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Review

Methods Employed in Cytofluorometric Assessment of Eryptosis, the Suicidal Erythrocyte Death

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Key Words
RBC • Calcium • Cell volume • Cell membrane scrambling • ROS • Ceramide • Glutathion • Caspases • Kinases • NSC-95397 • Bi-2536 • Fascaplycin • Lopinavir • Terfenadine • Fucoxanthin

Abstract
Suicidal erythrocyte death or eryptosis contributes to or even accounts for anemia in a wide variety of clinical conditions, such as iron deficiency, dehydration, hyperphosphatemia, vitamin D excess, chronic kidney disease (CKD), hemolytic-uremic syndrome, diabetes, hepatic failure, malignancy, arteriitis, sepsis, fever, malaria, sickle-cell disease, beta-thalassemia, Hb-C and G6PD-deficiency, Wilsons disease, as well as advanced age. Moreover, eryptosis is triggered by a myriad of xenobiotics and endogenous substances including cytotoxic drugs and uremic toxins. Eryptosis is characterized by cell membrane scrambling with phosphatidylserine exposure to the erythrocyte surface. Triggers of eryptosis include oxidative stress, hyperosmotic shock, and energy depletion. Signalling involved in the regulation of eryptosis includes Ca^{2+} entry, ceramide, caspases, calpain, p38 kinase, protein kinase C, Janus-activated kinase 3, casein kinase 1α, cyclin-dependent kinase 4, AMP-activated kinase, p21-activated kinase 2, cGMP-dependent protein kinase, mitogen- and stress-activated kinase MSK1/2, and ill-defined tyrosine kinases. Inhibitors of eryptosis may prevent anaemia in clinical conditions associated with enhanced eryptosis and stimulators of eryptosis may favourably influence the clinical course of malaria. Additional experimentation is required to uncover further clinical conditions with enhanced eryptosis, as well as further signalling pathways, further stimulators, and further inhibitors of eryptosis. Thus, a detailed description of the methods employed in the analysis of eryptosis may help those, who enter this exciting research area. The present synopsis describes the experimental procedures required for the analysis of phosphatidylserine exposure at the cell surface with annexin-V, cell volume with forward scatter, cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) with Fluo3, oxidative stress with 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), glutathione (GSH) with mercury orange 1(4-chloromercuryphenyl-azo-2-naphthol), lipid peroxidation with BODIPY 581/591 C11 fluorescence, and ceramide abundance with specific antibodies. The contribution of kinases and caspases is defined with the use of the respective inhibitors.

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It is hoped that the present detailed description of materials and methods required for the analysis of eryptosis encourages further scientists to enter this highly relevant research area.

Introduction

Suicidal death of mature red blood cells (RBCs) or erythrocytes is commonly named eryptosis. Similar to apoptosis of nucleated cells, eryptosis typically leads to cell shrinkage, cell membrane blebbing and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface [1-7]. The signalling triggering eryptosis includes in most cases the activation of Ca^{2+} permeable channels with increase of cytosolic Ca^{2+} activity [8, 9]. However, several further mechanisms may be involved, such as oxidative stress, energy depletion, excessive cell shrinkage, calpain, caspases, ceramide, eicosanoids, and diverse kinases [8, 9].

Eryptosis is a common clinical problem, as it is triggered by a myriad of xenobiotics as well as endogenous substances [1-3, 10-68] and accelerated eryptosis is observed in a variety of clinical disorders including iron deficiency [1-3], dehydration [69], hyper-cholesterolemia and enhanced oxysterol levels [70, 71], hyperphosphatemia [72], vitamin D excess [36], chronic kidney disease (CKD) [73-77], haemolytic-uremic syndrome [78], diabetes [79], hepatic failure [38, 80], malignancy [81, 82], arteritis [83], sepsis [84], fever [1-7], malaria [85], sickle-cell disease [1-3], beta-thalassemia [1-3], Hb-C and G6PD-deficiency [1-3], Wilsons disease [86], as well as advanced age [87]. Eryptosis further increases following erythrocyte storage for transfusion [88].

Accelerated eryptosis leads to anaemia as soon as its rate surpasses the formation of new erythrocytes by erythropoiesis [1-3]. Moreover, eryptotic erythrocytes adhere to the vascular wall thus triggering thrombosis and compromising microcirculation [1-3].

In view of the emerging clinical importance of eryptosis, a detailed description of the methods uncovering and characterizing eryptotic erythrocytes appears desirable. The present paper thus describes flow cytometric methods used in the analysis of eryptosis and the signalling mechanisms involved.

