Caffeine Inhibits Suicidal Erythrocyte Death

Elisa Floride^{1,2}, Michael Föller¹, Markus Ritter² and Florian Lang¹

¹Department of Physiology, University of Tübingen, ²Department of Physiology and Pathophysiology, Paracelsus Medical University Salzburg

Key Words

Apoptosis • Eryptosis • Phosphatidylserine • Calcium
• Energy depletion

Abstract

Eryptosis, the suicidal death of erythrocytes, is characterized by cell shrinkage and by cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Eryptosis is triggered by several stress conditions including isotonic cell shrinkage (Cl⁻ removal) and energy depletion (glucose removal). Both are effective through an increase in the cytosolic Ca²⁺ concentration. Phosphatidylserine-exposing erythrocytes are cleared from circulating blood. Enhanced eryptosis thus leads to anemia. Accordingly, drugs interfering with eryptosis may prove useful in the treatment of anemia. The present study explored, whether caffeine interferes with eryptosis. Erythrocyte phosphatidylserine exposure was estimated from annexin V-binding, cell volume from forward scatter and cytosolic Ca²⁺ activity from Fluo3 fluorescence. Under control conditions, eryptosis affected less than 5% of the erythrocytes and was not significantly modified by the presence of caffeine (50-500 μ M). Glucose depletion (for 48 hours) significantly increased Fluo3 fluorescence and

annexin V-binding and decreased forward scatter, effects partially reversed by caffeine (500 μ M). Low Cl⁻ solution (Cl⁻ exchanged by gluconate for 48 hours) similarly increased annexin V-binding and decreased forward scatter, effects again reversed by caffeine (50-500 μ M). In conclusion, caffeine inhibits Ca²⁺ entry following glucose depletion and thus counteracts eryptosis during isotonic cell shrinkage and energy depletion.

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Introduction

Eryptosis, the suicidal death of erythrocytes [1], is characterized by cell shrinkage and phosphatidylserine exposure at the cell surface [2-4].

Mechanisms involved in the triggering of eryptosis include activation of Ca²⁺-permeable erythrocyte cation channels [5], entry of Ca²⁺, activation of Ca²⁺-sensitive K⁺ channels, KCl exit, osmotic loss of cellular water and cell shrinkage [6, 7]. Ca²⁺ further triggers Ca²⁺-sensitive scrambling of the cell membrane [8] with phosphatidylserine exposure at the cell surface.

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Prof. Dr. F. Lang
Physiologisches Institut der Universität Tübingen
Gmelinstr. 5, D-72076 Tübingen (Germany)
Tel. +49 7071 29 72194, Fax +49 7071 29 5618
E-Mail florian.lang@uni-tuebingen.de

Cell membrane scrambling is further triggered by ceramide (acylsphingosine) [9] and by activation of protein kinase C [10, 11].

Phosphatidylserine-exposing erythrocytes are engulfed by macrophages [2-4, 12, 13]. Thus, similar to erythrocyte senescence [14-16] and neocytolysis [17], eryptosis precedes the clearance of erythrocytes from circulating blood. Phosphatidylserine-exposing erythrocytes further bind to receptors in the vascular wall and may thus impede microcirculation [18-20] and trigger haemostasis [18]. Enhanced trapping of annexin V-binding erythrocytes has indeed been observed in renal medulla following ischemia of the mouse kidney [21].

Enhanced eryptosis parallels a wide variety of anemic conditions such as iron deficiency [13], phosphate depletion [22], Hemolytic Uremic Syndrome [23], sepsis [24], malaria [25], Wilson's disease [26], sickle cell disease [27-29], thalassemia [28], and glucose-phosphate dehydrogenase deficiency [28]. Moreover, eryptosis may be stimulated by a variety of chemicals including cordycepin [30], Bay-5884 [31], curcumin [32], methylglyoxal [33], amyloid peptides [34], listeriolysin [35], paclitaxel [36], valinomycin [37], chlorpromazine [38], cyclosporine [39], lead [40], mercury [41] and copper [26].

Eryptosis is inhibited by adenosine [42], which is effective by blunting Ca^{2+} entry and thus Ca^{2+} -dependent cell membrane scrambling and cell shrinkage. The present experiments were performed to determine whether caffeine, which is known to interact with adenosine receptors, phosphodiesterases, channels, and intracellular Ca^{2+} release [43-46] similarly influences eryptosis. Caffeine has been shown to inhibit [47-49] or to stimulate [50-53] apoptosis of nucleated cells, an effect depending on the cell type and the presence of other challenges.

