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Original Paper

Stimulation of Erythrocyte Death by Phloretin

Rosi Bissinger[a] Salome Fischer[a] Kashif Jilani[a,b] Florian Lang[a]

[a]Department of Physiology, University of Tübingen, Germany, [b]Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan

Key Words
Phosphatidylserine • Phloretin • Calcium • Cell volume • Eryptosis

Abstract
Background: Phloretin, a natural component of apples, pears and strawberries, has previously been shown to stimulate apoptosis of nucleated cells. Erythrocytes may similarly enter suicidal death or eryptosis, which is characterized by cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), ceramide, ATP depletion, and activation of protein kinase C (PKC) as well as p38 mitogen activated protein kinase (p38 kinase). ***Methods:*** Phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, and ceramide abundance from binding of specific antibodies. ***Results:*** A 48 h exposure of human erythrocytes to phloretin significantly increased the percentage of annexin-V-binding cells (≥100 μM) without significantly influencing forward scatter. Phloretin did not significantly modify $[Ca^{2+}]_i$ and the stimulation of annexin-V-binding by phloretin (300 μM) did not require presence of extracellular Ca^{2+}. Phloretin did not significantly modify erythrocyte ATP levels, and the effect of phloretin on annexin-V-binding was not significantly altered by PKC inhibitor staurosporine (1 μM) or p38 kinase inhibitor SB2203580 (2 μM). However, phloretin significantly increased the ceramide abundance at the cell surface. ***Conclusions:*** Phloretin stimulates phospholipid scrambling of the erythrocyte cell membrane, an effect at least partially due to up-regulation of ceramide abundance.

Introduction

Phloretin (2',4',6'-trihydroxy-3-(4-hydroxyphenyl)-propiophenone), a component of apples, pears and strawberries [1, 2], is a dipolar small molecule interfering with diverse channels and transporters [3-10] including glucose transporters [1, 2]. Effects of Phloretin include decrease of oxidative stress [11], inhibition of inflammation [12], increase

Prof. Dr. Florian Lang
Physiologisches Institut der Universität Tübingen
Gmelinstr. 5, D-72076 Tübingen (Germany)
Tel. +49 7071 29 72194, Fax +49 7071 29 5618
E-Mail florian.lang@uni-tuebingen.de

endothelial nitric oxide production and vasodilation [13, 14], downregulation of endothelial adhesion molecules [15], and inhibition of platelet activation [15]. Some of the effects are due to estrogenic activity of the substance [13, 14, 16]. At low concentrations, phloretin has been shown to protect against apoptosis [17]. At high concentrations, phloretin may trigger apoptosis and thus counteract malignancy [1, 2, 18-22]. The stimulation of apoptosis may involve activation of p38 kinase [18].

Similar to apoptosis of nucleated cells, erythrocytes may enter suicidal cell death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with translocation of phosphatidylserine from the inner cell membrane leaflet to the cell surface [23]. Eryptosis may be triggered by increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), which leads to cell shrinkage due to activation of Ca^{2+}-sensitive K^+ channels with subsequent K^+ exit, hyperpolarization, Cl^- exit and thus cellular loss of KCl and water [24]. Increased $[Ca^{2+}]_i$ further triggers cell membrane scrambling with translocation of phosphatidylserine at the erythrocyte surface [23]. Even in the absence of increased $[Ca^{2+}]_i$, eryptosis may be triggered by ceramide [25]. Eryptosis is further stimulated by energy depletion, activated caspases [23, 26, 27] and deranged activities of AMP activated kinase AMPK [23], casein kinase 1α [28, 29], cGMP-dependent protein kinase [23], Janus-activated kinase JAK3 [23], protein kinase C [23], p38 kinase [23], PAK2 kinase [23], sorafenib sensitive kinases [30] and sunitinib sensitive kinases [31]. Eryptosis is triggered further by a wide variety of xenobiotics [23, 25, 30-64].

The present study explored, whether phloretin stimulates eryptosis. To this end, human erythrocytes from healthy individuals were treated with phloretin and phosphatidylserine surface abundance, cell volume, $[Ca^{2+}]_i$, and ceramide abundance at the erythrocyte surface determined by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 g for 20 min at 23 °C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 48 h. Where indicated, erythrocytes were exposed to phloretin (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations, whereby 25 mg phloretin were solved in 911,51 µl DMSO. For comparison, the effect of 3 µl DMSO/ml Ringer was tested.

Analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 µl cell suspension was washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 5 µM Fluo-3/AM. The cells were incubated at 37°C for 30 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 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Determination of ceramide abundance at the erythrocyte surface

To determine ceramide abundance, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37 °C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) at a dilution of 1:10. After two washing steps with PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analyzed by flow cytometric analysis at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored whether the antineoplastic alkaloid phloretin modifies eryptosis, the suicidal erythrocyte death, which is characterized by cell membrane scrambling with phosphatidylserine translocation from the cell interior to the cell surface.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding in flow cytometry. Annexin-V-binding was estimated by flow cytometry. Prior to measurements the erythrocytes were incubated for 48 hours in Ringer solution without or with phloretin (100 - 300 µM). As shown in Fig. 1, a 48 h exposure to phloretin increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 100 µM phloretin concentration. Thus, phloretin treatment leads to erythrocyte cell membrane scrambling with translocation of phosphatidylserine to the cell surface.

In order to test, whether phloretin leads to cell shrinkage, erythrocyte cell volume was estimated from forward scatter in flow cytometry following a 48 hours incubation of human erythrocytes in Ringer solution without or with phloretin (100 - 300 µM). As illustrated in Fig. 2, forward scatter was similar following incubation of human erythrocytes in Ringer solution without and with phloretin (100 - 300 µM).

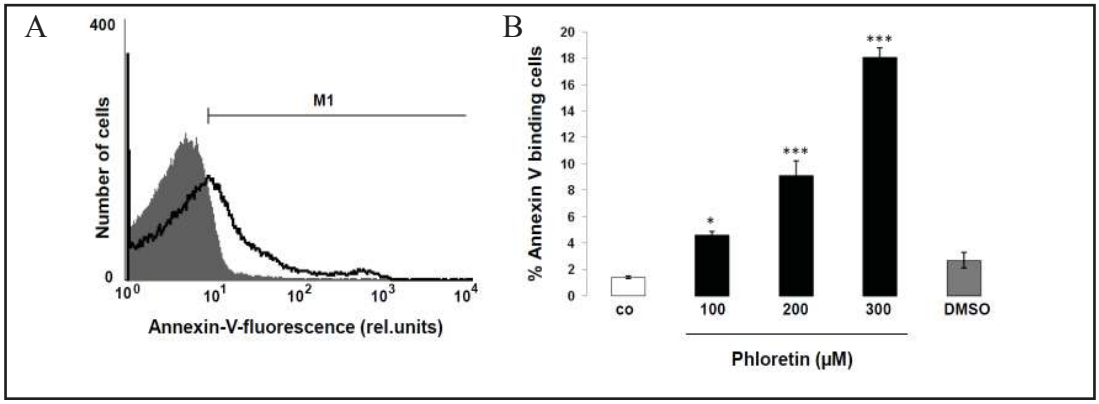

Fig. 1. Effect of phloretin on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 300 µM phloretin. B. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 10) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of phloretin (100-300 µM). For comparison, the effect of 3 µl DMSO/ml Ringer is shown (grey bar).*($p<0.05$), ***($p<0.001$) indicate significant difference from the absence of phloretin (ANOVA).

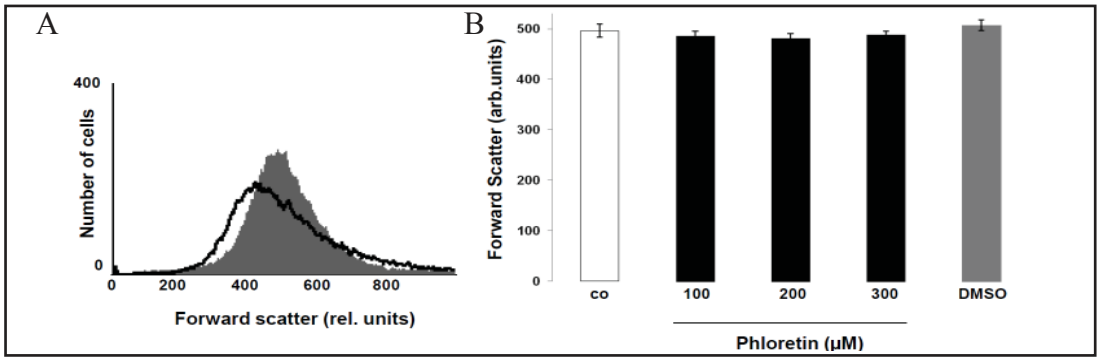

Fig. 2. Effect of phloretin on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 300 µM phloretin. B. Arithmetic means ± SEM (n = 10) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) phloretin (100 - 300 µM). For comparison, the effect of 3 µl DMSO/ml Ringer is shown (grey bar).

