Original Paper

Stimulation of Suicidal Erythrocyte Death by Rottlerin

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Calcium • Ceramide

Abstract

Background/Aims: The phytochemical polyphenol rottlerin is a potent activator of diverse Ca^{2+} -sensitive K^+ channels. Those channels play a decisive role in the execution of eryptosis, the suicidal death of erythrocytes, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling involved in the stimulation of eryptosis includes increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and ceramide. The present study explored, whether rottlerin induces eryptosis and, if so, to test for the involvement of Ca^{2+} entry and ceramide. **Methods:** Flow cytometry was employed to estimate phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, and ceramide abundance utilizing specific antibodies. Hemolysis was quantified by determination of haemoglobin concentration in the supernatant. **Results:** A 48 hours exposure of human erythrocytes to rottlerin (1–5 μM) significantly increased the percentage of annexin-V-binding cells, an effect paralleled by significant decrease of forward scatter. Up to 5 μM rottlerin failed to significantly increase average Fluo3-fluorescence. Rottlerin (5 μM) did, however, significantly increase the ceramide abundance. Rottlerin (5 μM) further significantly increased hemolysis. The effect of rottlerin (5 μM) on annexin-V-binding was virtually abolished by removal of extracellular Ca^{2+} . **Conclusions:** Rottlerin stimulates eryptosis with erythrocyte shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect paralleled by and at least in part due to Ca^{2+} entry and ceramide.

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Introduction

The natural polyphenol rottlerin has antioxidant, anti-inflammatory, antipsoriatic, anti-amyloid and anti-cancer potency [1-3]. The substance is partially effective by influencing the activity and/or expression of several enzymes, such as kinases, heme oxygenase, DNA

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methyltransferase, cyclooxygenase, and lipoxygenase, as well as transcription factors including NF- κ B and STAT [1]. Moreover, rottlerin may prevent aggregation of different amyloid precursors, such as α -synuclein, amyloid A β , prion proteins, and lysozyme [1-3]. Rottlerin has further been shown to stimulate Ca^{2+} -sensitive K^+ channels [4].

Activation of Ca^{2+} -sensitive K^+ channels is a key event in eryptosis [5], the suicidal death of erythrocytes characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface [6]. Eryptosis is stimulated by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [6] and ceramide [7]. Further stimulators of eryptosis include oxidative stress [6] and energy depletion [6]. Eryptosis may or may not involve activation of caspases depending on the respective trigger of eryptosis [6, 8, 9]. Kinases fostering eryptosis include casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and/or p38 kinase [6]. Kinases inhibiting eryptosis include AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases [6]. Eryptosis is stimulated by a myriad of xenobiotics [6, 10-43]. Enhanced eryptosis is observed in several clinical conditions including dehydration [44], hyperphosphatemia [45], chronic kidney disease (CKD) [46-49], hemolytic-uremic syndrome [50], diabetes [51], hepatic failure [52], malignancy [6], sepsis [53], sickle-cell disease [6], beta-thalassemia [6], Hb-C and G6PD-deficiency [6], as well as Wilsons disease [54].

The present study explored, whether rottlerin stimulates eryptosis. To this end, human erythrocytes drawn from healthy volunteers were exposed to rottlerin and phosphatidylserine abundance at the erythrocyte surface, erythrocyte volume, $[\text{Ca}^{2+}]_i$, and ceramide determined by flow cytometry. For comparison, hemolysis was quantified from hemoglobin release.

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 21 °C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* for 48 hours at 37°C and 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; pH 7.4), 5 glucose, 1 CaCl_2 without or with rottlerin (Santa Cruz Biotechnology, USA). Where indicated, CaCl_2 was removed and 1 mM EGTA added.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 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 15 min under protection from light. The annexin-V-abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and rottlerin treated erythrocytes. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

Hemolysis

Following incubation, the erythrocyte suspension was centrifuged for 3 min at 1600 rpm, 4°C, and the supernatant harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration in the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and 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. 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.

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 hour at 37°C with 1 µg/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. The cells were stained for 30 minutes 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. The samples were analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used.

Statistics

Data are expressed as arithmetic means $\pm$ 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 rottlerin is capable to trigger eryptosis, the suicidal erythrocyte death. Hallmarks of eryptosis are cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with rottlerin (1 - 5 µM). As illustrated in Fig. 1, rottlerin increased average erythrocyte forward scatter, an effect reaching statistical significance at 10 µM rottlerin concentration. A closer analysis of the histograms revealed that treatment with rottlerin increased the percentage of both, swollen and shrunken erythrocytes.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with rottlerin (1 - 5 µM). As shown in Fig. 2, a 48 hours exposure to rottlerin significantly increased the percentage of phosphatidylserine exposing erythrocytes at all concentrations tested.

Fig. 1. Effect of rottlerin on erythrocyte forward scatter. A. Original histograms of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 5 µM rottlerin. B. Arithmetic means $\pm$ SEM (*n* = 10) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) rottlerin (1 - 5 µM) or solvent DMSO alone (grey bar). *(*P* < 0.05), **(*p* < 0.01), ***(*p* < 0.001) indicate significant difference from the absence of rottlerin (ANOVA).