To explore the effect of caffeine on eryptosis, erythrocytes were exposed to energy depletion due to glucose withdrawal or to isotonic cell shrinkage due to Cl^- removal in the presence and absence of caffeine (50-500 μM).

Materials and Methods

Erythrocytes, solutions and chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers. The erythrocytes were kindly provided by the blood bank of the University of Tübingen. The volunteers provided informed consent. The study has been approved by the ethics committee of the University of Tübingen (184/2003V).

Ringer solution contained (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. Where indicated, Cl^- was replaced by gluconate or glucose removed from Ringer solution. Removal of Cl^- leads to exit of KCl and osmotically obliged water and thus to cell shrinkage, a well known trigger of eryptosis [1]. Caffeine and staurosporine were purchased from Sigma (Schnelldorf, Germany), and the Ca^{2+} dye Fluo-3/AM from Calbiochem (Bad Soden, Germany).

FACS analysis of annexin V-binding and forward scatter

FACS analysis was performed as described. After incubation, cells were washed in Ringer solution + 4 mM CaCl_2 . Erythrocytes were stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin-V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} measurements were performed as described previously [54]. Briefly, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer solution. Then, Ca^{2+} -dependent fluorescence intensity was measured in FL-1.

Statistics

Data are expressed as arithmetic means $\pm$ SEM, and statistical analysis was made by ANOVA.

Results

Annexin V was employed to identify suicidal (i.e. eryptotic) erythrocytes [1]. The percentage of erythrocytes exposing annexin V following incubation for 48 h in Ringer solution approached $2.8 \pm 0.4\%$ ($n = 8$). Addition of caffeine (50-500 μM) did not significantly modify the percentage of annexin V-binding erythrocytes following exposure to control Ringer (Fig. 1B).

Known triggers of eryptosis include energy depletion, which is accomplished by glucose removal [11]. Glucose depletion leads to Ca^{2+} entry, which stimulates scrambling of the cell membrane with phosphatidylserine exposure at the cell surface [11]. As illustrated in Fig. 1B, glucose withdrawal indeed significantly increased the percentage of annexin V-binding erythrocytes. Annexin V-binding was blunted in the presence of caffeine (500 μM).

Since protein kinase C (PKC) is known to mediate

Fig. 1. Stimulation of phosphatidylserine exposure by glucose depletion in the presence and absence of caffeine. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours without glucose in the absence (black line) or presence (red line) of 500 μ M caffeine. B. Arithmetic means $\pm$ SEM ($n = 4-8$) of the percentage of annexin V-binding erythrocytes following incubation for 48 hours in the presence (+ glu) or absence (- glu) of glucose without (white bar) or with (black bars) 50-500 μ M caffeine. ### indicates significant difference from the presence of glucose ($P \leq 0.001$), *** indicates significant difference from the absence of caffeine ($P \leq 0.001$). C. Arithmetic means $\pm$ SEM ($n = 12$) of the percentage of annexin V-binding erythrocytes following incubation for 48 hours in the presence (+ glu, left bars) or absence (- glu, right bars) of glucose without (white bars) or with (black bars) 500 nM PKC inhibitor staurosporine in the presence or absence of 500 μ M caffeine. ## indicates significant difference from the absence of staurosporine ($P \leq 0.01$), *** indicates significant difference from the absence of caffeine ($P \leq 0.001$). D. Arithmetic means $\pm$ SEM ($n = 8$) of the percentage of annexin V-binding erythrocytes following incubation in the presence (+ glu, left bar) or absence (- glu, right bars) of glucose without (white bars) or with (black bars) 500 μ M caffeine added the indicated numbers of hours prior to the end of the 48 hours incubation in glucose free Ringer. *** indicates significant difference from the absence of caffeine ($P \leq 0.001$).

Fig. 2. Decrease of forward scatter by glucose depletion in the presence and absence of caffeine. A. Histogram of erythrocyte forward scatter in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours without glucose in the absence (black line) or presence (red line) of 500 μ M caffeine. B. Arithmetic means $\pm$ SEM ($n = 11-12$) of the normalized erythrocyte forward scatter following incubation for 48 hours in the presence (+ glu) or absence (- glu) of glucose without (white bar) or with (black bars) 50-500 μ M caffeine. ### indicates significant difference from the presence of glucose ($P \leq 0.001$), *** indicates significant difference from the absence of caffeine ($P \leq 0.001$).

eryptosis due to energy depletion [11], annexin V-binding of erythrocytes was determined following glucose-depletion with or without caffeine in the presence or absence of the PKC inhibitor staurosporine (500 nM). As shown in Fig. 1C, in the absence of caffeine staurosporine significantly blunted eryptosis following energy depletion. In the presence of 500 μ M caffeine, staurosporine had only a slight additional effect on eryptosis following energy depletion (Fig. 1C).

