Licochalcone A Induced Suicidal Death of Human Erythrocytes

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract
Background: The anti-inflammatory, immunomodulatory, and antimicrobial *Glycyrrhiza inflata* extract component licochalcone A triggers apoptosis of tumor cells and is thus considered for the treatment of malignancy. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling involved in the triggering of eryptosis includes $\text{Ca}^{2+}$ entry with increase of cytosolic $\text{Ca}^{2+}$ activity ($[\text{Ca}^{2+}]_i$), and ceramide. The present study explored, whether and how licochalcone A induces eryptosis.

Methods: Human erythrocytes drawn from healthy individuals were exposed for 24 hours to 1–10 µg/ml licochalcone A. Flow cytometry was subsequently 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 utilizing specific antibodies. In addition, hemolysis was quantified from hemoglobin release.

Results: Licochalcone A significantly increased the percentage of annexin-V-binding cells ($\geq 5$ µg/ml), significantly decreased forward scatter (2.5 - 5 µg/ml), significantly increased Fluo3-fluorescence (≥ 7.5 µg/ml), and significantly increased ceramide abundance (10 µg/ml). The effect of licochalcone on annexin-V-binding was not significantly modified, but hemolysis significantly enhanced by removal of extracellular $\text{Ca}^{2+}$.

Conclusions: Licochalcone triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect independent from $\text{Ca}^{2+}$ entry and presumably in part due to ceramide.

Introduction

Licochalcone A, an anti-inflammatory [1-4], immunomodulatory [5, 6], and antimicrobial [3, 5, 7-9] polyphenol extracted from *Glycyrrhiza inflata* [1-3, 10], triggers apoptosis or inhibits proliferation and migration of tumor cells [11], including those of cervical cancer [12], oral squamous carcinoma [7, 9, 13-15], esophageal carcinoma [16], gastric cancer [4, 17, 18], hepatocellular carcinoma [2, 19], malignant pleural mesothelioma [20], bladder...
cancer [21, 22], prostate tumor [23-25], ovarian carcinoma [26, 27], breast cancer [24], lung cancer [28] and leukemia [29]. Licochalcone A is thus considered for the treatment of malignancy [12, 30]. Licochalcone A is partially effective by inducing oxidative stress [17, 19, 21, 26], mitochondrial depolarization [20, 22], caspase activation [13, 14, 25, 26], inhibition of phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of mTOR signaling [12, 25, 28, 31], suppression of nuclear factor-kappa B (NF-κB) [2, 9], interaction with the Jak/Stat pathway [29], suppression of cyclooxygenase (COX)-2 expression [4, 31], and inhibition of cytochrome P450 (CYP) enzymes [32].

On the other hand, licochalcone A has been shown exert cytoprotective effects by decreasing oxidative stress [33]. Licochalcone B and licochalcone D have been shown to counteract apoptosis of cardiomyocytes [34-36], an effect involving inhibition of inflammation and oxidative stress, suppression of NF-κB/p65 and p38 MAPK signaling and activation of the AKT pathway [35-37]. Licochalcone A is further effective against atopic dermatitis [38], asthma [39, 40], and osteoporosis [10, 41]. Licochalcone A further inhibits the liver X receptor α and thus interferes with lipogenesis [37, 42-44].

Similar to licochalcone A, licochalcone F counteracts inflammation [45]. The proapoptotic and antitumor effect of licochalcone A is shared by licochalcone B [46, 47] and E [48, 49], and the antimicrobial effect by licochalcone E [50].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal death of erythrocytes which is characterized by cell shrinkage and by phospholipid scrambling of the cell membrane leading to phosphatidylserine translocation to the cell surface [51]. Stimulators of eryptosis include energy depletion, increase of cytosolic Ca^{2+} activity ([Ca^{2+}]_i), ceramide, caspases, casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and p38 kinase [51]. Inhibitors of eryptosis include AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases [51].