In order to illustrate the results, the respective effects of several xenobiotics known to stimulate eryptosis are shown. Examples are provided by treatment of erythrocytes with the CDC25B phosphatase inhibitor NSC-95397 [35], the PLK1 kinase inhibitor BI-2536 [89], the protease inhibitor Lopinavir [22], the marine sponge-derived Fascaplycin [44], the selective histamine H1 receptor antagonist Terfenadine [58] and Fucoxanthin (a carotenoid from the chloroplasts of brown seaweeds) [25]. In parallel examples of substances interfering with eryptosis signalling, such as caspase inhibitor Z-VAD-FMK, p38 inhibitor SB203580, protein kinase C inhibitor staurosporine and casein kinase 1 CK1α inhibitor D4476 [9] are provided. In those experiments erythrocytes were incubated for 48 hours in Ringer solution without or with xenobiotics.

Materials

Erythrocytes

Blood samples are collected by venopuncture from healthy donors in lithium-heparin tubes. The tubes are kept at room temperature (20–25 °C). For this study blood was 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).

Flow cytometer

Cytofluorometer FACSCalibur (BD, San José, USA) equipped with a 488 nm-blue laser and controlled by the BD CellQuest™ Pro software (BD Biosciences).

Software

CellQuest™ Pro software (BD Biosciences), Microsoft Excel (Microsoft) for data analysis and GraphPad Prism (GraphPad) for test of statistical significance.

Reagents, compounds, dyes and antibodies

Solutions and chemicals employed include:

1. Ringer solution: highly purified water (distilled water filtered on 0.22µm) containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, and 1 $CaCl_2$.
2. Annexin-V wash solution: highly purified water containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, and 1 $CaCl_2$.
3. Phosphate buffered saline (PBS) (Sigma Aldrich, Hamburg, Germany).
4. 0.1% (w/v) Bovine serum albumin (BSA) (Sigma Aldrich, Hamburg, Germany) in PBS.
5. NSC95397 (Sigma Aldrich, Hamburg, Germany).
6. BI-2536 (Selleck Chemicals, München, Germany).
7. Lopinavir (Sigma Aldrich, Hamburg, Germany).
8. Fascaplycin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).
9. Fucoxanthin (Sigma Aldrich, Hamburg, Germany).
10. Terfenadine (Sigma Aldrich, Hamburg, Germany).
11. Ionomycin (Sigma Aldrich, Hamburg, Germany).
12. Necrostatin-1 (Sigma Aldrich, Hamburg, Germany).
13. D4476 (Sigma Aldrich, Hamburg, Germany).
14. zVAD (Tocris bioscience, Bristol, UK).
15. zIETD-fmk (Selleck Chemicals, München, Germany).
16. zDEVD-fmk (Selleck Chemicals, München, Germany).
17. SB203580 (Tocris bioscience, Bristol, UK).
18. Staurosporine (Sigma Aldrich, Hamburg, Germany).
19. Chelerythrine (Sigma Aldrich, Hamburg, Germany).
20. BAPTA-AM (Thermo Fisher Scientific, Darmstadt, Germany).
21. BODIPY 581/591 C11 (Thermo Fisher Scientific, MA, USA).
22. Fluo-3 AM (Biotium, Hayward, USA).
23. 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (Sigma, Schnelldorf, Germany).
24. Mercury orange (Sigma-Aldrich, Hamburg, Germany).
25. Annexin-V FITC (ImmunoTools, Friesoythe, Germany).
26. Anti-ceramide (clone MID 15B4; Alexis, Grünberg, Germany).
27. FITC-conjugated goat Anti-Mouse IgG (H+L) (BD Pharmingen, Hamburg, Germany).

Experimental procedures

Erythrocytes preparation

It is important to work with fresh blood to avoid any unspecific results due to storage and manipulation of erythrocytes.

1. Mix 1 ml of blood with 3 ml of Ringer solution into a 5 ml FACS tube
2. Centrifuge for 20 min at 120 g at 21°C with zero deceleration for an optimal separation of erythrocytes
3. Discard supernatant that contains Ringer, platelets and leukocytes, the rest is erythrocytes.
4. Slowly recuperate 300 µl from the bottom of the tube and transfer it into a 1.5 µl microcentrifuge tube and keep in fridge at 4°C for maximum 24h.