Further experiments were performed to investigate the time course of the caffeine action on eryptosis due to energy depletion. To this end, erythrocytes were exposed to Ringer and glucose-depleted Ringer for 48 h, and 500 μ M caffeine were added at different time points prior to the end of incubation. As shown in Fig. 1D, the presence of caffeine in the last 24 h of exposure to glucose-depleted solution was sufficient to significantly blunt eryptosis.

Fig. 3. Stimulation of phosphatidylserine exposure by Cl^- depletion in the presence and absence of caffeine. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours without Cl^- (replaced by gluconate) in the absence (black line) or presence (red line) of 50 μM caffeine. B. Arithmetic means $\pm$ SEM ($n = 11-12$) of the percentage of annexin V-binding erythrocytes following incubation for 48 hours in the presence (+ Cl^-) or absence (- Cl^-) of Cl^- without or with 50-500 μM caffeine. ### indicates significant difference from the presence of Cl^- ($P \leq 0.001$), *, ** indicate significant difference from the absence of caffeine ($P \leq 0.05$ and $P \leq 0.01$).

Fig. 4. Decrease of forward scatter by Cl^- depletion in the presence and absence of caffeine. A. Histogram of erythrocyte forward scatter in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours without Cl^- (replaced by gluconate) in the absence (black line) or presence (red line) of 250 μM caffeine. B. Arithmetic means $\pm$ SEM ($n = 12-24$) of the normalized erythrocyte forward scatter following incubation for 48 hours in the presence (+ Cl^-) or absence (- Cl^-) of Cl^- without or with 0-500 μM caffeine. ### indicates significant difference from the presence of Cl^- ($P \leq 0.001$).

The Ca^{2+} entry following glucose depletion further leads to activation of Ca^{2+} -sensitive K^+ channels and cell shrinkage [11]. As illustrated in Fig. 2, glucose withdrawal indeed decreased forward scatter as a measure of cell volume in FACS analysis. Caffeine significantly blunted the shrinking effect of glucose depletion.

Eryptosis is further triggered by hyperosmotic or isoosmotic cell shrinkage [1]. Isotonic cell shrinkage follows removal of extracellular Cl^- leading to cellular Cl^- and K^+ loss with osmotically obliged water [54]. Cl^- removal activates Ca^{2+} -permeable erythrocyte cation channels leading to Ca^{2+} entry and subsequent Ca^{2+} -sensitive cell membrane scrambling [54]. As illustrated in Fig. 3, Cl^- removal indeed increased annexin V-binding, an effect significantly blunted in the presence of 50-500 μM caffeine.

Cl^- removal further led to a significant decrease of forward scatter (Fig. 4). The decrease of forward scatter following Cl^- removal tended to be blunted in the presence of caffeine, an effect, however, not reaching statistical significance.

The cell shrinkage and cell membrane scrambling following glucose depletion is at least partially due to stimulation of Ca^{2+} entry [11]. As shown in Fig. 5, glucose removal indeed increased Fluo3 fluorescence of the erythrocytes reflecting an increase in intracellular Ca^{2+} concentration. The effect was significantly blunted in the presence of 500 μM caffeine (Fig. 5). Cl^- removal similarly led to the expected significant increase in Fluo3 fluorescence. The increase in Fluo3 fluorescence following Cl^- removal was, however, not significantly influenced by the presence of 500 μM caffeine (Fig. 6).

Fig. 5. Effect of glucose depletion on cytosolic Ca^{2+} activity in the presence and absence of caffeine. A. Histogram of Fluo3-dependent fluorescence in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours in the absence of glucose without (black line) or with (red line) 500 μM caffeine. B. Arithmetic means $\pm$ SEM ($n = 19$ -20) of Fluo3-dependent fluorescence in erythrocytes incubated for 48 hours in Ringer solution in the presence (+glu) or absence (-glu) of glucose without or with 500 μM caffeine. ### indicates significant difference from the presence of glucose ($P \leq 0.001$), *** indicates significant difference from the absence of caffeine ($P \leq 0.001$).

