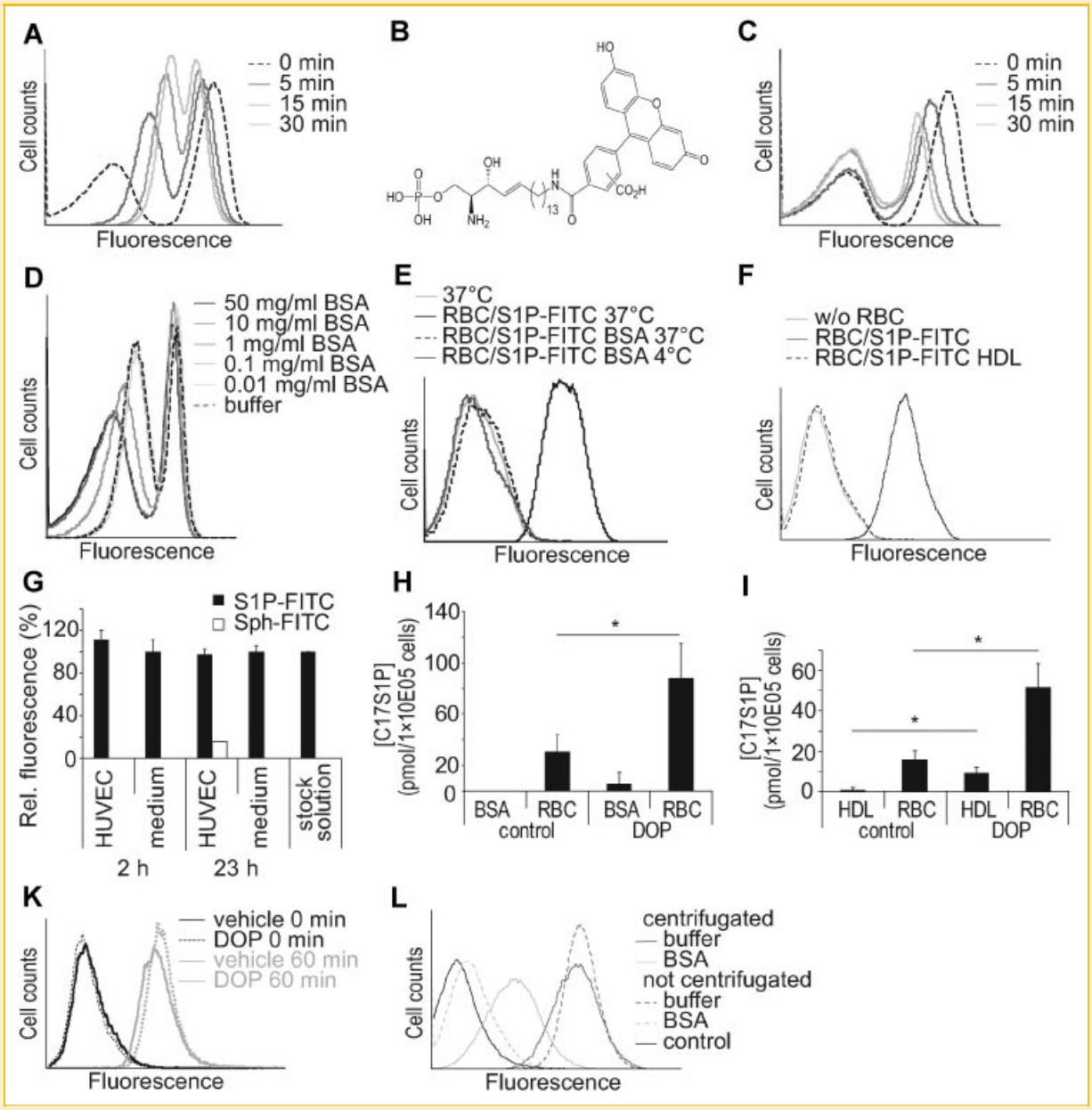

Fig. 4. Cellular exchange of S1P. A: RBC (8×10^6 cells) preloaded with 80 pmol S1P-FITC were coincubated with unloaded RBC. B: Chemical structure of S1P-FITC. C: RBC (8×10^6 cells) preloaded with 80 pmol S1P-FITC were coincubated with unloaded RBC in presence of 300 μg BSA. D: Coincubation of RBC (8×10^6 cells) loaded with 80 pmol S1P-FITC and unloaded RBC in presence of increasing BSA concentrations for 1 h at 37°C. E,F: Coincubation of HUVEC (1×10^5 cells) with 250 pmol S1P-FITC-loaded RBC (2×10^8 cells) in presence or absence of (E). 25 mg/ml BSA or (F). 5 mg/ml HDL for 2 h at 37°C. G: HUVEC (1×10^5 cells) were incubated with 0.25 μM S1P-FITC in FBS-free EGM-2 at 37°C and 5% CO_2 for 2 and 23 h. Cell-free incubations (medium) and the original S1P-FITC stock solution are shown as controls. The relative amounts of S1P-FITC and Sph-FITC in the supernatant were measured by HPLC. H: Coincubation of HUVEC (1×10^5 cells) with 2 nmol C17-S1P-loaded RBC (2×10^8 cells) and 2.5 mg BSA (preloaded with C17-S1P via 1 h incubation with C17-S1P-preloaded RBC) in the presence or absence of 0.5 mM DOP. I: Coincubation of HUVEC (1×10^5 cells) with 2 nmol C17-S1P-loaded RBC (2×10^8 cells) and 0.25 mg HDL (preloaded with C17-S1P via 1 h incubation with C17-S1P-preloaded RBC) in the presence or absence of 0.5 mM DOP. K: DOP-treated (DOP) and untreated (vehicle) HUVEC (2×10^5 cells) not incubated (0 min) and incubated for 60 min with 1 μM S1P-FITC. L: S1P-FITC uptake via forced cell–cell contact. HUVEC (4×10^5 cells) were incubated with RBC (8×10^7 cells), preloaded with 200 pmol S1P-FITC, for 30 min at 37°C in the presence or absence of 25 mg/ml BSA with and without constant centrifugation at 500*g*. Shown is one of two to five independent experiments.

