Original Paper

Stimulation of Suicidal Erythrocyte Death by Garcinol

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background/Aims: The benzophenone garcinol from dried fruit rind of *Garcinia indica* counteracts malignancy, an effect at least in part due to stimulation of apoptosis. The proapoptotic effect of garcinol is attributed in part to inhibition of histone acetyltransferases and thus modification of gene expression. Moreover, garcinol triggers mitochondrial depolarisation. Erythrocytes lack gene expression and mitochondria but are nevertheless able to enter apoptosis-like suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include oxidative stress, energy depletion and Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). The present study explored, whether and how garcinol induces eryptosis. **Methods:** To this end, phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, hemolysis from hemoglobin release, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, ROS formation from DCFDA dependent fluorescence and cytosolic ATP levels utilizing a luciferin-luciferase-based assay. **Results:** A 24 hours exposure of human erythrocytes to garcinol (2.5 or 5 μM) significantly increased the percentage of annexin-V-binding cells. Garcinol decreased (at 1 μM and 2.5 μM) or increased (at 5 μM) forward scatter. Garcinol (5 μM) further increased Fluo3-fluorescence, increased DCFDA fluorescence, and decreased cytosolic ATP levels. The effect of garcinol on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+} . **Conclusions:** Garcinol triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to stimulation of ROS formation, energy depletion and Ca^{2+} entry.

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Introduction

The benzophenone garcinol [1], a component of the dried fruit rind of *Garcinia indica* [1-3], has been shown to counteract oxidative stress [2-4], inflammation [2, 4, 5] and

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malignancy [2-17]. Garcinol is effective against malignancy at least in part by triggering suicidal cell death or apoptosis [8, 11, 12, 14, 16, 18-22]. On the other hand, garcinol could counteract apoptosis [23]. Cellular mechanisms involved in the effects of garcinol include inhibition of histone acetyltransferases and thus modification of gene expression [10, 13, 15, 18, 24-27], down-regulation of nuclear factor NF- κ B [5, 7, 8, 16, 18, 19, 23], signal transducer and activator of transcription 3 (STAT3) [5, 6, 28], cyclin-dependent kinase 2 (CDK2) [4], p38 kinase [4], and phosphoinositide 3 kinase (PI3K)/Akt signaling [21]. Garcinol treatment leads to growth arrest and expression of DNA damage-inducible gene 153 (GADD153) [29]. Garcinol further triggers mitochondrial depolarisation [29].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis [30], a suicidal death characterized by cell shrinkage [31] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [30]. Triggers of eryptosis include Ca^{2+} entry through Ca^{2+} permeable unselective cation channels with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Eryptosis is further stimulated by ceramide [32], oxidative stress [30], energy depletion [30], activated caspases [30, 33, 34], casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C and p38 kinase [30]. Eryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, and sorafenib/sunitinib sensitive kinases [30]. Eryptosis may be stimulated by a wide variety of xenobiotics [30, 35-60].

The present study explored whether and how garcinol triggers eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to garcinol and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$ and abundance of reactive oxygen species (ROS) 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 garcinol (Tocris bioscience, Bristol, UK) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 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 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 (3 min at 1600 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.

Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 100 μl suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnellendorf, Germany) in PBS containing DCFDA at a final concentration of 10 μM . Erythrocytes were incubated at 37°C for 30 min in the dark and then washed in PBS. The DCFDA-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).

Intracellular ATP concentration

For the determination of intracellular erythrocyte ATP, 80 μl of erythrocyte pellets were incubated for 24 h at 37°C in Ringer solution (final hematocrit 4.7%). All subsequent manipulations were performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of HClO_4 (6%). After centrifugation, an aliquot of the supernatant (400 μl) was adjusted to pH 7.7 by addition of saturated KHCO_3 solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin-luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l cytosol of erythrocytes.

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 tested, whether garcinol modifies eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. In order to uncover cell membrane scrambling, phosphatidylserine at the erythrocyte surface was visualized with annexin-V-binding, which was determined by flow cytometry. Prior to measurement, erythrocytes were incubated for 24 hours in Ringer solution without or with garcinol (1 – 5 μM). As shown in

Fig. 1. Effect of garcinol on phosphatidylserine exposure. (A) Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 5 μM garcinol. (B) Arithmetic means $\pm$ SEM (*n* = 12) of erythrocyte annexin-V-binding (black bars) following incubation for 24 hours to Ringer solution without or with presence of garcinol (1 – 5 μM). For comparison, arithmetic means $\pm$ SEM (*n* = 12) of hemolysis are shown (grey bars). * (*p* < 0.05), *** (*p* < 0.001), ### (*p* < 0.001) indicate significant difference from the absence of garcinol (ANOVA).

Fig. 2. Effect of garcinol on erythrocyte forward scatter. (A) Original histogram of forward scatter of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 2.5 μM garcinol. (B) Arithmetic means $\pm$ SEM ($n = 12$) of the erythrocyte forward scatter (FSC) following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) garcinol (1 - 5 μM). ** ($p < 0.01$), *** ($p < 0.001$) indicate significant difference from the absence of garcinol (ANOVA).

