Original Paper

Triggering of Erythrocyte Cell Membrane Scrambling by Emodin

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Key Words

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

Abstract

Background/Aims: The natural anthraquinone derivative emodin (1,3,8-trihydroxy-6-methylantraquinone) is a component of several Chinese medicinal herbal preparations utilized for more than 2000 years. The substance has been used against diverse disorders including malignancy, inflammation and microbial infection. The substance is effective in part by triggering suicidal death or apoptosis. Similar to apoptosis of nucleated cells erythrocytes may enter suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling involved in the triggering of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress and ceramide. The present study aimed to test, whether emodin induces eryptosis and, if so, to elucidate underlying cellular mechanisms. **Methods:** Phosphatidylserine abundance at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, ROS formation from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. **Results:** Exposure of human erythrocytes for 48 hours to emodin ($\geq 10 \mu\text{M}$) significantly increased the percentage of annexin-V-binding cells, and at higher concentrations ($\geq 50 \mu\text{M}$) significantly increased forward scatter. Emodin significantly increased Fluo3-fluorescence ($\geq 10 \mu\text{M}$), DCFDA fluorescence ($75 \mu\text{M}$) and ceramide abundance ($75 \mu\text{M}$). The effect of emodin on annexin-V-binding was significantly blunted but not abolished by removal of extracellular Ca^{2+} . **Conclusions:** Emodin triggers phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to stimulation of Ca^{2+} entry and paralleled by oxidative stress and ceramide appearance at the erythrocyte surface.

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Introduction

The natural anthraquinone emodin (1,3,8-trihydroxy-6-methylantraquinone) [1] is an active component of several Chinese medicinal herbs in use for more than 2000 years [2]. Effects of emodin include stimulation of diuresis [1], laxation [3], and vasodilation [1], as well as inhibition of bacterial/viral infection [1, 2, 4], peptic ulcers [1], inflammation [1, 2], and malignancy [1-3, 5-41].

The efficacy against cancer is at least in part due to stimulation of tumor cell apoptosis [3, 5-7, 16, 20, 22, 27, 38, 41-66]. Molecular mechanisms involved include tyrosine kinases [3, 31], casein kinase II [1], protein kinase C [47], AKT/mTOR [1, 28, 62], NF- κ B [1, 67, 68], HIF-1 α [1], STAT3 [1, 59], p53 [1, 21, 23, 32, 54], Wnt signaling [69], Bcl-2/Bax [21, 26, 28], mitochondria [38, 44, 45, 49, 51, 57, 63, 64], oxidative stress [21, 23, 32, 49, 57, 61, 70], and endoplasmic reticulum stress [38].

Despite their lack of mitochondria and nuclei, erythrocytes may enter apoptosis-like suicidal death or eryptosis [71]. Hallmarks of eryptosis are cell shrinkage [72] and cell membrane scrambling with translocation of phosphatidylserine to the cell surface [71]. Signaling involved in the stimulation of eryptosis includes increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [71], ceramide [73], oxidative stress [71], caspases [71, 74, 75], casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase. Signaling inhibiting eryptosis includes nitric oxide [71], AMP activated kinase AMPK [71], cGMP-dependent protein kinase [71], PAK2 kinase [71], and sorafenib/sunitinib sensitive kinases [71]. Eryptosis is triggered by energy depletion [71], and a large variety of xenobiotics [71, 76-117].

The present study tested, whether emodin is capable to stimulate eryptosis. To this end, erythrocytes were isolated from healthy volunteers, exposed to emodin and analysed by flow cytometry to determine phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, ROS formation, and ceramide abundance.