result was achieved with C17-S1P. HUVEC co-cultured with C17-S1P-loaded RBC incorporated significant amounts of C17-S1P, whereas HUVEC cultured with C17-S1P-loaded SA did not (Fig. 4H). The addition of HDL preparations to HUVEC also prevented the incorporation of C17-S1P (Fig. 4I). Inhibition of SGPL1 by the vitamin B_6 antagonist DOP increased the amount of incorporated cellular C17-S1P by 2.5-fold. Uptake of SGPL1-resistant S1P-FITC was not altered by DOP treatment (Fig. 4K), and DOP treatment did not change the fact that SA prevented uptake of C17-S1P (Fig. 4H). The blocking effect of SA for cellular incorporation of

RBC-associated S1P-FITC was circumvented by tight cell–cell contact between RBC and HUVEC during centrifugation (Fig. 4L). Those conditions, which were to resemble close contacts of RBC with vascular EC in capillaries, allowed cellular S1P transfer even in the presence of SA. RBC-associated S1P is, therefore, transferred to EC during tight RBC-EC contact and in the absence of BSA, and subsequently metabolized by the SGPL1 in EC.

CELLULAR LOCALIZATION OF S1P

RBC store S1P predominantly in their cell membrane (Fig. 2E). Confocal microscopy of HUVEC co-cultured with S1P-FITC-loaded RBC revealed that RBC-associated S1P-FITC was incorporated by HUVEC. It was predominantly located in the perinuclear space of cells and not in the cell membrane (Fig. 5A). S1P-FITC loaded to BSA did not result in cellular uptake, but remained outside the cells (Fig. 5B). Removal of BSA-bound S1P-FITC from cells after 3 h incubation showed no indication of S1P-FITC incorporation, but clearly reduced the amount of S1P-FITC in the medium (Fig. 5C). Receptor-available SA-bound S1P-FITC is therefore not incorporated by HUVEC, whereas RBC-associated S1P-FITC is taken up by cells during co-culture in the absence of SA and stored intracellularly in the perinuclear space. Although S1P-FITC principally behaved similar to unlabeled S1P in previous experiments, the observed distribution of S1P-FITC may not be identical to that of native S1P.