Fig. 2. Effect of rottlerin on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 5 μM rottlerin. B. Arithmetic means ± SEM (n = 10) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) rottlerin (1 - 5 μM) or solvent DMSO alone (grey bar). ***($p < 0.001$) indicates significant difference from the absence of rottlerin (ANOVA).

Fig. 3. Effect of rottlerin on hemolysis. Arithmetic means ± SEM (n = 15) of the percentage hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) rottlerin (5 μM) or solvent DMSO alone (grey bar). **($p < 0.01$), indicates significant difference from the absence of rottlerin (ANOVA).

Fig. 4. Effect of rottlerin on cytosolic Ca^{2+} activity. A. Original histograms of Fluo-3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 5 μM rottlerin. B. Arithmetic means ± SEM (n = 10) of Fluo-3 fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) rottlerin (1 - 5 μM) or solvent DMSO alone (grey bar).

Hemoglobin concentration in the supernatant was taken as a measure of hemolysis. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with rottlerin (5 μM). As illustrated in Fig. 3, a 48 hours exposure to 5 μM rottlerin significantly increased the percentage of hemolytic erythrocytes.

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with rottlerin (1 - 5 μM). As illustrated in Fig. 4A,B, a 48 hours incubation with rottlerin did not significantly modify the average Fluo3 fluorescence. Higher concentrations of rottlerin (10 μM) significantly ($p < 0.05$) increased Fluo-3 fluorescence (from 13.4 ± 1.3 to 18.2 ± 3.1 n = 10).

A next series of experiments explored whether the rottlerin-induced translocation of phosphatidylserine required entry of extracellular Ca^{2+} . To this end, erythrocytes were incubated for 48 hours in the absence or presence of 5 μM rottlerin in the presence or

Fig. 5. Ca^{2+} sensitivity of rottlerin-induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) rottlerin ($5 \mu\text{M}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 10$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) rottlerin ($5 \mu\text{M}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of rottlerin, ###($p < 0.001$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 6. Effect of rottlerin on ceramide abundance. A. Original histograms of ceramide abundance at the erythrocyte surface following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of $5 \mu\text{M}$ rottlerin. B. Arithmetic means $\pm$ SEM ($n = 10$) of ceramide abundance at the erythrocyte surface following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) rottlerin ($5 \mu\text{M}$). ***($p < 0.001$) indicates significant difference from the absence of rottlerin (ANOVA).

nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 5, removal of extracellular Ca^{2+} significantly blunted the effect of rottlerin on annexin-V-binding. In the absence of extracellular Ca^{2+} , rottlerin did not significantly increase the percentage annexin-V-binding erythrocytes. Thus, rottlerin-induced cell membrane scrambling was in large part dependent on entry of extracellular Ca^{2+} .

Ca^{2+} sensitivity of cell membrane scrambling could be enhanced by ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As shown in Fig. 6, a 48 hours exposure to $5 \mu\text{M}$ rottlerin significantly increased the ceramide abundance.

Discussion

The present study discloses a novel effect of rottlerin, i.e. the stimulation of eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The rottlerin concentrations required for stimulation of eryptosis were in the range of plasma concentrations ($2.9 \pm 0.4 \mu\text{M}$) observed in mice following rottlerin treatment [55]. Similar concentrations have previously been shown to stimulate apoptosis of tumor cells [56].

The effect of rottlerin on cell membrane scrambling was in large part dependent on Ca^{2+} entry from the extracellular space, as it was markedly decreased by removal of extracellular Ca^{2+} . Up to 5 μM rottlerin did not significantly increase the average Fluo3 fluorescence suggesting that rottlerin did not increase average cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Only higher concentrations of rottlerin did increase average Fluo3 fluorescence. It thus appears that rottlerin enhances the Ca^{2+} sensitivity of cell membrane scrambling rather than increasing $[\text{Ca}^{2+}]_i$. Rottlerin treatment increased the abundance of ceramide, which is known to enhance the Ca^{2+} sensitivity of erythrocyte cell membrane scrambling [6].

Rottlerin treatment increased both, the percentage of shrunken and swollen cells. The cell shrinkage is presumably due to Ca^{2+} entry from the extracellular space with subsequent increase of $[\text{Ca}^{2+}]_i$, activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [6]. The mechanisms accounting for swelling of a subset of erythrocytes remained elusive.

The cell swelling presumably accounts for the increase of hemolysis following rottlerin treatment. Hemolysis leads to release of hemoglobin, which may pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus cause renal failure [57]. Eryptosis may accomplish clearance of defective erythrocytes prior to hemolysis thus preventing release of hemoglobin. Eryptosis may further trigger clearance of erythrocytes, which are infected with the malaria pathogen *Plasmodium*. Along those lines, stimulation of eryptosis may favourably influence the clinical course of malaria [6].

The clearance of eryptotic erythrocytes from circulating blood may, on the other hand, result in anemia to the extent that the loss of erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [6]. Phosphatidylserine exposing erythrocytes may further compromise microcirculation [7, 58-62] by adherence to the vascular wall [63], stimulation of blood clotting and triggering of thrombosis [58, 64, 65].

In conclusion, rottlerin triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect involving Ca^{2+} entry and ceramide.

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Disclosure Statement

None.

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