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 supernatants were 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, and 1 CaCl_2 , at 37°C for 12 – 72 hours. Where indicated, emodin (Sigma Aldrich, Hamburg, Germany) was added to the Ringer solution, or CaCl_2 was removed and 0.5 mM EGTA added to achieve nominally Ca^{2+} free solutions.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 μ l 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 emodin 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

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) after incubation under the respective experimental conditions and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of 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 μ M 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 oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μ l suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnellendorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μ l 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 μ g/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 cytometry 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 the effect of emodin on phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface as well as on erythrocyte volume.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with emodin (10 – 75 μ M). As shown in Fig. 1, a 48 hours exposure to emodin significantly increased the percentage of phosphatidylserine exposing erythrocytes at all emodin concentrations tested (10 – 75 μ M). As illustrated in Fig. 1C, the effect of 75 μ M emodin reached statistical significance within 24 hours and the effect of 25 μ M emodin exposure reached statistical significance within 48 hours.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with emodin (10 – 75 μ M). As illustrated in Fig. 2, emodin increased erythrocyte forward scatter, an effect reaching statistical significance at 50 μ M emodin concentration.

A further series of experiments explored whether emodin influences hemolysis. The percentage of hemolytic erythrocytes was determined utilizing hemoglobin concentration in

Fig. 1. Effect of emodin 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 75 μ M emodin. B. Arithmetic means $\pm$ SEM (n = 15) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) emodin (10 - 75 μ M). For comparison, the effect of the solvent DMSO is shown (grey bar). C. Time course of annexin-V-binding of erythrocytes following exposure to Ringer solution without (white bars) and with presence of 25 μ M emodin (grey bars) or 75 μ M emodin (black bars). *(p<0.05), **(p<0.01) and *** (p<0.001) indicates significant difference from the absence of emodin (ANOVA).

Fig. 2. Effect of emodin on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 75 μ M emodin. B. Arithmetic means $\pm$ SEM (n = 15) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) emodin (10 - 75 μ M). For comparison, the effect of the solvent DMSO is shown (grey bar). *(p<0.05), *** (p<0.001) indicates significant difference from the absence of emodin (ANOVA).

Fig. 3. Effect of emodin on erythrocyte hemolysis. Arithmetic means $\pm$ SEM (n = 15) of the percentage hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) emodin (10 - 75 μ M). For comparison, the effect of the solvent DMSO is shown (grey bar). *** (p<0.01) indicates significant difference from the absence of emodin (ANOVA).

the supernatant. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with emodin (10 - 75 μ M). As illustrated in Fig. 3, emodin had little effect on hemolysis up to 50 μ M emodin, but significantly increased hemolysis at 75 μ M emodin.

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Prior to loading with the fluorescent dye, the erythrocytes were again incubated for 48 hours in Ringer solution without or with emodin (10 - 75 μ M). As illustrated in Fig. 4, emodin increased Fluo3 fluorescence at all emodin concentrations tested (10 - 75 μ M).

Fig. 4. Effect of emodin on cytosolic Ca^{2+} activity. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 75 μM emodin. B. Arithmetic means $\pm$ SEM ($n = 15$) of Fluo3 fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) emodin (10 - 75 μM). For comparison, the effect of the solvent DMSO is shown (grey bar). ***($p < 0.001$) indicates significant difference from the absence of emodin (ANOVA).

Fig. 5. Ca^{2+} sensitivity of emodin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) emodin (75 μM) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 15$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with emodin (25 μM , grey bars, and 75 μM , black bars) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . *($p < 0.05$) and ***($p < 0.001$) indicates significant difference from the absence of emodin (ANOVA), ##($p < 0.01$) and ###($p < 0.001$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

In order to test whether the emodin-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 25 μM or 75 μM emodin 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 emodin on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , emodin significantly increased the percentage of annexin-V-binding erythrocytes (Fig. 5). Thus, emodin-induced cell membrane scrambling was in part, but not exclusively triggered by entry of extracellular Ca^{2+} .

Erythrocyte cell membrane scrambling is further stimulated by oxidative stress [118]. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 6, emodin (75 μM) significantly increased DCFDA fluorescence. Thus, emodin induced oxidative stress. In order to test, whether oxidative stress was required for the effect of emodin on phosphatidylserine translocation, emodin was applied in the absence and presence of antioxidant N-acetylcysteine (1 mM). As a result, the percentage of annexin-V-binding erythrocytes increased following exposure to 25 μM or 75 μM emodin for 48 hours to similar values in the absence (from 1.4 ± 0.4 % to 12.8 ± 1.2 % or 26.1 ± 2.2 %, $n = 9$ each) and presence (from 2.1 ± 0.1 % to 13.1 ± 1.6 % or 24.3 ± 3.8 %).