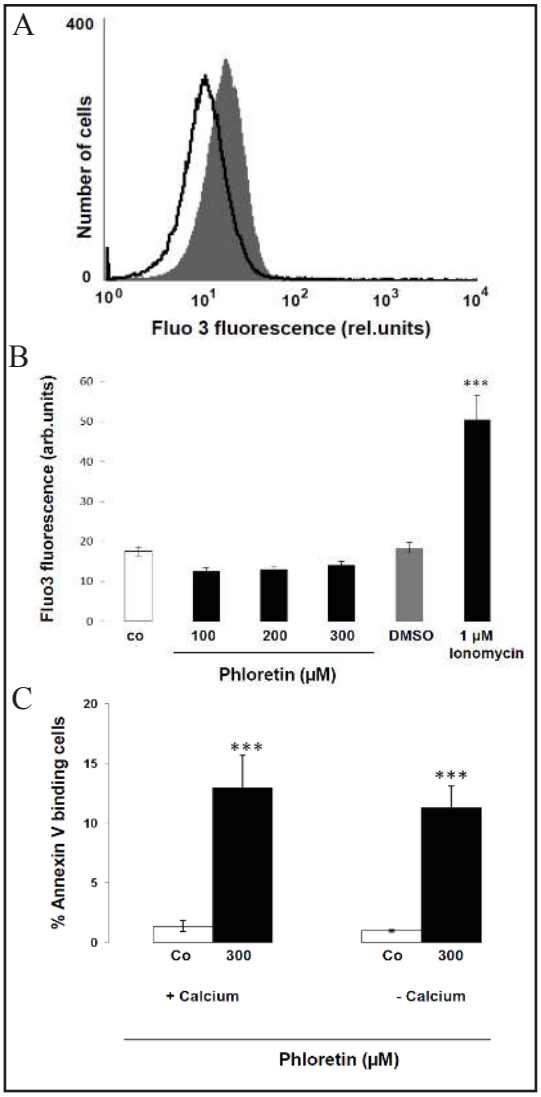

Fig. 3. Effect of phloretin on erythrocyte Ca^{2+} activity and Ca^{2+} dependence of phloretin- induced phosphatidylserine exposure. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of phloretin (300 µM). B. Arithmetic means ± SEM (n = 10) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) phloretin (100-300 µM). For comparison, the effect of 3 µl DMSO/ml Ringer (grey bar) and Ca^{2+} ionophore ionomycin (1 µM, 60 min, black bar) is shown. C. Arithmetic means ± SEM (n = 4) of erythrocyte forward scatter after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 300 µM phloretin in the presence (left bars, + calcium) and absence (right bars, -calcium) of calcium. ***($p<0.001$) indicates significant difference from the absence of phloretin.

Fluo3 fluorescence was employed to test whether phloretin affected cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As shown in Fig. 3, a 48 h exposure to phloretin (100 - 300 µM) did not significantly modify Fluo3 fluorescence. Thus, phloretin apparently did not significantly alter cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As illustrated in Fig. 3, $[Ca^{2+}]_i$ was increased by treatment of the erythrocytes with Ca^{2+} ionophore ionomycin (1 µM, 1 h). A further series of experiments tested whether the phloretin-induced cell membrane scrambling required entry of extracellular Ca^{2+}. To this end, erythrocytes were exposed for 48 h to 300 µM phloretin in the presence or nominal absence of extracellular Ca^{2+}. As shown in Fig. 3C, phloretin up-regulated annexin-V-