Fig. 3. Effect of garcinol on erythrocyte Ca^{2+} activity. (A) Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of garcinol (5 μM). (B) Arithmetic means $\pm$ SEM ($n = 12$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 24 hours to Ringer solution without (white bar) or with (black bars) garcinol (1 - 5 μM). *** ($p < 0.001$) indicate significant difference from the absence of garcinol (ANOVA).

Fig. 1, a 24 hours exposure to garcinol increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 2.5 μM garcinol. For comparison, hemolysis was quantified from the hemoglobin concentration in the supernatant. As a result, the percentage of hemolytic erythrocytes was significantly higher following exposure to 5 μM garcinol than in the absence of garcinol (Fig. 1). However, the percentage of hemolytic erythrocytes remained lower than the percentage of annexin-V-binding erythrocytes (Fig. 1).

Erythrocyte volume was estimated from forward scatter, which was determined utilizing flow cytometry. As illustrated in Fig. 2, a 24 hours incubation in Ringer solution with 1 and 2.5 μM garcinol was followed by a significantly lower erythrocyte forward scatter than a 24 hours incubation in Ringer solution without garcinol. In contrast, a 24 hours incubation in Ringer solution with 5 μM garcinol was followed by a significantly higher erythrocyte forward scatter than a 24 hours incubation in Ringer solution without garcinol. Thus, 1 μM and 2.5 μM garcinol shrank but 5 μM garcinol swelled erythrocytes.

In order to quantify cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), Fluo3 fluorescence was measured. As illustrated in Fig. 3, a 24 hours exposure to garcinol increased the Fluo3 fluorescence, an effect reaching statistical significance at 5 μM garcinol.

In order to test whether garcinol-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 μM garcinol in the presence or nominal absence of

Fig. 4. Ca²⁺ sensitivity of garcinol-induced phosphatidylserine exposure. (A, B) Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of garcinol (5 µM) in the presence (A) and absence (B) of extracellular Ca²⁺. (C) Arithmetic means ± SEM (n = 12) of annexin-V-binding of erythrocytes after a 24 hours treatment with Ringer solution without (white bars) or with (black bars) garcinol (5 µM) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. *** (P<0.001) indicates significant difference from the absence of garcinol, ##(p<0.01) indicate significant difference from the presence of Ca²⁺ (ANOVA).

Fig. 5. Bay 11-7082 sensitivity of garcinol-induced phosphatidylserine exposure. (A, B) Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of garcinol (5 µM) in the absence (A) and presence (B) of Bay 11-7082 (20 µM). (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) garcinol (5 µM) in the absence (left bars, -BAY) and presence (right bars, +BAY) of Bay 11-7082 (20 µM). *** (P<0.001) indicates significant difference from the absence of garcinol (ANOVA).

extracellular Ca²⁺. As shown in Fig 4, removal of extracellular Ca²⁺ slightly, but significantly blunted the effect of garcinol on annexin-V-binding. However, garcinol significantly increased the percentage of annexin-V-binding erythrocytes even in the absence extracellular Ca²⁺. Garcinol-induced cell membrane scrambling was thus partially but not fully due to entry of extracellular Ca²⁺.

Removal of extracellular Ca²⁺ did not significantly blunt the effect of 5 µM garcinol on forward scatter. In the absence of garcinol the forward scatter was virtually identical in the presence (437 ± 8, n = 12) and in the absence (437 ± 5, n = 12) of extracellular Ca²⁺ and incubation with 5 µM garcinol increased the forward scatter to similar values in the presence (473 ± 6, n = 12) and in the absence (480 ± 5, n = 12) of extracellular Ca²⁺.

Additional experiments explored whether the effect of garcinol was modified by the NF-κB inhibitor Bay 11-7082. As a result, Bay 11-7082 (20 µM) tended to increase annexin-V-binding of erythrocytes in the presence and absence of 5 µM garcinol, an effect, however, not reaching statistical significance (Fig. 5).

In order to quantify oxidative stress, reactive oxygen species (ROS) was determined with 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). As shown in Fig. 6, a 24 hours

Fig. 6. Effect of garcinol on erythrocyte ROS formation. (A) Original histogram of DCFDA fluorescence in erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of garcinol (5 μM). (B) Arithmetic means ± SEM (n = 12) of the DCFDA fluorescence (arbitrary units) in erythrocytes exposed for 24 hours to Ringer solution without (white bar) or with (black bars) garcinol (1–5 μM). *** (p<0.001) indicate significant difference from the absence of garcinol (ANOVA).

Fig. 7. N-Acetylcysteine insensitivity of garcinol-induced phosphatidylserine exposure. (A, B) Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of garcinol (5 μM) in the absence (A) and presence (B) of N-acetylcysteine (3 mM). (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) garcinol (5 μM) in the absence (left bars, -N-acetylcysteine) and presence (right bars, +N-acetylcysteine) of N-acetylcysteine (3 mM). *** (P<0.001) indicates significant difference from the absence of garcinol (ANOVA).