Fig. 6. Effect of Cl^- depletion on cytosolic Ca^{2+} activity in the presence and absence of caffeine. A. Histogram of Fluo3-fluorescence in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours in the absence of Cl^- without (black line) or with (red line) 500 μM caffeine. B. Arithmetic means $\pm$ SEM ($n = 12$) of Fluo3-fluorescence in erythrocytes incubated for 48 hours in Ringer solution in the presence (+ Cl^-) or absence (- Cl^-) of chloride without or with 500 μM caffeine. *** indicates significant difference from the presence of Cl^- ($P \leq 0.001$).

Discussion

The present experiments confirm earlier observations [1] that energy depletion following glucose removal and isotonic erythrocyte shrinkage following Cl^- removal trigger eryptosis.

As shown previously [1], glucose depletion and Cl^- withdrawal lead to triggering of cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface. The cell membrane scrambling is at least partially due to Ca^{2+} entry.

Glucose depletion and Cl^- removal further lead to cell shrinkage reflected by a decrease of erythrocyte forward scatter. Erythrocyte shrinkage following Cl^- removal is largely due to exit of cellular Cl^- along with K^+ and osmotically obliged water. The cell shrinkage following glucose depletion is at least partially due to entry of Ca^{2+} , which activates Ca^{2+} -sensitive K^+ channels with subsequent exit of K^+ , hyperpolarization and Cl^- exit [6, 7].

More importantly, the present observations disclose that those events are blunted in the presence of caffeine. Thus, caffeine protects erythrocytes against those two triggers of eryptosis. The concentrations of caffeine needed for a significant antieryptotic effect following chloride depletion (50 μM) are within the range of plasma concentrations (100 μM) encountered following caffeine ingestion [55-57]. Thus, caffeine might protect erythrocytes from stress-induced suicidal death.

Previously, adenosine has similarly been shown to interfere with eryptosis [42]. At least in case of Cl^- removal, adenosine, similar to caffeine, did not significantly interfere with the increase in cytosolic Ca^{2+} activity, and was thus considered to be partially effective downstream of Ca^{2+} entry. The inhibition of Ca^{2+} entry may contribute to but does not fully account for the effect of adenosine and caffeine on eryptosis. The effect of adenosine could not be modified by inhibition of the nucleoside transporter with S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) [42, 58-61] or by abrogation of purinergic receptors utilizing the

unspecific purinergic receptor antagonist CGS-15943 [62], the adenosine A1-receptor antagonist 8-cyclopentyl-3-N-[3-((3-(4-(fluorosulfonyl) benzoyl)-oxy)-propyl)-1-N-propyl-xanthine (FSCPX) [63] or the adenosine A3 receptor antagonist N-(2-methoxyphenyl)-N'-(2-(3-pyridyl)quinazolin-4-yl)urea (VUF5574) [64]. Thus, the mechanism involved in the antiapoptotic effect of adenosine remained elusive. Similarly, the effect of caffeine may involve mechanisms other than purinergic receptor stimulation. Along those lines, the antiapoptotic effect of caffeine in neurons was similarly independent of adenosine receptors [47]. In theory, caffeine could be effective through inhibition of the phosphodiesterase [43]. However, dibutyryl-cAMP does not significantly modify eryptosis (own unpublished observations). The inhibition of neuronal apoptosis by caffeine may be related to its inhibitory action on ATM kinase with subsequent suppression of p53 phosphorylation [65].

Whatever the underlying mechanism, in diseases with enhanced eryptosis the inhibition of eryptosis by caffeine may prevent the accelerated clearance of erythrocytes from circulating blood and thus the development of anemia. Moreover, caffeine may counteract the adherence of phosphatidylserine-exposing erythrocytes to the vascular wall and thus interfere with haemostasis [18-20].

The observed inhibition of Ca^{2+} entry may relate to further effects of caffeine, which has been shown to interact with Ca^{2+} transport [66-68]. Moreover, the antiapoptotic effects of caffeine in some types of

nucleated cells [47, 49] may involve similar mechanisms as its antiapoptotic effect. Interestingly, caffeine significantly inhibited the Ca^{2+} entry following energy depletion but did not significantly modify the Ca^{2+} entry following Cl^- removal. This discrepancy may reflect differences in cation channels involved or their activation mechanisms. Energy depletion is effective through activation of PKC [11], whereas Cl^- removal may be effective through some other mechanism. Caffeine presumably interferes with the PKC dependent activation of the cation channels. How caffeine interferes with phosphatidylserine exposure following Cl^- removal, remains elusive.

In conclusion, caffeine inhibits the suicidal death of erythrocytes. The present observations thus disclose a novel action of this widely used nutrient and drug.

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