DISCUSSION

SA and HDL are major components of blood plasma [Tirumalai et al., 2003]. Both molecules are associated with S1P, and S1P bound to SA and HDL is able to activate S1P receptors [Goetzl and An, 1998; Nofer et al., 2004]. SA- and HDL-associated S1P can, therefore, be considered as being "bioactive" in that these bound forms are active signaling molecules. SA and HDL not only bind S1P, they also trigger the release of S1P from RBC (Figs. 1 and 2). It has been suggested that HDL-associated S1P fulfils a unique function in activating Akt and eNOS via $S1P_3$, presumably by co-activation of the scavenger receptor class B type 1 (SR-B1) [Nofer et al., 2004]. Association of S1P with either SA or HDL may, therefore, account for different signaling outcomes depending on its plasma protein association. The data presented also demonstrate that HDL extracts S1P from RBC in blood (Fig. 1), which at least contributes to its association with S1P and may complete its assembly.

Association of S1P with SA is thought to have stabilizing and signal enhancing functions [Goetzl and An, 1998]. S1P receptor activation, however, also works in the absence of SA, and the latter had no enhancing effect on S1P receptor stimulation in vitro (Fig. 3C). Additionally, SA did not protect S1P from degradation by HUVEC [Hänel et al., 2007]. Based on the presented data, binding of S1P to SA has a different function than previously suggested. It is required for S1P extraction from RBC and serves as an uncommitted carrier that does not interfere with its signaling properties or with its metabolism. Whereas RBC have protective qualities, SA makes S1P accessible for receptors and metabolizing enzymes.

In this study, we used fluorescently labeled S1P and Sph to trace and to visualize these lipids in cells (Figs. 2F and 4B). Labeled lipids may behave differently compared to their native relatives. In fact, S1P-FITC was much more stable than unlabeled S1P in medium, demonstrating a much lower susceptibility to metabolizing enzymes like phosphatases (Fig. 4G). Although differences in membrane integration and cellular localization between labeled and unlabeled derivatives cannot be fully excluded, all relevant experiments were done with both labeled and unlabeled forms and showed similar outcomes. Fluorescently labeled sphingolipids are therefore useful tools to study their distribution and cellular localization in combination with related control experiments using unlabeled derivatives.

It was found recently that S1P release from RBC is reliant on an ATP-dependent transporter [Kobayashi et al., 2009]. The authors also found that SA was required in all their experiments to obtain efficient S1P release, demonstrating the unique role of this S1P-binding molecule for S1P secretion. The mechanism presented here works independently from active transporters (Fig. 6). The phosphate analog and inhibitor of ABC transporters vanadate did not inhibit S1P release in our experimental setting (Fig. 2H), in contrast to the above-mentioned study using inside-out membrane vesicles. It is possible, however, that ATP-driven processes are indeed relevant for S1P to cross membranes, for example, by specific transporters or flippases, which may account for the different

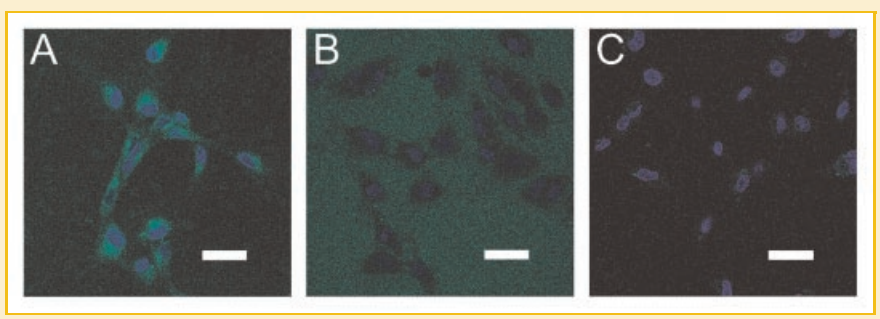

Fig. 5. Confocal microscopy of HUVEC. A,B: HUVEC (5×10^4 cells) were incubated for 3 h at 37°C with 15 μl of (A) RBC or (B) BSA preloaded with S1P-FITC to a final concentration of 1.5 μM. RBC were incubated in cell medium, whereas BSA was incubated in cell medium containing 25 mg/ml BSA. C: Cells shown in (B). were washed three times and analyzed for their cellular S1P-FITC content (scale bar = 40 μm). Shown is one of three independent experiments.

