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Sphingosine but not Sphingosine-1-phosphate Stimulates Suicidal Erythrocyte Death

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Key Words

Sphingosine • Sphingosine phosphate • Oxidative stress • Cell volume • Annexin • Eryptosis • Calcium • Phosphatidylserine

Abstract

Sphingosine kinase 1 phosphorylates sphingosine, which is converted to ceramide by ceramide synthetase. Ceramide triggers eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phosphatidylserine (PS) exposure at the erythrocyte surface. Erythrocytes lack sphingosine phosphate-degrading enzymes and thus store large quantities of sphingosine phosphate. The present study explored the influence of sphingosine and sphingosine phosphate on eryptosis. $[Ca^{2+}]_i$ was estimated from Fluo3 fluorescence, cell volume from forward scatter and PS exposure from annexin V-binding in FACS analysis. Sphingosine (0.1 – 10 μM) but not sphingosine-1- phosphate (0.1 – 10 μM) increased $[Ca^{2+}]_i$, decreased cell volume and increased PS-exposure. The observations disclose sphingosine, but not sphingosine-1-phosphate, as a strong inducer of eryptosis.

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Introduction

Sphingosine kinase 1 catalyzes the phosphorylation of sphingosine leading to formation of sphingosine-1-phosphate (S1P) [1-4]. Erythrocytes express low levels of sphingosine kinase but lack S1P-degrading enzymes, such as S1P lyase or S1P phosphohydrolase [5]. As a result, the conversion of sphingosine to sphingosine phosphate prevails [6] and erythrocytes store large amounts of S1P [1, 5]. S1P may bind to specific receptors expressed in a wide variety of tissues and regulate diverse functions including cardiovascular performance and immunity [1, 2, 7-12]. Sphingosine may be converted by ceramide synthetase to ceramide [1], which is known to trigger suicidal death of a wide variety of cells [13] including erythrocytes [14-16]. Accordingly, deranged sphingosine metabolism participates in the pathophysiology of a variety of diseases [17, 18]. Sphingosine has been shown to enhance the permeability of model membranes [19], but little is known about the role of sphingosine, sphingosine phosphate and sphingosine kinase in erythrocyte death or survival.

Suicidal erythrocyte death or eryptosis [20] is characterized by cell shrinkage, cell membrane blebbing and cell membrane scrambling resulting in

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exposure of phosphatidylserine at the erythrocyte surface [21-24]. The cell membrane scrambling could be stimulated by cytosolic Ca^{2+} [22-24], which enters into erythrocytes through Ca^{2+}-permeable cation channels [25]. Ca^{2+} further activates Ca^{2+}-sensitive K^+ channels [26, 27], leading to exit of KCl with osmotically obliged water [21]. The cellular loss of KCl contributes to suicidal erythrocyte death [28]. The Ca^{2+}-permeable cation channels [25] and the cell membrane scrambling [29] are stimulated by oxidative stress. The Ca^{2+} sensitivity of erythrocyte cell membrane scrambling is enhanced by ceramide [30]. Eryptosis is triggered in several clinical disorders and by a wide variety of substances, effects due to increase of cytosolic Ca^{2+} activity and/or stimulation of ceramide formation [31-45].

The present study explored, whether sphingosine and the derivative sphingosine-1-phosphate are capable of inducing suicidal erythrocyte death.

Materials and Methods

Human erythrocytes

Leukocyte-depleted erythrocytes from concentrates provided by the blood bank of the University of Tübingen were used. The volunteers providing erythrocytes gave informed consent. The study has been approved by the Ethical Commission of the University of Tübingen.

Incubations and solutions

For the *in vitro* experiments on suicidal death of erythrocytes incubations were carried out at 37°C in Ringer solution (in mM: 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 $CaCl_2$) at a hematocrit of 0.4% in a total volume of 200 µl. D-sphingosine and sphingosine-1-phosphate (both Sigma, Schnelldorf, Germany) were used at a concentration of 0.1, 1 and 10 µM. Where indicated K6PC-5 (N-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide) (NeoPharm, DaeJeon, South Korea) was used at a concentration of 1 and 10 µM.