Quantification of cell size

To explore the cell size, erythrocyte volume is estimated from forward scatter (FSC). A histogram SSC-linear *vs.* FSC-linear is made and the value of Geo Mean of FSC to around

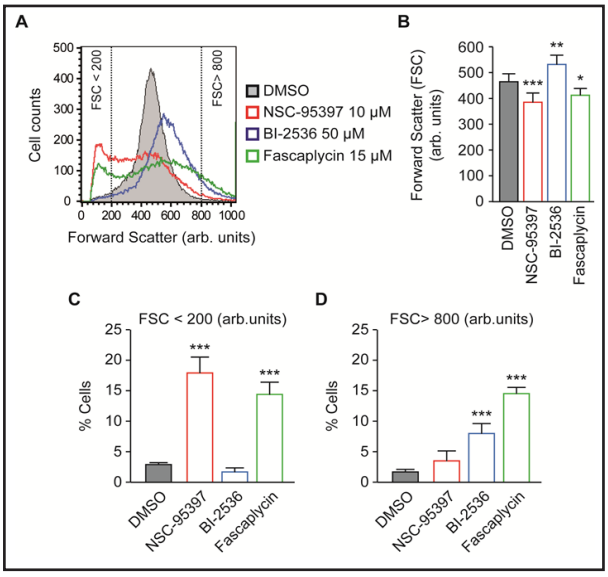

Fig. 1. Effect of NSC-95397, Bi-2536 and Fascaplycin on erythrocyte forward scatter. A. Original histograms of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with presence of 10 µM NSC-95397 (red line), 50 µM Bi-2536 (blue line) or 15 µM Fascaplycin (green line). B. Arithmetic means ± SEM (n = 12) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (grey bar) or with (white bars) 10 µM NSC-95397 (red line), 50 µM Bi-2536 (blue line) or 15 µM Fascaplycin (green line). C,D. Percentage of erythrocytes with (C) FSC< 200 or (D) FSC > 800 following incubation for 48 hours to Ringer solution without (grey bar) or with (white bars) 10 µM NSC-95397 (red line), 50 µM Bi-2536 (blue line) or 15 µM Fascaplycin (green line). *(p<0.05), **(p<0.01), ***(p<0.001) indicates significant difference from the absence of drugs treatment (ANOVA).

500 (arbitrary units) fixed. Cell shrinkage is reflected by a decrease and cell swelling by an increase in the geo mean of forward scatter.

1. Prepare the eryptosis inducing agents at the desired concentrations in Ringer solution (NSC-95397 at 10 µM, BI-2536 at 50 µM and Fascaplycin at 15 µM).
2. For each concentration prepare 1 tube of 1 ml solution.
3. For each tube add 4 µl of erythrocytes and mix well by pipetting then gentle vortex.
4. Incubate tubes in standard culture condition (37°C, 5% CO2).
5. Upon 48 h of incubation mix gently the cells and transfer 150 µl of each tube into a 1 ml FACS tube.
6. Place the "1 ml FACS tube" into a 5 ml FACS tube.
7. Acquire and analyze samples by means of a classic cytofluorometer allowing the acquisition of light scattering data. The data could be displayed as arithmetic means or as the percentage of erythrocytes undergoing shrinkage or swelling, whereby a threshold for shrunken (e.g. <200) and swollen (e.g.>800) erythrocytes is defined.

Example: Treatment with the CDC25B inhibitor NSC-95397 is followed by a decrease of cell volume reflected by a decrease of forward scatter (Fig 1A,B). Moreover, the substance increases the percentage of severely shrunken cells (Fig. 1C). Similarly, Fascaplycin treatment is followed by decrease of average forward scatter (Fig. 1B) and increase of the percentage of shrunken cells (Fig. 1C). In contrast, the PLK1 inhibitor BI-2536 leads to increase of average forward scatter (Fig. 1B) and increases the percentage of swollen cells (Fig. 1D). Please note that treatment with Fascaplycin increases both, the percentage of shrunken and the percentage of swollen erythrocytes (Fig. 1A, C and D).

Quantification of phosphatidylserine exposure

The measurement of scrambling and phosphatidylserine exposure to the cell surface is performed using annexin-V-binding. For staining with annexinV-FITC 150 µl erythrocyte suspension are washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-FITC (1:200 dilution) in this solution at 37°C for 15 min under protection from light. Annexin-V-binding is measured by FACS with an excitation wave length of 488 nm (blue laser) and an emission wave length of 530 nm (FL1 channel). The percentage of annexin-V-binding erythrocytes is defined by the shift of the FITC signal on the FL1-log channel in treated cells compared to control.