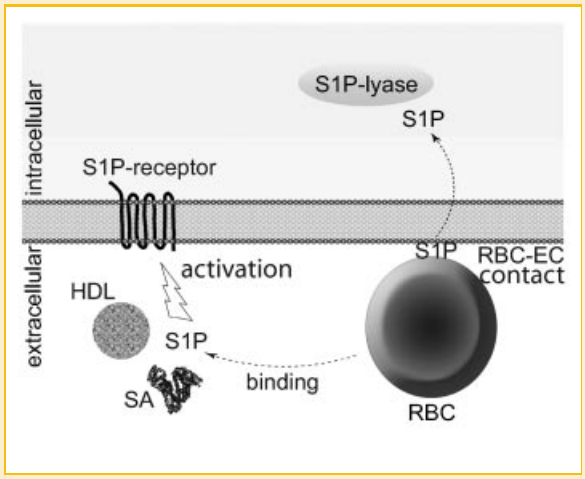

Fig. 6. Model for cellular uptake and effector functions of S1P in blood. Extracellular Sph is taken up and phosphorylated by RBC, which are able to accumulate high amounts of S1P without degradation. It is stored in the cell membrane of RBC, where it can be extracted by the S1P-binding molecules SA and HDL. S1P in plasma is bound to SA and HDL and can stimulate S1P receptors on the surface of endothelial cells and lymphocytes, modulating vascular permeability and lymphocyte circulation. S1P transferred from RBC via cell–cell contact enters cells and is accessible for cellular metabolizing enzymes such as SGPL1, resulting in further transfer, distribution, and degradation.

observations in inside-out membrane vesicles. The present data suggest that scrambling activity is important for efficient S1P release (Fig. 2K).

The effect of reduced RBC counts in anemic patients was a statistically significant reduction of S1P in plasma with unaltered S1P levels in blood cells (Fig. 3A,B). The total amount of S1P associated with RBC is, therefore, one critical determinant for the amount of S1P released in plasma. Vascular endothelial cells, which were proposed as an alternative source for S1P in blood [Venkataraman et al., 2008], were obviously not able to replenish plasma S1P levels, emphasizing the dominant role of RBC as the major source for S1P in blood plasma. The fact that no major differences were observed in cell-associated S1P levels indicates that RBC from anemic patients are not deficient in cellular S1P. Since the amount of released S1P from RBC can be up to 10-fold higher after ex vivo incubation compared with that found in freshly drawn blood [Hänel et al., 2007], it is likely that S1P release, metabolism, and distribution establish a steady-state condition with RBC serving as a reservoir for S1P with buffering capabilities.

We recently identified RBC as a major source for S1P in human blood [Hänel et al., 2007]. This observation was supported by inducible SK1/2-deficient mice [Pappu et al., 2007] and challenged by mouse studies that identified vascular endothelium as a contributor of plasma S1P [Venkataraman et al., 2008]. In the latter study, Venkataraman and colleagues did not find any significant differences in plasma S1P levels of normal and anemic mice. This result may reflect different regulatory mechanisms in mouse and man, but may also have experimental reasons. The cellular content of S1P in RBC was not determined in this study, and it is unclear whether or not RBC from anemic mice had less cellular S1P than normal controls. RBC release S1P into plasma even in isolated blood [Hänel et al., 2007], and immediate cooling of blood samples is essential for comparable results. The reason for this S1P-releasing activity is the presence of SA and HDL in plasma (Fig. 1). While we were focusing on anemic patients (Fig. 3A, B), a different study was able to correlate plasma S1P with RBC parameters even in different healthy subjects, showing significantly higher S1P concentrations in men than in women [Ohkawa et al., 2008]. Our groups of patients and healthy volunteers were probably too small to see significant differences between men and women, but clearly identified a difference between healthy controls and anemic patients (Fig. 3B).

RBC act as donors for extracellular S1P with SA and HDL as endogenous acceptor molecules presenting it to receptors and metabolizing enzymes (Figs. 1–3). This extracellular S1P is rapidly degraded by tissue cells independently from SGPL1 expression [Peest et al., 2008]. Furthermore, RBC also contribute to the cellular S1P pool of EC and tissue cells via direct cell–cell contact and subsequent transcellular transportation across EC and tissue cells (Figs. 4 and 5). The latter predominantly occurs during tight RBC-EC contact or in the absence of binding molecules such as SA and HDL, resembling environmental conditions in capillaries and interstitium. The contribution to cellular S1P is SGPL1 accessible and accumulates after inhibition of the SGPL1 by DOP (Fig. 4H), in contrast to extracellular S1P, which is not affected by the SGPL1 [Peest et al., 2008]. Both S1P distribution pathways are, therefore, clearly distinguishable by their different localization and degradation pathways.