Fig. 6. Effect of emodin on ROS formation. A. Original histogram of DCFDA fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of emodin (75 μ M). B. Arithmetic means $\pm$ SEM (n = 15) of the DCFDA fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) emodin (75 μ M). ***($p < 0.001$) indicates significant difference from the absence of emodin (ANOVA).

Fig. 7. Effect of emodin 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 (black line) presence of emodin (75 μ M). B. Arithmetic means $\pm$ SEM (n = 15) of the ceramide abundance (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) emodin (75 μ M). ***($p < 0.001$) indicates significant difference from the absence of emodin (ANOVA).

%, n = 9 each) of N-acetylcysteine. The effect of emodin thus apparently does not require oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As illustrated in Fig. 7, emodin (75 μ M) significantly increased the ceramide abundance at the erythrocyte surface.

Discussion

The present observations reveal that emodin induces cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The emodin concentration required for the effect is well in the range of plasma concentrations observed *in vivo* [119]. Cell membrane scrambling is the most important hallmark of eryptosis, the suicidal erythrocyte death [71]. However, emodin did not induce cell shrinkage, the other hallmark of eryptosis [71]. Instead, higher concentrations of emodin triggered significant cell swelling.

The stimulating effect of emodin on cell membrane scrambling was paralleled by an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Moreover, the emodin-induced cell membrane scrambling was in part dependent on Ca^{2+} entry from the extracellular space, as removal of extracellular Ca^{2+} significantly blunted emodin induced cell membrane scrambling. However, even in the nominal absence of extracellular Ca^{2+} , emodin significantly induced cell membrane scrambling, an observation pointing to involvement of additional mechanisms. Emodin triggered oxidative stress, a well known stimulator of eryptosis [71]. The antioxidant N-acetylcysteine did, however, not significantly blunt the effect of emodin on annexin-V-binding. Emodin could thus apparently trigger erythrocyte cell membrane scrambling even in the absence of oxidative stress. Emodin further significantly enhanced the abundance of ceramide, which could sensitize cells for the scrambling effect of Ca^{2+} [71].

Despite the increase of $[\text{Ca}^{2+}]_i$, emodin did not lead to cell shrinkage. The increase of $[\text{Ca}^{2+}]_i$ were expected to activate Ca^{2+} sensitive K^+ channels leading to K^+ exit, cell membrane

hyperpolarization, Cl^- exit and thus cellular loss of KCl with water. The underlying mechanism of emodin-induced erythrocyte swelling remained elusive. Potential mechanisms include impairment of Na^+/K^+ ATPase activity which would decrease the cellular K^+ content and thus interfere with Ca^{2+} induced hyperpolarization.

High concentrations of emodin triggered hemolysis thus leading to 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 [120].

Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood and stimulation of eryptosis may lead to anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [71]. Along those lines, eryptosis may contribute to anemia in several clinical conditions, such as iron deficiency [71], dehydration [121], hyperphosphatemia [122], chronic kidney disease (CKD) [123-126], hemolytic-uremic syndrome [127], diabetes [128], hepatic failure [129], malignancy [71], sepsis [130], sickle-cell disease [71], beta-thalassemia [71], Hb-C and G6PD-deficiency [71], as well as Wilsons disease [131].

Phosphatidylserine exposing erythrocytes may further interfere with microcirculation [73, 132-136], as they adhere to the vascular wall [137], stimulate blood clotting and trigger thrombosis [132, 138, 139].

Conclusion

Emodin triggers cell membrane scrambling, an effect paralleled by increase of cytosolic Ca^{2+} activity and partially dependent on Ca^{2+} entry, and in addition paralleled by oxidative stress and increase of ceramide abundance at the erythrocyte surface.

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Disclosure Statement

None.

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