Phosphatidylserine exposure and forward scatter

FACS analysis was performed as described earlier [46]. After incubation, erythrocytes were washed once in Ringer solution containing 5 mM $CaCl_2$. The cells were then stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analyzed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo-3/AM [47]. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Whole-cell patch clamp

Measurements were done at room temperature. Borosilicate glass patch pipettes were pulled with a horizontal DMZ puller (Zeitz). Pipettes with high resistance from 15 to 18 MOhm were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech) and by using Pulse software (HEKA). For current measurements erythrocytes were held at a holding potential (V_h) of -10 mV, and 200 ms pulses from -100 to +100 mV were applied in increments of 20 mV. The original whole-cell current traces are depicted without filtering (acquisition frequency of 3 kHz). The currents were analyzed by averaging the current values measured between 90 and 190 ms of each square pulse (I-V relationship). The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The offset potentials between both electrodes were zeroed before sealing. The liquid junction potentials between bath and pipette solution and between the bath solutions and the salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch. Data were corrected for liquid junction potentials. The pipette solution contained (in mM): 125 Na-gluconate, 10 NaCl, 1 MgATP, 1 EGTA, and 10 HEPES/NaOH (pH 7.4). The cells were suspended in standard NaCl bath solution containing (in mM): 145 NaCl, 5 KCl, 10 HEPES/NaOH, 5 D-glucose, 2 $MgCl_2$, 1 $CaCl_2$ (pH 7.4). Sphingosine and sphingosine phosphate (10 µM) were added acutely. Whole-cell recordings were obtained after 1-2 minutes of perfusion with 10 µM sphingosine/ sphingosine phosphate dissolved in NaCl bath solution. Cation conductance was calculated for the inward currents by linear regression.

Statistics

Data are expressed as arithmetic means ± SEM and statistical analysis was made by ANOVA, as appropriate, $p<0.05$ was considered as statistically significant.

Results

To determine, whether sphingosine modifies the cytosolic Ca^{2+} concentration, the Fluo3 fluorescence was determined in FACS analysis. As illustrated in Fig. 1, the treatment of human erythrocytes with sphingosine (0.1 – 10 µM) was followed within 48 hours by a marked in-

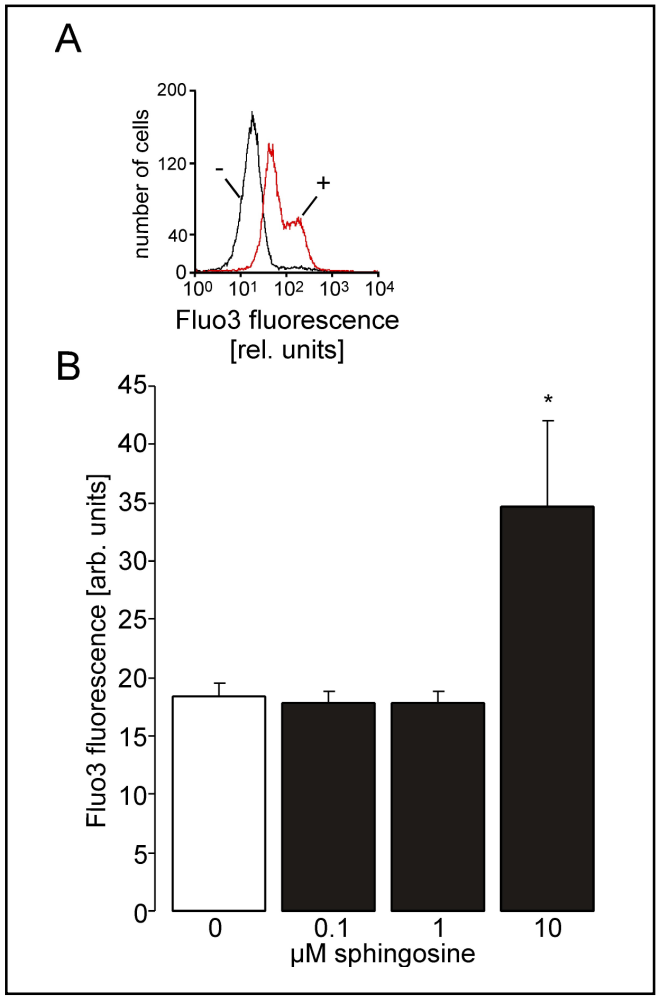


Fig. 1. Effect of sphingosine on the cytosolic Ca^{2+} concentration in human erythrocytes A. Histogram of Fluo3 fluorescence as an indicator of cytosolic Ca^{2+}-concentration in a representative experiment of erythrocytes exposed for 48 hours to Ringer without (-, black line) or with (+, red line) 10 µM sphingosine. B. Arithmetic means ± SEM (n = 12) of Fluo3 fluorescence of erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) 0.1 µM - 10 µM sphingosine. * indicates significant ($p<0.05$) difference from Ringer alone (ANOVA).