The present study explored whether licochalcone triggers eryptosis. To this end, human erythrocytes from healthy volunteers were treated with licochalcone and phosphatidylserine surface abundance, cell volume, [Ca^{2+}], and ceramide abundance 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 24 h. Where indicated, erythrocytes were exposed to licochalcone A (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 100 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. The annexin V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). 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 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 once 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.

Ceramide abundance

To determine the ceramide abundance at the erythrocyte surface, a monoclonal antibody 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 (BD 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 may be differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study addressed the putative effect of licochalcone on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding which was quantified by flow cytometry. The erythrocytes were analysed following a 24 hours incubation in Ringer solution without or with licochalcone (1 - 10 µg/ml). As illustrated in Fig. 1, a 24 hours exposure to licochalcone increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 5 µg/ml licochalcone.

In order to estimate erythrocyte volume, forward scatter was determined by flow cytometry. Again, the measurement was made after a 24 hours incubation in Ringer solution without or with licochalcone (1 – 10 µg/ml). As shown in Fig. 2, exposure of erythrocytes to 2.5 or 5 µg/ml licochalcone decreased erythrocyte forward scatter. No significant alterations of cell volume were observed at lower (1 µg/ml) and higher concentrations (7.5 and 10 µg/ml of licochalcone.

Fluo3-fluorescence was taken as measure of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)). As illustrated in Fig. 3, a 24 hours exposure to licochalcone increased the Fluo3-fluorescence, an effect reaching statistical significance at 7.5 µg/ml licochalcone.

In order to test whether licochalcone-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca\(^{2+}\), erythrocytes were incubated for 24 hours in the absence or presence of 5 or 10 µg/ml licochalcone in the presence or nominal absence of extracellular Ca\(^{2+}\). As illustrated in Fig. 4, removal of extracellular Ca\(^{2+}\) did not significantly modify the effect of 5 µg/ml and 10 µg/ml licochalcone. Thus, licochalcone-induced cell membrane scrambling was not dependent on entry of extracellular Ca\(^{2+}\).

As shown in Fig. 5, following removal of extracellular Ca\(^{2+}\), 5 or 10 µg/ml licochalcone still tended to decrease forward scatter, an effect, however not reaching statistical significance.
In order to quantify hemolysis, the hemoglobin concentration in the supernatant was determined by photometry. As shown in Fig. 6, a 24 hours incubation with licochalcone increased the percentage of hemolysed erythrocytes. Removal of extracellular Ca$^{2+}$ did not significantly modify the effect of 5 µg/ml licochalcone on hemolysis. However, removal of
extracellular Ca\(^{2+}\) significantly augmented the stimulating effect of 10 µg/ml licochalcone on hemolysis. Ca\(^{2+}\) independent triggers of eryptosis include ceramide. Thus, specific antibodies have been employed to quantify the ceramide abundance at the erythrocyte surface. As illustrated

Fig. 4. Ca\(^{2+}\) insensitivity of licochalcone-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of licochalcone (5 and 10 µg/ml) in the presence (A) and absence (B) of extracellular Ca\(^{2+}\). C. Arithmetic means ± SEM (n = 8) of annexin-V-binding of erythrocytes after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) licochalcone (5 or 10 µg/ml) in the presence (left bars, +Ca\(^{2+}\)) and absence (right bars, -Ca\(^{2+}\)) of Ca\(^{2+}\). *(p<0.05), ***(p<0.001) indicates significant difference from the absence of licochalcone.

Fig. 5. Impact of Ca\(^{2+}\) removal on licochalcone-induced alterations of erythrocyte volume. A,B. Original histograms of erythrocyte forward scatter following exposure for 24 hours to Ringer solution without (grey areas) and with (black lines) presence of licochalcone (10 µg/ml) in the presence (A) and absence (B) of extracellular Ca\(^{2+}\). C. Arithmetic means ± SEM (n = 8) of erythrocyte forward scatter after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) licochalcone (5 or 10 µg/ml) in the presence (left bars, +Ca\(^{2+}\)) and absence (right bars, -Ca\(^{2+}\)) of Ca\(^{2+}\). *(p<0.05), ***(p<0.001) indicates significant difference from the absence of licochalcone.

