Original Paper

Ca²⁺ Entry, Oxidative Stress, Ceramide and Suicidal Erythrocyte Death Following Diosgenin Treatment

Morena Mischitelli^a Mohamed Jemaà^a Mustafa Almasry^a Caterina Faggio^b
Florian Lang^{a,c}

^aDepartments of Cardiology, Vascular Medicine and Physiology, Eberhard-Karls-University of Tuebingen, Tuebingen, Germany; ^bDepartment of Chemical, Biological, Pharmaceutical and Environmental Sciences-University of Messina Viale Ferdinando Stagno d'Alcontres S. Agata-Messina, Italy; ^cDepartment of Molecular Medicine II, Heinrich Heine University Duesseldorf, Duesseldorf, Germany

Key Words

Phosphatidylserine • Cell volume • Eryptosis • Calcium • Oxidative stress • Ceramide

Abstract

Background/Aims: The bioactive steroid sapogenin diosgenin is considered for a wide variety of applications including treatment of malignancy. The substance counteracts tumor growth in part by stimulating apoptosis of tumor cells. Similar to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, 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 Ca²⁺ activity ([Ca²⁺]_i), oxidative stress and ceramide. The present study explored, whether diosgenin induces eryptosis and, if so, to decipher cellular mechanisms involved. **Methods:** Flow cytometry was employed to estimate phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, [Ca²⁺]_i from Fluo3-fluorescence, ROS formation from DCF dependent 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 diosgenin significantly increased the percentage of annexin-V-binding cells (≥ 5 μM), significantly decreased forward scatter (15 μM), significantly increased Fluo3-fluorescence (≥ 10 μM), significantly increased DCF fluorescence (15 μM), significantly increased ceramide abundance (15 μM) and significantly increased hemolysis (15 μM). The effect of diosgenin (15 μM) on annexin-V-binding was significantly blunted but not abolished by removal of extracellular Ca²⁺. **Conclusions:** Diosgenin stimulates eryptosis with erythrocyte shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect paralleled by and at least in part due to Ca²⁺ entry, oxidative stress and ceramide.

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Florian Lang

Department of Physiology and Cardiology and Vascular Medicine,
University of Tuebingen, Gmelinstr. 5, 72076 Tuebingen (Germany)
Tel. +49 7071 29-72194, Fax +49 707129-5618, E-Mail florian.lang@uni-tuebingen.de

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Introduction

Diosgenin, a bioactive steroid sapogenin from legumes and yams [1-4], has anticancer, cardiovascular protective, anti-diabetes, neuroprotective, immunomodulatory, estrogenic, and skin protective potency [1-13]. Moreover, diosgenin may foster male fertility [14]. Diosgenin is in part effective by triggering apoptosis [4, 5, 7, 8, 10, 12, 13, 15-43]. Under appropriate experimental conditions, diosgenin may counteract apoptosis [44]. The stimulation of apoptosis by diosgenin involves oxidative stress [27, 28].

In analogy to apoptosis of nucleated cells, erythrocytes may enter eryptosis [45], the suicidal death of erythrocytes [46]. Hallmarks of eryptosis include cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface [45]. Signaling stimulating eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [45], ceramide [47], oxidative stress [45], energy depletion [45], and activated caspases [45, 48, 49]. Eryptosis may be stimulated by activation of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, p38 kinase and/or PAK2 kinase [45] and is inhibited by activation of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [45]. Eryptosis is stimulated by a wide variety of xenobiotics [45, 50-91].

The present study explored, whether diosgenin is capable to stimulate eryptosis. To this end, human erythrocytes drawn from healthy volunteers were exposed to diosgenin and phosphatidylserine abundance at the erythrocyte surface, erythrocyte volume, $[\text{Ca}^{2+}]_i$, ROS abundance and ceramide 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 21°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; pH 7.4), 5 glucose, 1 CaCl_2 , at 37°C for 48 hours. Where indicated, erythrocytes were exposed to diosgenin (MedChem Express, Princeton, 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 diosgenin 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 supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants 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.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCF). After incubation, a 150 μl suspension of erythrocytes was washed in Ringer solution and stained with DCF (Sigma, Schnelldorf, Germany) in Ringer solution containing DCF at a final concentration of 10 μM . Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCF-loaded erythrocytes were resuspended in 200 μl Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

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 $\mu\text{g}/\text{ml}$ 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 elucidated the effect of diosgenin on 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 diosgenin (5 – 15 μM). As illustrated in Fig. 1, diosgenin decreased average erythrocyte forward scatter, an effect reaching statistical significance at 15 μM diosgenin concentration. A closer analysis of the histograms revealed that treatment with diosgenin decreased the percentage of both, swollen (15 μM diosgenin) and shrunken (5 μM diosgenin) 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 diosgenin (5 – 15 μM). As shown in Fig. 2, a 48 hours exposure to diosgenin increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 5 μM diosgenin.

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 diosgenin (5 – 15 μM). As illustrated in Fig. 3, a 48 hours exposure to Diosgenin increased the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 15 μM diosgenin.