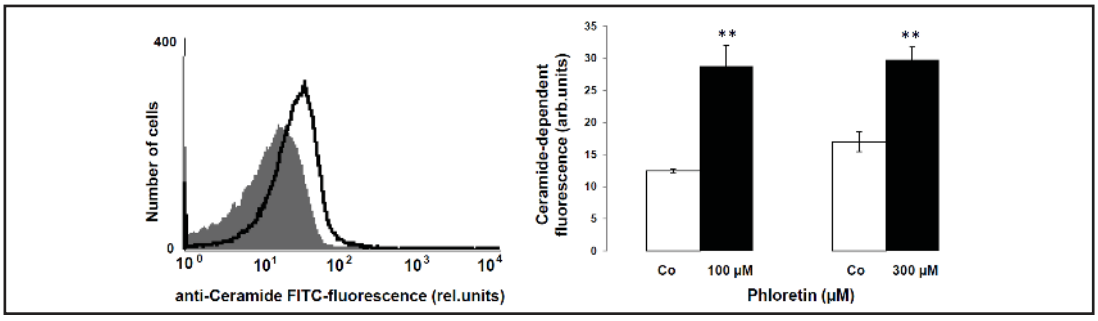

Fig. 4. Effect of phloretin on ceramide formation. A. Original histogram of ceramide surface abundance of erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) and with (black line) presence of 300 µM phloretin. B. Arithmetic means ± SEM (n= 4) of ceramide abundance after a 48 h incubation in Ringer solution without (white bars) or with 100 µM and 300 µM phloretin (black bars). ** ($p< 0.01$) indicates significant difference from the absence of phloretin (*t* test).

binding significantly and to a similar extent in the absence and presence of extracellular Ca^{2+}. Thus, the effect of phloretin on phosphatidylserine translocation did not depend on entry of extracellular Ca^{2+}.

In order to test, whether the triggering of cell membrane scrambling was secondary to activation of p38 mitogen activated protein kinase, erythrocytes were exposed for 48 h to phloretin (300 µM) in the absence and presence of the p38 kinase inhibitor SB203580 (2 µM). As a result, the phloretin treatment increased the percentage of annexin-V-binding erythrocytes from 1.9 ± 0.2 to 19.3 ± 1.9 % (n = 8) in the absence of SB203580, and from 1.7 ± 0.1 to 18.1 ± 1.6 % (n = 8) in the presence of SB203580. The increase was not significantly different between absence and presence of SB203580. As a positive control, the percentage of phoshatidylserine exposing erythrocytes was, following hyperosmotic shock (addition of 550 mM sucrose, 6 hours), significantly lower in the presence (11.9 ± 0.8 , n = 8) than in the absence (15.0 ± 0.6, n = 8) of SB203580 (2 µM, $p< 0.001$).

Further experiments addressed whether the triggering of cell membrane scrambling was secondary to activation of protein kinase C. To this end, erythrocytes were exposed for 48 h to phloretin (300 µM) in the absence and presence of the protein kinase C inhibitor staurosporine (1 µM). As a result, the phloretin treatment increased in this series of experiments the percentage of annexin-V-binding erythrocytes from 1.8 ± 0.3 to 23.6 ± 2.1 % (n = 8) in the absence of staurosporine, and from 2.1 ± 0.3 to 23.4 ± 2.0 % (n = 8) in the presence of staurosporine. Again, the increase was not significantly different between absence and presence of staurosporine. As a positive control, the phoshatidylserine exposure following glucose deprivation (48 hours) was significantly lower in the presence (17.2 ± 0.8, n = 8) than the absence (22.7 ± 1.0, n = 8) of staurosporine (1 µM, $p<0.001$).

As cell membrane scrambling could result from energy depletion, further experiments tested, whether phloretin decreased the erythrocyte ATP content. As a result, the ATP level approached 0.57 ± 0.10 mM (n = 4) following a 48 h incubation in the absence of phloretin and 0.61 ± 0.11 mM (n = 4) following a 48 h incubation exposure of erythrocytes in the presence of phloretin (300 µM). The respective values were not significantly different. In contrast, a 48 h incubation of erythrocytes in the absence of glucose was followed by a significant ($p<0.05$) decrease of the erythrocyte ATP level (0.18 ± 0.07 mM (n = 4).

As cell membrane scrambling is known to be stimulated by ceramide, which is effective without requirement to increase $[Ca^{2+}]_i$, the ceramide abundance at the erythrocyte surface was determined utilizing a specific anti-ceramide antibody. As shown in Figure 4, a 48 h exposure of erythrocytes to 100 µM or 300 µM phloretin significantly increased the abundance of ceramide at the erythrocyte surface. The increase of ceramide abundance following treatment of erythrocytes with 300 µM phloretin was not significantly modified by acid sphingomyelinase inhibitors, which approached 24.2 ± 2.1 u.a. (n = 3) in the absence of acid sphingomyelinase inhibitors, 23.7 ± 1.6 u.a. (n = 3) in the presence of 0.01 µM amitriptyline

and 24.8 ± 1.8 u.a. (n = 3) in the presence of 10 μM zoledronic acid. The phloretin (300 μM) induced increase of phosphatidylserine exposure was similarly not significantly modified by the acid sphingomyelinase inhibitors and approached 14.0 ± 2.3 u.a. (n = 4) in the absence of inhibitors, 13.8 ± 2.3 u.a. (n = 4) in the presence of 0.01 μM amitriptyline and 12.0 ± 1.5 u.a. (n = 4) in the presence of 10 μM zoledronic acid.