Fig. 6. Impact of Ca\(^{2+}\) removal of licochalcone-induced hemolysis. Arithmetic means ± SEM (n = 4) of annexin-V-binding of erythrocytes after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) licochalcone (5 or 10 µg/ml) in the presence (left bars, +Ca\(^{2+}\)) and absence (right bars, -Ca\(^{2+}\)) of Ca\(^{2+}\). ***(p<0.001) indicates significant difference from the absence of licochalcone, ###(p<0.001) indicates significant difference from the presence of Ca\(^{2+}\) (ANOVA).
in Fig. 7, a 24 hours exposure to licochalcone (10 µg/ml) significantly increased the ceramide abundance.

Discussion

The present study discloses a novel effect of licochalcone, i.e. the triggering of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to licochalcone results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for the effect are in the range of those required to induce apoptosis of tumor cells [17].

The effect of licochalcone on cell membrane scrambling and cell shrinkage was paralleled by increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)). Ca\(^{2+}\) entry from the extracellular space appears not to be required, however, for the effect of licochalcone on cell membrane scrambling. Removal of extracellular Ca\(^{2+}\) disrupted, however, the licochalcone induced cell shrinkage. An increase of [Ca\(^{2+}\)]\(_i\), activates Ca\(^{2+}\) sensitive K\(^+\) channels which contributes to or even accounts for the cell shrinkage due to K\(^+\) exit, cell membrane hyperpolarization, Cl\(^-\) exit and thus cellular loss of KCl with water [52]. Following an increase of licochalcone concentration to 7.5 or 10 µg/ml the licochalcone induced cell shrinkage disappeared despite an increasing [Ca\(^{2+}\)], Possibly, licochalcone has a dual effect on erythrocyte cell volume, a Ca\(^{2+}\) dependent cell shrinkage and a Ca\(^{2+}\) independent cell swelling. Possibly due to the latter effect, licochalcone triggers hemolysis, an effect augmented by removal of extracellular Ca\(^{2+}\). It is actually the purpose of the Ca\(^{2+}\) sensitive K\(^+\) channels to counteract swelling of defective erythrocytes [51]. The phosphatidylserine exposure is followed by rapid clearance of the defective erythrocytes from circulating blood [51]. The preceding eryptosis is thus supposed to prevent hemolysis. The hemolysis would otherwise lead to release of hemoglobin, which is filtered in renal glomerula, precipitates in the acidic lumen of renal tubules and thus occludes nephrons [53].

Excessive eryptosis may, on the other hand, lead to anemia, if the formation of new erythrocytes cannot match the loss of eryptotic erythrocytes [51]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [54], stimulate blood clotting and trigger thrombosis [55-57]. Thus, triggering of eryptosis may impair microcirculation [55, 58-62].

It is expected that the sensitivity to the eryptotic effect of licochalcone is increased by clinical conditions associated with enhanced eryptosis, such as dehydration [63],
hyperphosphatemia [64] chronic kidney disease (CKD) [65-68], hemolytic-uremic syndrome [69], diabetes [70], hepatic failure [71], malignancy [51], sepsis [72], sickle-cell disease, beta-thalassemia, Hb-C and G6PD-deficiency [51], as well as Wilsons disease [73]. In those disorders lower licochalcone concentrations may be sufficient to trigger eryptosis. Moreover, eryptosis is stimulated by a wide variety of xenobiotics [63, 64, 66, 74-96]. Combination of licochalcone with those xenobiotics may lead to additive effects.

In conclusion, licochalcone triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect not depending on Ca\(^{2+}\) activity but paralleled by and presumably in part due to increased ceramide abundance.

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**Disclosure Statement**

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