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without

Fig. 1. Effect of Diosgenin 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 15 μ M Diosgenin. 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) Diosgenin (5 - 15 μ M). C,D. Percentage of erythrocytes with (C) FSC < 200 or (D) FSC > 800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Diosgenin (1 - 10 μ M). ** (p < 0.01), *** (p < 0.001) indicate significant difference from the absence of Diosgenin (ANOVA).

Fig. 2. Effect of Diosgenin 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 (black line) presence of 15 μ M Diosgenin. B. Arithmetic means $\pm$ SEM (n = 10) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Diosgenin (5 - 15 μ M). *** (p < 0.001) indicates significant difference from the absence of Diosgenin (ANOVA).

or with diosgenin (5 - 15 μ M). As illustrated in Fig. 4A,B, a 48 hours incubation with diosgenin increased the Fluo3 fluorescence, an effect reaching statistical significance at 10 μ M diosgenin.

A next series of experiments explored whether the diosgenin-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 15 μ M diosgenin in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 5, removal of extracellular Ca^{2+} significantly blunted the effect of diosgenin on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , diosgenin significantly decreased the percentage annexin-V-binding erythrocytes.

Fig. 3. Effect of Diosgenin on hemolysis. Arithmetic means $\pm$ SEM (n = 10) of the percentage hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Diosgenin (5 - 15 μ M). *** (p < 0.001) indicates significant difference from the absence of Diosgenin (ANOVA).

Fig. 4. Effect of Diosgenin 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 15 μM Diosgenin. B. Arithmetic means $\pm$ SEM ($n = 10$) of Fluo-3 fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Diosgenin (5 - 15 μM). * ($p < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of Diosgenin (ANOVA).

Fig. 5. Ca^{2+} sensitivity of Diosgenin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Diosgenin (15 μM) 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) Diosgenin (15 μM) 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 Diosgenin, ### ($p < 0.001$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Thus, diosgenin-induced cell membrane scrambling was in part but not completely due to entry of extracellular Ca^{2+} .

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCF). As shown in Fig. 6, a 48 hours exposure to 15 μM diosgenin significantly increased the DCF fluorescence. Thus, diosgenin induced oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As shown in Fig. 7, a 48 hours exposure to 15 μM diosgenin significantly increased the ceramide abundance.

Discussion

The present observations reveal that the phytochemical diosgenin is capable to trigger eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The diosgenin

Fig. 6. Effect of Diosgenin on reactive oxygen species. A. Original histograms of DCF fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 15 μ M Diosgenin. B. Arithmetic means $\pm$ SEM (n = 10) of DCF fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Diosgenin (15 μ M). ***($p < 0.001$) indicates significant difference from the absence of Diosgenin (ANOVA).

Fig. 7. Effect of Diosgenin 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 15 μ M Diosgenin. 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) Diosgenin (15 μ M). ***($p < 0.001$) indicates significant difference from the absence of Diosgenin (ANOVA).

concentrations required for stimulation of eryptosis were slightly lower than the concentrations (30–40 μ M) triggering apoptosis [5, 15, 32, 34, 39]. The present study was performed with erythrocytes from healthy individuals. The sensitivity to the effects of diosgenin may be enhanced in clinical conditions with accelerated eryptosis including dehydration [92], hyperphosphatemia [93], chronic kidney disease (CKD) [94–97], hemolytic-uremic syndrome [98], diabetes [99], hepatic failure [100], malignancy [45], sepsis [101], sickle-cell disease [45], beta-thalassemia [45], Hb-C and G6PD-deficiency [45], as well as Wilsons disease [102].

The effect of diosgenin on cell membrane scrambling was in large part dependent on Ca^{2+} entry from the extracellular space, as it was paralleled by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), and was significantly blunted by removal of extracellular Ca^{2+} . However, diosgenin significantly triggered erythrocyte cell membrane scrambling even in the nominal absence of extracellular Ca^{2+} . Thus, additional mechanisms must have contributed to the stimulation of cell membrane scrambling following treatment of erythrocytes with diosgenin. The treatment apparently increased the abundance of reactive oxygen species and of ceramide, both powerful stimulators of eryptosis [45].

The effect of diosgenin on cell shrinkage could similarly have been secondary to Ca^{2+} entry from the extracellular space, which is known to trigger cell shrinkage due to increase of $[\text{Ca}^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [45].

At high concentrations, diosgenin triggers hemolysis [45]. In vivo, hemolysis is followed by release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and thus may lead to renal failure [103]. To the extent that eryptosis precedes hemolysis, it accomplishes clearance of defective erythrocytes prior to release of hemoglobin. Eryptosis accomplishes the clearance of erythrocytes, which are infected with the malaria pathogen *Plasmodium*. Thus, stimulation of eryptosis may favourably influence the clinical course of malaria [45].

The rapid clearance of phosphatidylserine exposing erythrocytes from circulating blood may, however, lead to anemia as soon as the loss of erythrocytes outrides the formation of new erythrocytes by erythropoiesis [45]. Phosphatidylserine exposing erythrocytes may further adhere to the vascular wall [104], stimulate blood clotting and trigger thrombosis [105-107] and thus compromise microcirculation [47, 105, 108-111].

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

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Disclosure Statement

All authors declare that there are no conflicts of interest .

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