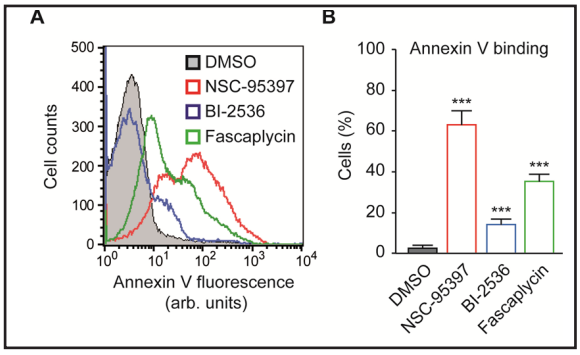

Fig. 2. Effect of NSC-95397, Bi-2536 and Fascaplycin on phosphatidylserine exposure. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with presence of 10 µM NSC-95397 (red line), 50 µM Bi-2536 (blue line) or 15 µM Fascaplycin (green line). B. Arithmetic means ± SEM (n = 12) of erythrocytes samples annexin-V-binding following incubation for 48 hours to Ringer solution without (grey bar) or with (white bars) 10 µM NSC-95397 (red line), 50 µM Bi-2536 (blue line) or 15 µM Fascaplycin (green line). ***(p<0.001) indicates significant difference from the absence of drugs treatment (ANOVA).

1. Prepare and incubate the sample tubes as described above (*Erythrocytes preparation*).
2. Upon 48 h of incubation mix gently the cells and transfer 150 µl of the each tube into a well of 96-well plate.
3. Centrifuge the plate for 3 min at 120 g at 21°C.
4. Remove supernatant and add for each well 100 µl solution of annexin-V FITC diluted in annexin-V wash buffer (dilution 1:200).
5. Mix well by pipetting.
6. Transfer the cells into a 1 ml FACS tube.
7. Incubate tubes for 20 min in standard culture condition (37°C, 5% CO2) under protection from light.
8. Place the 1 ml FACS tube into a 5 ml FACS tube and mix well.
9. Acquire and analyze samples by means of a classic cytofluorometer allowing the acquisition of light scattering data and fluorescence recording.

Example: As illustrated in Fig. 2, NSC-95397, BI-2536 and Fascaplycin all markedly enhance the percentage of erythrocytes undergoing cell membrane scrambling. It should be pointed out that none of the three substances triggers hemolysis, which in theory may yield false positive binding of annexin-V.

Quantification of intracellular Ca^{2+}

Cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) is determined utilizing Fluo3 fluorescence [35]. After cells staining with the Fluo-3 AM dye (Biotium, Hayward, USA) (excitation wave length of 488 nm and emission wavelength of 530 nm corresponding to the green channel FL1), the Geo mean and the shift of the signal between control and treated cells were evaluated on the FL1-log channel.

1. Prepare and incubate the sample tubes as described above.
2. Remove supernatant and add for each well 100 µl solution of 5 µM Fluo-3/AM diluted in Ringer buffer.
3. Mix well by pipetting.
4. Transfer the cells into a 1 ml FACS tube.
5. Incubate tubes for 20 min in standard culture condition (37°C, 5% CO2) under protection from light.
6. Place the 1 ml FACS tube into a 5 ml FACS tube and mix well.
7. Acquire and analyze samples by means of a classic cytofluorometer allowing the acquisition of light scattering data and fluorescence recording.

Examples: Cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) is determined utilizing Fluo3 fluorescence [35]. A histogram and bar graph is shown (Fig. 3 A and B). To confirm the involvement of cytosolic Ca^{2+} activity in the eryptosis, cells are incubated in Ringer solution with or without calcium and the difference of death with annexin-V staining in each case quantified (Fig. 3 C and D). Moreover, the cells can be co-treated with the Ca^{2+} chelator 1,2-Bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) BAPTA-AM (Thermo Fisher

