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Gossypol-induced suicidal erythrocyte death

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ABSTRACT

Side effects of gossypol, a polyphenolic component of *Gossypium*, with male contraceptive, anticancer, antimicrobial and antiviral activities include anemia due to accelerated demise of erythrocytes. Erythrocytes may be cleared from circulating blood following apoptosis-like suicidal death or eryptosis. Hallmarks of eryptosis are cell shrinkage and cell membrane scrambling with subsequent phosphatidylserine-exposure at the cell surface. Stimulators of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$). The present study explored, whether gossypol stimulates eryptosis of human erythrocytes. Utilizing flow cytometry, $[Ca^{2+}]_i$ was estimated from Fluo-3 fluorescence, cell volume from forward scatter, phosphatidylserine-exposure from annexin-V-binding, and hemolysis from hemoglobin release. A 48 h exposure to gossypol (0.75 μM) significantly increased $[Ca^{2+}]_i$, decreased forward scatter and increased annexin-V-binding. Gossypol exposure was followed by a slight but significant increase of hemolysis. Removal of extracellular Ca^{2+} significantly blunted the effect of gossypol (1 μM) on annexin-V-binding. The present observations reveal a novel effect of gossypol on human erythrocytes, which contributes to or even accounts for the triggering of anemia by this substance.

1. Introduction

Gossypol, a component of the cotton plant *Gossypium* (Wang et al., 2009), has been used as male contraceptive (Abou-Donia et al., 1989; Aneja et al., 2003; Dodou et al., 2005; Gafvels et al., 1984; Heywood et al., 1986; Kovacic, 2003) and shown to exert anticancer, antioxidant, antiviral, antibacterial, antitrypanosomal, and antimalarial activities (Dodou et al., 2005; Kalla, 1990; Kovacic, 2003; Wang et al., 2009). The use of gossypol is limited by several side effects including inhibition of 11β-hydroxysteroid dehydrogenase (Ma et al., 2011; Reidenberg, 2000) and hemolytic anemia with hyperbilirubinemia (Akingbemi et al., 1995; Aneja et al., 2003).

At least in theory, the gossypol-induced hemolytic anemia could result from apoptosis-like suicidal erythrocyte death or eryptosis. Eryptosis is elicited by a wide variety of xenobiotics (Bhavsar et al., 2010a,b, 2011; Eberhard et al., 2010; Felder et al., 2011; Ganesan et al., 2012; Gatidis et al., 2011; Ghashghaeinia et al., 2011; Lang et al., 2011; Lupescu et al., 2012; Nguyen et al., 2011; Qadri et al., 2011a,b,c; Zelenak et al., 2012) and contributes to accelerated erythrocyte loss in several clinical disorders (Lang et al., 2008), including hemolytic uremic syndrome (Lang et al., 2008), sepsis (Lang et al., 2008), renal insufficiency (Lang et al., 2008), diabetes (Calderon-Salinas et al., 2011; Maellaro et al., in press), sickle cell disease (Lang et al., 2008), malaria (Bobbala et al., 2010; Siraskar et al., 2010), Wilson's disease (Lang et al., 2008), iron deficiency (Lang et al., 2008), phosphate depletion (Lang et al., 2008) and presumably metabolic syndrome (Zappulla, 2008).

Hallmarks of eryptosis include cell membrane scrambling and cell shrinkage (Lang et al., 2008). Signaling of eryptosis includes Ca^{2+} entry through Ca^{2+}-permeable cation channels (Foller et al., 2008b, 2009b). The increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) leads to activation of Ca^{2+}-sensitive K^+ channels (Brugnara et al., 1993) resulting in K^+ exit, hyperpolarization and Cl^- exit (Lang et al., 2003). The cellular KCl loss together with osmotically obliged water results in cell shrinkage (Lang et al., 2003). Increased $[Ca^{2+}]_i$ further triggers cell membrane scrambling with phosphatidylserine exposure at the cell surface (Berg et al., 2001). The $[Ca^{2+}]_i$ sensitivity of erythrocyte cell membrane scrambling is enhanced by ceramide, which is similarly able to trigger eryptosis (Lang et al., 2010). Eryptosis may be further stimulated by energy depletion (Klarl et al., 2006) and activation of caspases (Bhavsar et al., 2010a; Lang et al., 2008; Lau et al., in press; Maellaro et al., in press). Signaling governing eryptosis further includes AMP activated kinase AMPK (Foller et al., 2009b), cGMP-dependent protein kinase (Foller et al., 2008a) and Janus-activated kinase JAK3 (Bhavsar et al., 2011).

The present study explored, whether gossypol stimulates eryptosis and, if so, to elucidate the signaling involved.