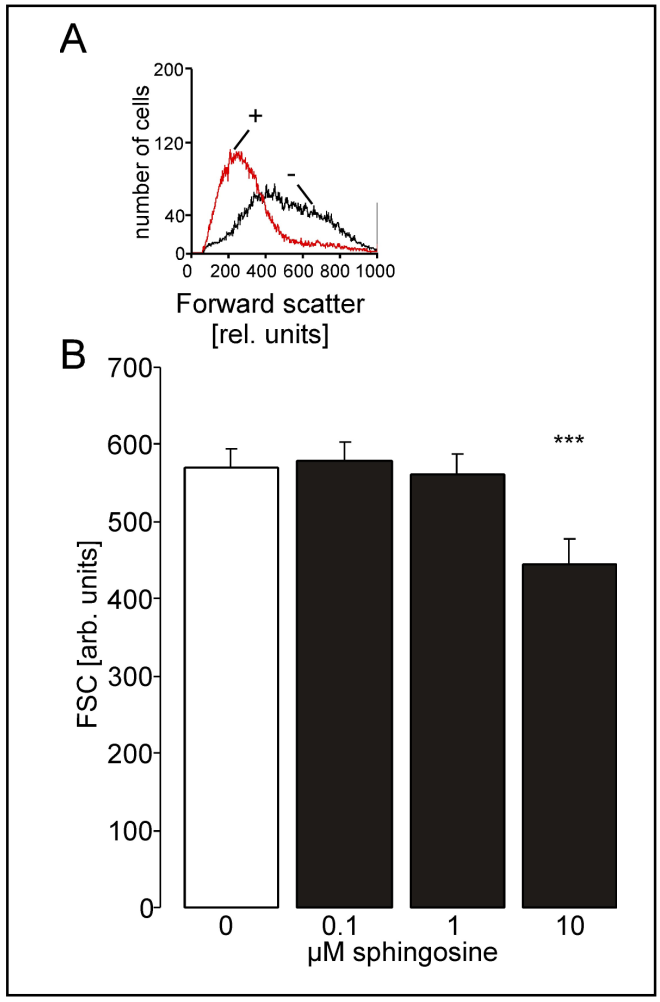


Fig. 2. Effect of sphingosine on forward scatter of human erythrocytes A. Histogram of forward scatter reflecting cell volume in a representative experiment of erythrocytes exposed for 48 hours to Ringer without (-, black line) or with (+, red line) 10 µM sphingosine. B. Arithmetic means ± SEM (n = 12) of the forward scatter of erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) 0.1 µM - 10 µM sphingosine. *** indicates significant ($p<0.001$) difference from Ringer alone (ANOVA).

crease in Fluo3 fluorescence pointing to an increase in the cytosolic Ca^{2+} concentration.

An increase in the cytosolic Ca^{2+} concentration is expected to stimulate Ca^{2+}-sensitive K^+ channels, thus triggering exit of K^+, hyperpolarisation, Cl^- exit, osmotic water loss and cell shrinkage [21]. To estimate alterations of cell volume, the signal of the foward scatter was determined in FACS analysis. As shown in Fig. 2, sphingosine (0.1 – 10 µM) significantly decreased within 48 hours the signal of the forward scatter indicating that sphingosine treatment was followed by cell shrinkage.

An increase in the cytosolic Ca^{2+} concentration is further known to trigger erythrocyte cell membrane scrambling resulting in exposure of phosphatidylserine at the erythrocyte surface [21, 23, 24]. Annexin V-binding was employed to estimate phosphatidylserine at the erythrocyte surface. The application of sphingosine (0.1 – 10 µM) was followed within 48 hours by a significant increase in the percentage of annexin V-binding

erythrocytes pointing to erythrocyte cell membrane scrambling (Fig. 3).