Discussion

The present study reveals that exposure of erythrocytes to phloretin is followed by cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Cell membrane scrambling is the hallmark of eryptosis, the suicidal death of erythrocytes. The phloretin concentration (100 μM) required for stimulation of erythrocyte cell membrane scrambling was similar to that stimulating tumor cell apoptosis [1, 2]. A dosage of 10 mg/kg was shown to counteract tumor growth in vivo [1]. The *in vivo* concentrations following application of this dosage have not been reported [1]. Lower concentrations of phloretin are rather protective against apoptosis [17].

Phloretin exposure did not trigger erythrocyte shrinkage, another hallmark of eryptosis. During eryptosis, cell shrinkage is mainly due to increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [24]. According to Fluo3 fluorescence, phloretin did not increase $[Ca^{2+}]_i$. Moreover, the stimulating effect of phloretin on cell membrane scrambling did not require presence and thus entry of extracellular Ca^{2+}.

The cell membrane scrambling following phloretin exposure was further not secondary to energy depletion, since, other than glucose depletion, phloretin did not decrease the erythrocyte ATP level. Moreover, other than the eryptosis following energy depletion [23], the phloretin-induced cell membrane scrambling was not blunted by the protein kinase C inhibitor staurosporine. Pharmacological inhibition of the p38 kinase, which participates in the triggering of eryptosis following osmotic shock [23], did again not significantly interfere with cell membrane scrambling following phloretin exposure.

According to our observations, the stimulation of cell membrane scrambling is paralleled by an enhancement of ceramide abundance in the erythrocyte cell membrane. The increase could not be inhibited by amitriptyline or zoledronic acid and may thus be due to translocation rather than formation of ceramide. Ceramide is a powerful stimulator of eryptosis [23]. To the best of our knowledge, an increase of ceramide abundance at the cell surface following phloretin exposure has never been shown before.

Phosphatidylserine exposure at the erythrocyte surface serves the function to trigger elimination of defective erythrocytes and thus to protect against hemolysis [23]. Hemolysis of defective erythrocytes inevitably leads to release of hemoglobin, which may be filtered at the renal glomeruli with subsequent precipitation in the acidic lumen of renal tubules and thus occlusion of the affected nephrons [65]. Removal of phosphatidylserine exposing erythrocytes is further of advantage in malaria [66]. The malaria pathogen *Plasmodium* invades erythrocytes and subsequently induces oxidative stress, which in turn activates several ion channels of the host cell membrane including Ca^{2+}-permeable erythrocyte cation channels [23, 67]. Ca^{2+} entry through those channels triggers eryptosis with rapid clearance of the infected erythrocytes from circulating blood [66]. Accelerated eryptosis counteracts the development of parasitemia and thus favourably influences the clinical course of malaria [66]. Accordingly, several genetic erythrocyte disorders including sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and homozygous G6PD-deficiency protect against a severe course of malaria at least partially due to accelerated eryptosis [23, 68-70]. The clinical course of malaria is further favourably influenced by clinical conditions with accelerated eryptosis, such as iron deficiency [71], and by xenobiotics triggering eryptosis including lead [72], chlorpromazine [73] or NO synthase inhibitors [74]. In theory, phloretin may similarly influence eryptosis of Plasmodium infected erythrocytes. Beyond that, phloretin is known to interfere with several transport processes in the parasite and/or host erythrocyte [75-81].

Accelerated eryptosis may be of pathophysiological significance, as the phagocytosis and subsequent removal of phosphatidylserine exposing erythrocytes may result in anemia as soon as the accelerated clearance of erythrocytes during stimulated eryptosis is not matched by a similarly increased formation of new erythrocytes [23]. Moreover, phosphatidylserine exposing erythrocytes bind to CXCL16/SR-PSO at the apical membrane of endothelial cells in the vascular wall [82]. Phosphatidylserine exposing erythrocytes further stimulate blood clotting and thrombosis [83-85]. Thus, stimulation of phosphatidylserine translocation to the erythrocyte surface may lead to impairment of microcirculation [25, 83, 86-89].

In conclusion, phloretin stimulates erythrocyte cell membrane scrambling, an effect paralleled by and possibly due to increase of ceramide abundance at the ersythrocyte surface.

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