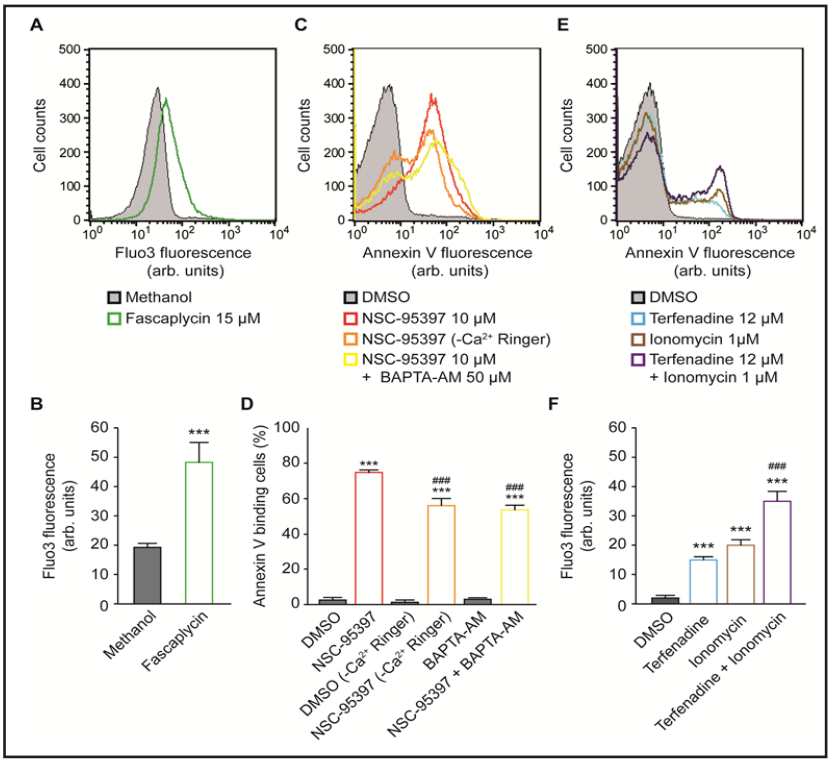

Fig. 3. Effect of drug treatment on erythrocyte cytosolic Ca^{2+} activity. A. Original histograms of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (green line) presence of 15 µM Fascaplycin. B. Arithmetic means ± SEM (n = 12) of Fluo3 fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (grey bar) or with (green line) presence of 15 µM Fascaplycin. C. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with presence of 10 µM NSC-95397 (red line), 10 µM NSC-95397 in Ca^{2+} free solution (orange line) or 10 µM NSC-95397 and Ca^{2+} chelator BAPTA-AM (yellow line). D. Arithmetic means ± SEM (n = 12) of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey bar) and with presence of 10 µM NSC-95397 (white bars and red line), 10 µM NSC-95397 in Ca^{2+} free solution (orange line) or 10 µM NSC-95397 and Ca^{2+} chelator BAPTA-AM (yellow line). E. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with presence of 12 µM Terfenadine (blue line), exposure for 30 minutes to 1 µM Ionomycin (brown line) or pre-exposure for 48 hours with 12 µM Terfenadine then 30 minutes to 1 µM Ionomycin (purple line). F. Arithmetic means ± SEM (n = 12) of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey bar) and with presence of 12 µM Terfenadine (white bar and blue line), exposure for 30 minutes to 1 µM Ionomycin (white bar and brown line) or pre-exposure for 48 hours with 12 µM Terfenadine then 30 minutes to 1 µM Ionomycin (white bar and purple line).***(p<0.001) indicates significant difference from the absence of treatment (ANOVA). ###(p<0.001) indicates significant difference from the presence of Ca^{2+} or absence of Ca^{2+} chelator BAPTA-AM after 10 µM NSC-95397 treatment and significant difference from presence of Ionomycin after 12 µM Terfenadine treatment (ANOVA).

Scientific, Darmstadt, Germany) [35] and the difference of death with annexin-V between treated cells and co-treated with BATA-AM evaluated. The NSC-95397 and BAPTA-AM co-treatment decrease eryptosis due to the CDC25B phosphatase inhibitor (Fig. 3 C and D). In contrast, treatment of the cells for 30 minutes with the ionophore Ionomycin [58] after Terfenadine 48 hours pre-incubation increases the cell death and confirms the role of Ca^{2+} entry as death pathway (Fig. 3 E and F).

Modulation of intracellular Ca^{2+}

For decreasing $[Ca^{2+}]_i$:

1. Prepare the eryptosis inducing agent(s) at the desired concentrations (10 µM of NSC-95397) in the Ringer solution with and without calcium or with addition of Ca^{2+} chelator BAPTA-AM.

2. For each condition prepare 1 tube of 1 ml solution and perform the annexin-V assay as described above.

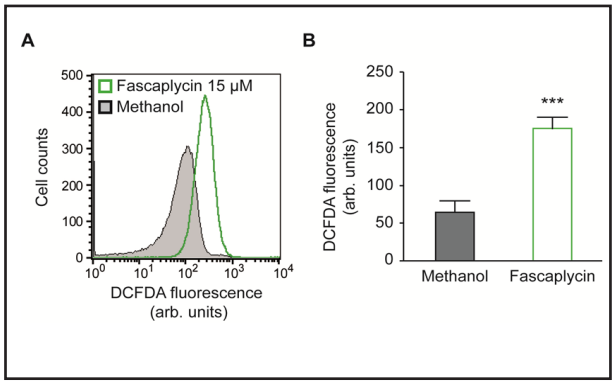

Fig. 4. Effect of Fascaplycin on ROS formation. A. Original histogram of DCF fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (green line) presence of Fascaplycin (15 µM). B. Arithmetic means ± SEM (n = 12) of the DCF fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (grey bar) or with (white bar, green line) Fascaplycin (15 µM). ***(p<0.001) indicates significant difference from the absence of Fascaplycin (ANOVA).