A further series of experiments was performed to investigate whether the effects of sphingosine on the erythrocyte Ca^{2+} concentration, on cell volume and on the externalization of phosphatidylserine were mimicked by the sphingosine derivative sphingosine-1-phosphate. Sphingosine-1-phosphate failed to significantly modify the intracellular Ca^{2+} concentration and erythrocyte phosphatidylserine exposure. The percentage of annexin V-binding was 3.9 ± 0.4, 4.0 ± 0.5, 4.4 ± 0.5 and 4.8 ± 0.6 (n = 9), respectively, for treatment with sphingosine-1-phosphate (0, 0.1, 1 and 10 µM, respectively). Fluo3 fluorescence on incubation with sphingosine-1-phosphate was 16.4 ± 0.6, 16.6 ± 1.0, 16.9 ± 1.1 and 17.1 ± 1.1 (n = 9), respectively, for treatment with sphingosine-1-phosphate (0, 0.1, 1 and 10 µM, respectively).

To investigate whether the sphingosine kinase activator K6PC-5 influences eryptosis induced by sphingosine, a further series of experiments was performed. The percentage of annexin V-binding was in untreated erythrocytes 0.8 ± 0.3 (n = 4), in erythrocytes treated with sphingosine (10 µM) 22.0 ± 4.7 (n = 4), and in erythrocytes treated with K6PC-5 (1 and 10 µM) 0.7 ± 0.2 and 0.8 ± 0.21 (n = 4). Addition of K6PC-5 (1 and 10 µM) did not significantly change the eryptosis induced by sphingosine (10 µM). The percentage of annexin V-binding following the addition of K6PC-5 (1 and 10 µM) was 21.8 ± 2.8 and 30.2 ± 6 respectively (n = 4).

Furthermore, whole-cell patch clamp experiments revealed a slight increase in cation conductance of erythrocytes following acute perfusion with 10 µM sphingosine from 75 ± 10 pS to 86 ± 23 pS (n=6). Acute application of 10 µM sphingosine-1-phosphate failed to increase cation conductance in human erythrocytes (68 ± 22 pS, n=4, in the absence and 64 ± 22 pS, n=4, in the presence of 10 µM sphingosine-1-phosphate).

The *in vitro* data suggests that sphingosine is a strong inducer of eryptosis whereas the closely related sphingosine-1-phosphate fails to stimulate eryptosis.