Mechanistically, there is strong evidence that the binding capabilities of HDL and SA are critical for S1P release from RBC. Antibodies against S1P are also able to extract S1P from RBC (Fig. 2D), although in contrast to HDL and SA, anti-S1P antibodies greatly annihilate S1P-induced cellular responses [Sabbadini, 2006; Visentin et al., 2006; Caballero et al., 2008; Swaney et al., 2008]. S1P is mainly stored in the cell membrane of RBC (Fig. 2E), making it accessible for solubilized molecules in their microenvironment. Therefore, we propose a model in which Sph is incorporated from an unknown source and phosphorylated by SK1 and SK2 in RBC. Both kinases were shown to be active in blood [Billich et al., 2003]. S1P is stored in the plasma membrane of RBC, where it is protected from degradation, but accessible for binding molecules (Fig. 6). SA and HDL bind S1P in plasma and extract it from RBC. In its bound form, S1P stimulates S1P receptors and serves as substrate for extracellular and cell surface-bound S1P-metabolizing enzymes. On the other hand, the unique membrane location of S1P in RBC enables cellular uptake via tight RBC-cell contact, and cellular S1P is distributed to other tissue cells via transcellular transportation (Fig. 6). RBC thus also contribute to cellular S1P in EC and tissue cells, which serves as substrate for SGPL1. The amount of RBC-associated S1P and the concentration of extracellular S1P determine the extent of S1P release. Metabolism, distribution, and release constitute a steady-state condition that determines the actual S1P concentration in tissues and blood plasma. Modulating any of these parameters may ultimately change S1P levels in blood and tissues and could serve as potential therapeutic

intervention for treatment of cardiovascular as well as immune diseases and cancer.

ACKNOWLEDGMENTS

The authors thank Anika Münk for her excellent technical assistance and Dr. Roger Sabbadini for the generous gift of the anti-S1P antibody Sphingomab™. This work was supported by the German Research Foundation (DFG), grants GR-1943/1-4 of the Emmy Noether Program, GR-1943/2-1 of the priority program 1267, and GR-1943/3-1, and by the National Institutes of Health (NIH), grant HL-083187.

REFERENCES

Allende ML, Dreier JL, Mandala S, Proia RL. 2004. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. J Biol Chem 279:15396–15401.

Andréani P, Gräler MH. 2006. Comparative quantification of sphingolipids and analogs in biological samples by high-performance liquid chromatography after chloroform extraction. Anal Biochem 358:239–246.

Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N, Baumruker T. 2003. Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. J Biol Chem 278:47408–47415.

Burstein M, Scholnick HR, Morfin R. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J Lipid Res 11:583–595.

Caballero S, Swaney J, Moreno K, Afzal A, Kielczewski J, Stoller G, Cavalli A, Garland W, Hansen G, Sabbadini R, Grant MB. 2008. Anti-sphingosine-1-phosphate monoclonal antibodies inhibit angiogenesis and sub-retinal fibrosis in a murine model of laser-induced choroidal neovascularization. Exp Eye Res 88:367–377.

Cantley LC Jr, Resh MD, Guidotti G. 1978. Vanadate inhibits the red cell (Na+, K+) ATPase from the cytoplasmic side. Nature 272:552–554.

Chandrasekhar S, Babu BN, Reddy NR, Chandraiah L. 2005. Towards the synthesis of pyloricidins: Synthesis of (2*S*,3*R*,4*R*,5*S*)-5-(*tert*-butyloxycarbonyl)amino-2,3,4,6-tetrahydroxyhexanoyl-ß-D-phenylalanine methyl ester. Arkivoc ix:40–47.

Goetzl EJ, An S. 1998. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. FASEB J 12:1589–1598.

Hagen N, Van Veldhoven PP, Proia RL, Park H, Merrill AH Jr, van Echten-Deckert G. 2009. Subcellular origin of sphingosine 1-phosphate is essential for its toxic effect in lyase-deficient neurons. J Biol Chem 284:11346–11353.

Hänel P, Andréani P, Gräler MH. 2007. Erythrocytes store and release sphingosine 1-phosphate in blood. FASEB J 21:1202–1209.

Hara A, Radin NS. 1978. Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem 90:420–426.

Havel RJ, Eder HA, Bragdon JH. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 34:1345–1353.