For increasing $[Ca^{2+}]_i$:

1. Prepare the eryptosis inducing agent at the desired concentration in Ringer solution (Terfenadine at 12 µM) and incubate the sample tubes as described above.

2. Upon 48 h of incubation mix gently the cells and for each tube add 1 µM of the ionophore Ionomycin.

3. Incubate tubes for 30 min in standard culture condition (37°C, 5% CO_2) under protection from light.

4. Perform the annexin-V assay as described above

Quantification of oxidative stress

To quantify oxidative stress, erythrocytes are stained with the 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Sigma, Schnelldorf, Germany) to measure the reactive oxygen species (ROS) [90]. DCFDA is non-fluorescent, but in the presence of ROS, it is oxidized and becomes green fluorescent. Geo mean is used to quantify ROS on the FL1-log channel.

1. Prepare the eryptosis inducing agent at the desired concentrations in Ringer solution (Fascaplycin at 15 µM).

2. Incubate the sample tubes as described above.

3. Remove supernatant and add for each well 100 µl solution of 10 µM DCFDA diluted in PBS.

4. Mix well by pipetting.

5. Transfer the cells into a 1 ml FACS tube.

6. Incubate tubes for 20 min in standard culture condition (37°C, 5% CO_2) under protection from light.

7. Place the 1 ml FACS tube into 5 ml FACS tube and mix well.

8. Acquire and analyze samples by means of a classic cytofluorometer allowing the acquisition of light scattering data and fluorescence recording.

Example: Original histogram and arithmetic means ± SEM of DCF fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without and with presence of Fascaplycin (15 µM) is shown in Fig. 4.

Quantification of reduced glutathione abundance

Erythrocytes depend on protection of antioxidant glutathione (GSH) against oxidative stress and reactive oxygen species [91]. A decrease of GSH can be a hallmark of eryptosis due to oxidative stress [92]. To quantify GSH, mercury orange 1(4-chloromercuryphenyl-azo-2-naphthol) (Sigma-Aldrich, Hamburg, Germany) [93] is used. The reaction between mercury orange and GSH leads to emitted intense red fluorescence when excited with the blue laser (excitation wavelength of 488 nm). The fluorescence can be measured on the FL2-log channel (emission wavelength of 576 nm). Geo mean is used to calculate the value of GSH in control or in the treated cells.

1. Prepare the eryptosis inducing agent at the desired concentrations in Ringer solution (Lopinavir at 40 µM).

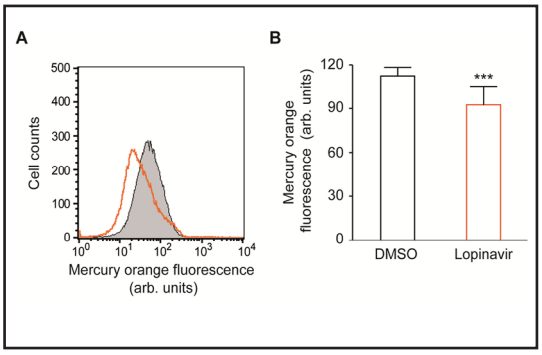

Fig. 5. Effect of Lopinavir on erythrocyte GSH level. A. Original histogram of mercury orange fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (orange line) presence of Lopinavir (40 µM). B. Arithmetic means ± SEM (n = 12) of the mercury orange fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (white bar and grey line) and with (white bar and orange line) presence of Lopinavir (40 µM). ***(p<0.001) indicates significant difference from the absence of Lopinavir (ANOVA).

2. Incubate the sample tubes as described above.

3. Remove supernatant and add for each well 100 µl solution of 40 µM mercury orange diluted in PBS.

4. Mix well by pipetting.

5. Transfer the cells into a 1 ml FACS tube.

6. Incubate tubes for 20 min in standard culture conditions (37°C, 5% CO_2) under protection from light.

7. Place the 1 ml FACS tube into 5 ml FACS tube and mix well.

8. Acquire and analyze samples by means of a classic cytofluorometer allowing the acquisition of light scattering data and fluorescence recording.