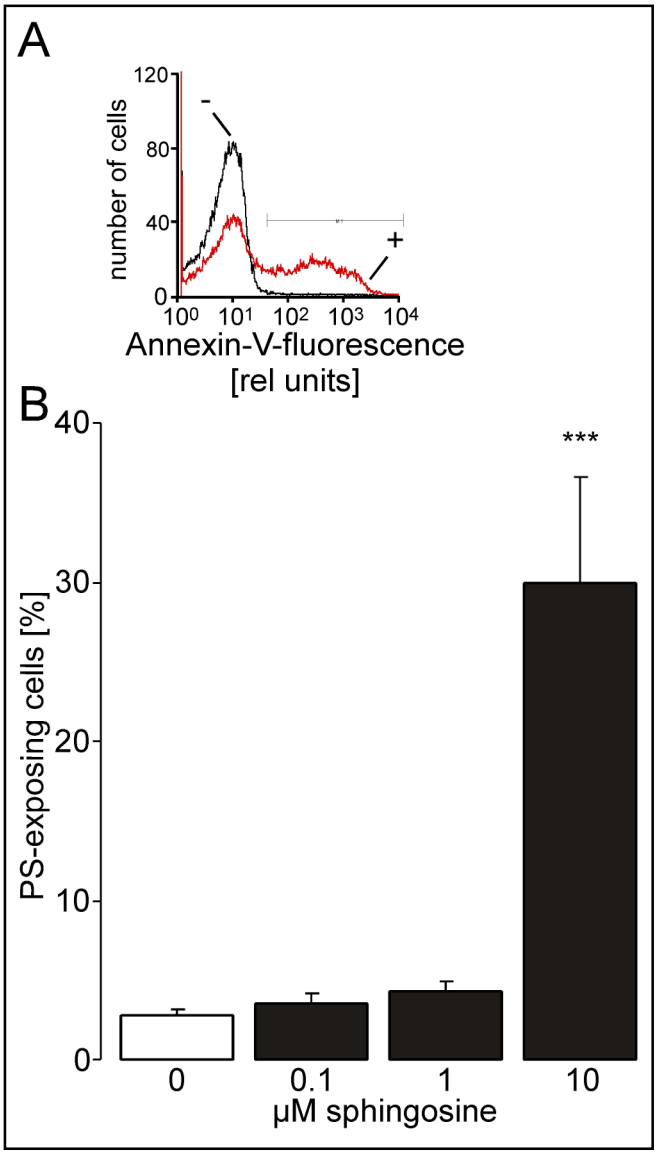


Fig. 3. Effect of sphingosine on annexin V-binding of human erythrocytes A. Histogram of annexin V-binding as an indicator of cell membrane scrambling in a representative experiment of erythrocytes exposed for 48 hours to Ringer without (-, black line) or with (+, red line)10 µM sphingosine. B. Arithmetic means ± SEM (n = 12) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) 0.1 µM - 10 µM sphingosine. *** indicates significant ($p<0.001$) difference from Ringer alone (ANOVA).

Discussion

The present observations disclose a novel function of sphingosine, i.e. the stimulation of suicidal erythrocyte death. Sphingosine increases the cytosolic Ca^{2+} activity, decreases forward scatter and leads to cell membrane scrambling with phosphatidylserine exposure at the cell surface, effects not shared by sphingosine-1-phosphate. It is therefore intriguing to speculate that conversion of sphingosine into sphingosine-1-phosphate by sphingosine kinase protects the cells from sphingosine-induced suicidal cell death.

Sphingolipids participate in the regulation of cell survival, proliferation, apoptosis, chemotherapeutic resistance, differentiation, endoplasmic reticulum stress response, autophagy, senescence, migration, adhesion and angiogenesis [17, 48-66]. Ceramide stimulates the death of a wide variety of cells including neurons [67-69], T-lymphocytes [70], hepatocytes [71], epithelial cells [72] and erythrocytes [30, 42, 44, 71, 73]. Sphingolipids participate in cancer [64, 74-79], inflammatory disease [52, 56, 80, 81] including lung inflammation and infection [82], cystic fibrosis [83], cardiovascular disease [84-86], Wilson´s disease [71], diabetes [87-90] and several neurological diseases [17, 18, 67, 91-94].

Sphingolipids modify cell function through G proteins [62, 64, 95], kinases [76], nitric oxide [63] and modification of gene expression [77]. In erythrocytes, nitric oxide is a powerful inhibitor of suicidal cell death [96].

Sphingosine kinase 1 has been shown to exert antiapoptotic activity in mesangial cells [97] and vascular smooth muscle cells [98]. On the other hand, sphingosine kinase 1 contributes to vascular injury in hyperglycemia [99]. Ceramide and sphingosine trigger apoptosis or cell growth arrest, whereas sphingosine 1-phosphate rather fosters cell proliferation, cell survival and angiogenesis [78, 100-102]. The immunosuppressive substance FTY720, which stimulates the sphingosine-1-phosphate (S1P) receptor induces, however, apoptosis of a variety of nucleated cells [11] and suicidal erythrocyte death [73].

Sphingosine induced eryptosis is expected to result in anemia, as phosphatidylserine-exposing erythrocytes are bound by the respective receptors of macrophages [103], which engulf phosphatidylserine-exposing cells [104]. Eryptotic cells are thus rapidly cleared from circulating blood, whereby they are mainly trapped in the spleen [105].

Besides its influence on erythrocyte survival, eryptosis may affect the microcirculation. Phosphatidylserine-exposing erythrocytes may bind to the vascular wall [106-110]. In addition, phosphatidylserine exposure stimulates the assembly of prothrombinase and tenase and thus favours the generation of thrombin and clotting [106, 111, 112]. Trapping of eryptotic erythrocytes has previously been observed in renal medulla following ischemia of the mouse kidney. Moreover, suicidal erythrocytes may contribute to vascular injury of metabolic syndrome [113].

In conclusion, in contrast to sphingosine phosphate, sphingosine stimulates Ca^{2+} entry into erythrocytes leading to cell shrinkage and cell membrane scrambling, key events in suicidal erythrocyte death.

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