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2. Materials and methods

2.1. Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes 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 (184/2003V). 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), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37 °C for 48 h. Where indicated, erythrocytes were exposed to gossypol (Enzo, Lörrach, Germany) at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA).

2.2. FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental condition, 50 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. In the following, the forward scatter (FSC) of the cells was determined, and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

2.3. Measurement of 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 2 μM Fluo-3/AM. The cells were incubated at 37 °C for 30 min and washed twice 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 in fluorescence channel FL-1 in FACS analysis.

2.4. Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (3 min at 400 × *g*, room temperature) after incubation, 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.

2.5. 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 are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

3. Results

In a first series of experiments, cytosolic Ca^{2+} activity was estimated in erythrocytes from Fluo3 fluorescence in flow cytometry. As shown in Fig. 1, treatment of human erythrocytes with gossypol was followed by an increase of Fluo-3 fluorescence, an effect reaching statistical significance at 0.75 μM gossypol concentration. Thus, gossypol increased cytosolic Ca^{2+} concentration in human erythrocytes.

Increased cytosolic Ca^{2+} concentration has previously been shown to activate Ca^{2+}-sensitive K^+ channels with subsequent exit of KCl and osmotically obliged water (Lang et al., 2008). The increase of cytosolic Ca^{2+} concentration was thus expected to result in erythrocyte shrinkage. In order to test, whether gossypol exposure was followed by cell shrinkage, cell volume was estimated in a second series of experiments utilizing forward scatter in flow cytometry. As illustrated in Fig. 2, gossypol treatment decreased forward scatter, an effect reaching statistical significance at 0.25 μM gossypol concentration.

According to previous studies, increased cytosolic Ca^{2+} activity further leads to cell membrane scrambling with phosphatidylserine exposure at the cell surface (Lang et al., 2008). In order to test whether gossypol treatment results in erythrocyte cell membrane scrambling, phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, which was determined by flow cytometry in a third series of experiments. As shown in Fig. 3, a 48 h exposure to gossypol increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 0.75 μM gossypol concentration.

A fourth series of experiments addressed the putative effect of gossypol on hemolysis. To this end, the percentage of hemolysed erythrocytes was estimated from hemoglobin release into the supernatant. As shown in Fig. 3, exposure of erythrocytes for 48 h to gossypol was followed by an increase of hemoglobin concentration in the supernatant, an effect reaching statistical significance at 1.0 μM (Fig. 3B). The percentage of hemolysed erythrocytes remained, however, one order of magnitude lower than the percentage of phosphatidylserine exposing erythrocytes.

A fifth series of experiments explored whether the gossypol induced cell membrane scrambling was a consequence of the effect of gossypol on Ca^{2+} entry. To this end, erythrocytes

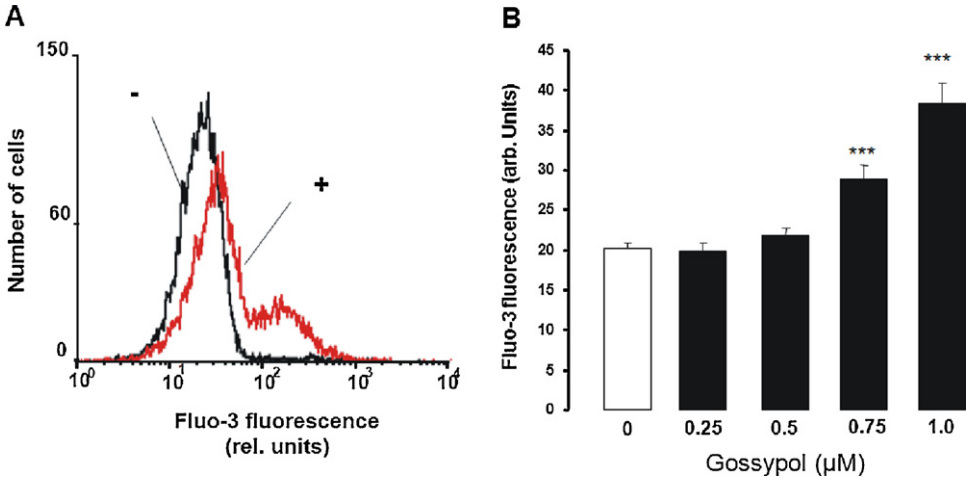

Fig. 1. Effect of gossypol on erythrocyte cytosolic Ca^{2+} concentration. (A) Original histogram of Fluo-3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, red line) presence of 1 μM gossypol. (B) Arithmetic means ± SEM ($n = 12$) of the normalized geo means (geometric mean of the histogram in arbitrary units) of Fluo-3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) gossypol (0.25–1 μM). ***$p < 0.001$ indicates significant difference from the absence of gossypol (ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)