Example: Lopinavir treatment [22] induces oxidative stress and reduction of antioxidant glutathione as shown in Fig. 5.

Quantification of lipid peroxidation

Oxidative stress induces also damage on erythrocytic membrane lipids [94]. The polyunsaturated fatty acids are subject to oxidization by lipid peroxyl radicals and ROS, resulting in the formation of lipid hydroperoxides [95]. This reaction is commonly named lipid peroxidation [96]. In order to quantify this membrane damage, the dye BODIPY 581/591 C11 (Thermo Fisher Scientific, MA, USA) is used [97]. This dye is a fatty acid analogue with particular fluorescent properties. After excitation with the blue laser (488 nm), it emits in the red range of visible spectrum (maximum of 595 nm) but once ROS are oxidized, it shifts into the green range (510 nm). The lipid peroxidation is quantified by measuring the Geo mean on both FL1-log and FL2-log channel.

1. Prepare the eryptosis inducing agent at the desired concentrations in Ringer solution (Fucoxanthin at 80 µM) and incubate the sample tubes as described above.

2. Upon 48 h of incubation mix gently the cells and for each tube add 5 µM BODIPY 581/591 C11.

3. Incubate tubes for 30 min in standard culture condition (37°C, 5% CO_2) under protection from light.

4. Mix gently the cells and transfer 150 µl of the each tube into 1 ml FACS tube.

5. Place the 1 ml FACS tube into a 5 ml FACS tube and mix well.

6. Acquire and analyze samples by means of a classic cytofluorometer allowing the acquisition of light scattering data and fluorescence recording.

Example: Original histogram and arithmetic means ± SEM of BODIPY 581/591 C11 fluorescence at 530 nm emission wavelength following exposure for 48 hours to Ringer solution without and with presence of 80 µM Fucoxanthin is shown in Fig. 6.

Quantification of ceramide abundance

Ceramide is another important stimulator of the eryptosis [98]. Ceramide is generated by breakdown of cell membrane sphingomyelin and sensitizes erythrocytes to the enhanced intracellular calcium concentration [99]. The abundance of ceramide the erythrocyte surface can be measured utilizing specific antibodies.

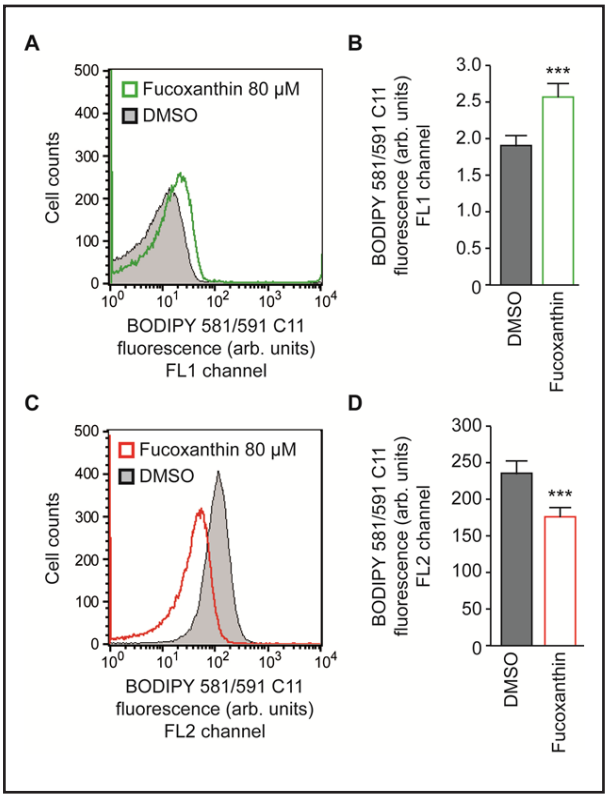

Fig. 6. Effect of Fucoxanthin on lipid peroxidation. A. Original histogram of BODIPY 581/591 C11 fluorescence at 530 nm emission wavelength (FL1 channel) following exposure for 48 hours to Ringer solution without (grey area) and with (green line) presence of 80 µM Fucoxanthin. B. Arithmetic means ± SEM (n = 12) of the BODIPY 581/591 C11 fluorescence at 530 nm emission wavelength (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (grey bar) or with (white bar and green line) 80 µM Fucoxanthin. C. Original histogram of BODIPY 581/591 C11 fluorescence at 585 nm emission wavelength (FL2 channel) following exposure for 48 hours to Ringer solution without (white bar and grey line) and with (red line) presence of 80 µM Fucoxanthin. D. Arithmetic means ± SEM (n = 12) of the BODIPY 581/591 C11 fluorescence at 585 nm emission wavelength (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (grey bar) or with (white bar and red line) 80 µM Fucoxanthin. ***(p<0.001) indicates significant difference from the absence of Fucoxanthin (ANOVA).