Fig. 8. Effect of garcinol on erythrocyte ATP concentration. Arithmetic means ± SEM (n = 5) of the cytosolic ATP concentrations (arb. units) in erythrocytes exposed for 24 hours to Ringer solution without (white bar) or with (black bars) garcinol (5 μM). * (p<0.05) indicate significant difference from the absence of garcinol (t-test).

exposure to garcinol (1–5 μM) significantly increased the DCFDA fluorescence. Accordingly, garcinol induced oxidative stress.

Additional experiments explored whether the effect of garcinol was modified by the antioxidant N-acetylcysteine (3 mM). As a result, N-acetylcysteine did not significantly modify the effect of 5 μM garcinol on annexin-V-binding of erythrocytes (Fig. 7).

In order to explore whether garcinol triggers energy depletion, ATP levels were determined utilizing a luciferin–luciferase assay. As illustrated in Fig. 8, a 24 hours exposure to garcinol decreased the cytosolic ATP levels, an effect reaching statistical significance at 5 μM garcinol concentration.

Discussion

The present study reveals that exposure of human erythrocytes to garcinol is followed by stimulation of cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Moreover, lower concentrations of garcinol (1 and 2.5 μ M) trigger cell shrinkage, the second hallmark of eryptosis, the suicidal erythrocyte death. The concentrations required for the effect are lower than those required to counteract growth of tumor cells [20]. In theory, lower garcinol concentrations may be required for triggering of eryptosis in clinical conditions associated with enhanced eryptosis susceptibility of the erythrocytes, such as dehydration [48], hyperphosphatemia [58] chronic kidney disease (CKD) [40, 61-63], hemolytic-uremic syndrome [64], diabetes [65], hepatic failure [66], malignancy [30], sepsis [67], Sickle-cell disease [30], beta-thalassemia [30], Hb-C and G6PD-deficiency [30], as well as Wilsons disease [68]. In those disorders lower garcinol concentrations may be sufficient to trigger eryptosis.

The effect of garcinol on cell membrane scrambling was in small part due to increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), which is known to trigger cell membrane scrambling by activating an illdefined scramblase [30]. Accordingly, removal of extracellular Ca^{2+} significantly blunted cell membrane scrambling. Ca^{2+} entered presumably through Ca^{2+} permeable cation channels, which are known to be activated by oxidative stress [30]. DCFDA fluorescence indeed revealed that high concentrations of garcinol increased the abundance of reactive oxidant species.

An increase of $[\text{Ca}^{2+}]_i$ could further trigger erythrocyte shrinkage by activation of Ca^{2+} sensitive K^+ channels with subsequent cell shrinkage due to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [31]. However, higher concentrations of garcinol are required to appreciably increase $[\text{Ca}^{2+}]_i$ and higher concentrations of garcinol increase rather than decrease erythrocyte volume. The increase of cell volume may result from the observed decline of ATP levels, which should compromise the function of the Na^+/K^+ ATPase with subsequent increase of cytosolic Na^+ , as well as decline of cytosolic K^+ with subsequent depolarization and gain of Cl^- with osmotically obliged water [69].

The most important functional significance of eryptosis is removal of defective erythrocytes prior to hemolysis [30]. Hemolysis triggers release of hemoglobin, which is filtered in renal glomerula with subsequent precipitation in the acidic lumen of renal tubules and thus occlusion of nephrons [70]. Eryptosis further fosters the removal of erythrocytes infected with the malaria pathogen *Plasmodium*. *Plasmodia* induce oxidative stress with subsequent activation of Ca^{2+} -permeable erythrocyte cation channels [30, 71]. Several genetic erythrocyte disorders, such as sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency enhance the susceptibility to triggers of eryptosis and thus accelerate the removal of infected erythrocytes. As a result, the disorders blunt parasitemia and thus protect against a severe course of malaria [30, 72-74]. Eryptosis is further fostered and thus increase of parasitemia slowed by iron deficiency [75] as well as treatment with lead [75], chlorpromazine [76] or NO synthase inhibitors [76]. In theory, garcinol may similarly foster eryptosis of *plasmodium* infected erythrocytes.

On the other hand, eryptosis may lead to anemia as soon as the loss of erythrocytes is not matched by a similar increase of erythrocyte formation [30]. Moreover, phosphatidylserine exposing erythrocytes may adhere to the vascular wall [77] and stimulate clotting as well as thrombosis [78-80]. Accordingly, excessive eryptosis may interfere with microcirculation [32, 78, 81-84].

Conclusion

Garcinol triggers cell membrane scrambling and cell shrinkage. At higher concentrations, garcinol leads to cell swelling, energy depletion, oxidative stress and increase of cytosolic Ca^{2+} activity.

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Disclosure Statement

The authors state that they have nothing to disclose.

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