Kamp D, Sieberg T, Haest CW. 2001. Inhibition and stimulation of phospholipid scrambling activity. Consequences for lipid asymmetry, echinocytosis, and microvesiculation of erythrocytes. Biochemistry 40:9438–9446.

Kobayashi N, Yamaguchi A, Nishi T. 2009. Characterization of the ATP-dependent sphingosine-1-phosphate transporter in rat erythrocytes. J Biol Chem 284:21192–21200.

Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL. 2000. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J Clin Invest 106:951–961.

Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG. 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 427:355–360.

Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. 2005. Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol 25:11113–11121.

Mizugishi K, Li C, Olivera A, Bielawski J, Bielawska A, Deng CX, Proia RL. 2007. Maternal disturbance in activated sphingolipid metabolism causes pregnancy loss in mice. J Clin Invest 117:2993–3006.

Murata N, Sato K, Kon J, Tomura H, Yanagita M, Kuwabara A, Ui M, Okajima F. 2000. Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. Biochem J 352 Pt 3:809–815.

Nofer JR, van der Giet M, Tolle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J, Levkau B. 2004. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. J Clin Invest 113:569–581.

Ohkawa R, Nakamura K, Okubo S, Hosogaya S, Ozaki Y, Tozuka M, Osima N, Yokota H, Ikeda H, Yatomi Y. 2008. Plasma sphingosine-1-phosphate measurement in healthy subjects: Close correlation with red blood cell parameters. Ann Clin Biochem 45:356–363.

Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, Camerer E, Zheng YW, Huang Y, Cyster JG, Coughlin SR. 2007. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science 316:295–298.

Peest U, Sensken SC, Andréani P, Hänel P, Van Veldhoven PP, Gräler MH. 2008. S1P-lyase independent clearance of extracellular sphingosine 1-phosphate after dephosphorylation and cellular uptake. J Cell Biochem 104:756–772.

Peters C, Billich A, Ghobrial M, Hogenauer K, Ullrich T, Nussbaumer P. 2007. Synthesis of borondipyrromethene (BODIPY)-labeled sphingosine derivatives by cross-metathesis reaction. J Org Chem 72:1842–1845.

Sabbadini RA. 2006. Targeting sphingosine-1-phosphate for cancer therapy. Br J Cancer 95:1131–1135.

Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG. 2005. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science 309:1735–1739.

Sudhakar N, Kumar AR, Prabhakar A, Jagadeesh B, Rao BV. 2005. The first synthesis of the anhydrophytosphingosine pachastrissamine (jaspine B) from Garner's aldehyde. Tetrahedron Lett 46:325–327.

Swaney JS, Moreno KM, Gentile AM, Sabbadini RA, Stoller GL. 2008. Sphingosine-1-phosphate (S1P) is a novel fibrotic mediator in the eye. Exp Eye Res 87:367–375.

Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. 2003. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2:1096–1103.

Venkataraman K, Lee YM, Michaud J, Thangada S, Ai Y, Bonkovsky HL, Parikh NS, Habrukowich C, Hla T. 2008. Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circ Res 102:669–676.

Visentin B, Vekich JA, Sibbald BJ, Cavalli AL, Moreno KM, Matteo RG, Garland WA, Lu Y, Yu S, Hall HS, Kundra V, Mills GB, Sabbadini RA. 2006. Validation of an anti-sphingosine-1-phosphate antibody as a potential therapeutic in reducing growth, invasion, and angiogenesis in multiple tumor lineages. Cancer Cell 9:225–238.

Vogel P, Donoviel MS, Read R, Hansen GM, Hazlewood J, Anderson SJ, Sun W, Swaffield J, Oravecz T. 2009. Incomplete inhibition of sphingosine 1-phosphate lyase modulates immune system function yet prevents early lethality and non-lymphoid lesions. PLoS ONE 4:e4112.

Yatomi Y, Ruan F, Hakomori S, Igarashi Y. 1995. Sphingosine-1-phosphate: A platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood 86:193–202.

Yatomi Y, Igarashi Y, Yang L, Hisano N, Qi R, Asazuma N, Satoh K, Ozaki Y, Kume S. 1997. Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. J Biochem (Tokyo) 121:969–973.

Yatomi Y, Ozaki Y, Ohmori T, Igarashi Y. 2001. Sphingosine 1-phosphate: Synthesis and release. Prostaglandins 64:107–122.