1. Prepare the eryptosis inducing agent at the desired concentrations in Ringer solution (Fascaplycin at 15 µM) and incubate the sample tubes as described above.

2. Remove supernatant and add for each well 100 µl of a solution containing the primary anti-ceramide antibody diluted 1:10 in 0.1% (w/v) BSA in PBS or with 100 µL of 1% (w/v) BSA in PBS as negative staining control.

3.Incubate tubes for 60 min in standard culture condition (37°C, 5% CO_2) under protection from light.

4. Wash twice with 0.1% (w/v) PBS-BSA by centrifuging for 3 min at 120 g at room temperature.

5. Remove supernatant and add 100 µL of a solution containing FITC-conjugated goat anti-mouse antibody diluted 1:50 in 0.1% (w/v) BSA in PBS.

6.Incubate tubes for 30 min in standard culture condition (37°C, 5% CO_2) under protection from light.

7. Wash twice with 0.1% (w/v) PBS-BSA by centrifuging for 3 min at 120 g at room temperature.

8. Transfer the cells into a 1 ml FACS tube.

9. Place the 1 ml FACS tube into 5 ml FACS tube and mix well.

10. Acquire and analyze samples by means of a classic cytofluorometer allowing the acquisition of light scattering data and fluorescence recording.

Example: Original histogram and arithmetic means ± SEM of ceramide abundance in erythrocytes following exposure for 48 hours to Ringer solution without and with presence of Fascaplycin (15 µM) is shown in Fig. 7.

Uncovering involvement of kinases in the signaling of eryptosis

Erythrocytes harbor a variety of functional enzymes that stimulate eryptosis, such as caspase 8 and 3 [100], casein kinase 1α [101], protein kinase C [102], and p38 kinase [2]. Moreover, eryptosis and RBC necroptosis share several features [103]. To evaluate the involvement of enzymes in eryptosis, cells are cotreated with death inducing agents and the

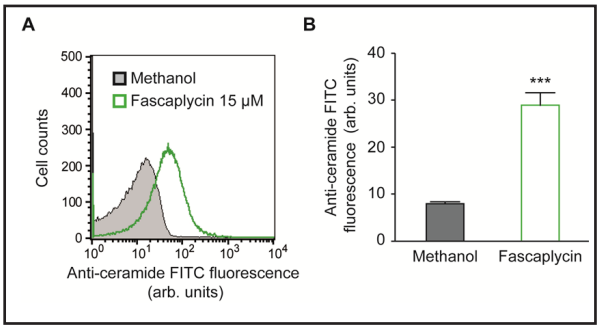

Fig. 7. Effect of Fascaplycin on ceramide abundance at the erythrocyte surface. A. Original histogram of ceramide abundance in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (green line) presence of Fascaplycin (15 µM). B. Arithmetic means ± SEM (n = 12) of the ceramide abundance (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (grey bar) or with (white bar and green line) Fascaplycin (15 µM). ***(p<0.001) indicates significant difference from the absence of Fascaplycin (15 µM).

respective inhibitors, such as 10 µM of the pan-caspase inhibitor zVAD, 50 µM of the caspase 8 inhibitor zIETD-fmk, 50 µM of the caspase 3 inhibitor zDEVD-fmk, 10 µM of the casein kinase 1α inhibitor D4476, 2 µM of the p38 kinase inhibitor SB203580, 1 µM of the protein kinase C inhibitor staurosporine, 10 µM of the protein kinase C inhibitor chelerythrine, or 100 µM of the necroptosis inhibitor necrostatin-1. Phosphatidylserine exposure at the surface is measured after 48 hours of incubation of the cells with the respective chemicals using annexin-V-binding (see above).

Conclusions

Eryptosis is the suicidal death of erythrocytes, cells lacking nuclei and mitochondria. Eryptosis shares some hallmarks of nucleated cell apoptosis, essentially cell shrinkage, phosphatidylserine exposure, membrane blebbing, and elevated ROS (reactive oxygen species) levels [2, 7, 90, 102]. The present paper outlines protocols that can be routinely used to assess eryptosis by Flow Cytometry, a powerful technique, allowing analysis of a large number of individual cells within short times. We highly recommend the described technology for further studies on triggers, inhibitors and mechanisms of this fundamental biological process with highest clinically relevance.

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Disclosure Statement

The authors declare no conflict of interest.

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