Stimulation of Erythrocyte Cell Membrane Scrambling by Quinine

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Key Words
Phosphatidylserine • Quinine • Eryptosis • Oxidative stress • Ceramide • Calcium • Casein kinase

Abstract
Background/Aims: The analkaloid drug quinine is utilized mainly for the chemoprophylaxis of malaria. The multiple side effects of quinine include hemolytic anemia and hemolytic uremic syndrome, disorders involving suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling contributing to stimulation of eryptosis include increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)), oxidative stress, ceramide and D4476 sensitive casein kinase. The present study explored the putative effect of quinine on eryptosis and elucidated cellular mechanisms involved. Methods: Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, [Ca\(^{2+}\)] from Fluo3-fluorescence, ROS formation from DCF dependent fluorescence, and ceramide abundance utilizing specific antibodies. Results: A 48 hours exposure of human erythrocytes to quinine (≥ 50 µM) significantly increased the percentage of annexin-V-binding cells without significantly affecting forward scatter. Quinine significantly increased Fluo3-fluorescence, DCF fluorescence and ceramide abundance. The effect of quinine on annexin-V-binding was significantly blunted by removal of extracellular Ca\(^{2+}\) and by addition of D4476 (10 µM). Conclusions: Quinine triggers phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to Ca\(^{2+}\) entry, oxidative stress, ceramide and D4476 sensitive casein kinase.

Introduction
Quinine, an alkaloid drug extracted from cinchona bark [1], was and is still widely employed for the chemoprophylaxis of malaria [1-21]. Quinine is further effective on Loa loa microfilaremia [22]. Moreover, quinine is used against muscle cramps [23-28] and has
antiepileptic effects in animals but apparently not in humans [29]. Side effects of quinine include chills, fever, hypotension, painful acral cyanosis, disseminated intravascular coagulation, hemolytic anemia, thrombocytopenia, neutropenia, bronchiolitis obliterans, organizing pneumonia, respiratory failure, hemolytic uremic syndrome, acute kidney injury, rhabdomyolysis, liver toxicity, vomiting, cardiotoxicity, hypoglycemia, ototoxicity, ocular toxicity, and toxic epidermal necrolysis [12, 20, 30-40].

Hemolytic anemia and hemolytic uremic syndrome could result from stimulation of eryptosis [41], the suicidal death of erythrocytes characterized by cell shrinkage [42] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [41]. Signaling involved in the stimulation of eryptosis include increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_{i}\)) [41], ceramide [43], oxidative stress [41], energy depletion [41], activated caspases [41, 44, 45], and stimulation of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and/or p38 kinase [41]. Eryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, and sorafenib/sunitinib sensitive kinases [41]. Eryptosis could be stimulated by a wide variety of xenobiotics [41, 46-87].

The present study explored, whether treatment of human erythrocytes with quinine impacts on eryptosis. Erythrocytes drawn from healthy volunteers were thus exposed to quinine and phosphatidylserine surface abundance, cell volume, [Ca\(^{2+}\)], ROS formation, 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 48 hours. Where indicated, erythrocytes were exposed for 48 hours to quinine (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In order to define the impact of extracellular Ca\(^{2+}\), CaCl\(_2\) was removed and 1 mM EGTA added. To test for an involvement of casein kinase 1α, erythrocytes were exposed for 48 hours to a combination of quinine and D4476 (Tocris bioscience, Bristol, UK). Ethanol was used as solvent.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 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 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 quinine 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”.

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 µM 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 (DCF) diacetate. After incubation, a 150 µl suspension of erythrocytes was washed in Ringer solution and stained with DCF (Sigma, Schnelldorf, Germany) in Ringer solution containing DCF at a final concentration of 10 µM. Erythrocytes
were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCF-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).

**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 µg/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 cytometric analysis 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 ± 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 addressed the putative effect of quinine on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Phosphatidylserine-exposing erythrocytes were identified by flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with quinine (10 – 100 µM). As illustrated in Fig. 1, a 48 hours exposure to quinine increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 50 µM quinine.

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 quinine (10 – 100 µM). As a result, following a 48 hours incubation the forward scatter was similar in the absence of quinine (494 ± 23, n = 10), and...
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the presence of 10 µM quinine (510 ± 16, n = 10), 50 µM quinine (507 ± 16, n = 10), as well as 100 µM quinine (504 ± 18, n = 10). Thus, quinine did not significantly affect forward scatter reflecting cell volume.

Hemolysis was estimated from hemoglobin concentration in the supernatant. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with quinine (10 – 100 µM). As a result, following a 48 hours incubation the percentage hemolytic cells was similar in the absence of quinine (3.3 ± 0.2 %, n =10), and the presence of 10 µM quinine (3.2 ± 0.2 %, n = 10), 50 µM quinine (3.6 ± 0.2 %, n = 10), as well as 100 µM quinine (3.5 ± 0.3 %, n = 10). Thus, quinine did not significantly modify hemolysis.
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Fluo3 fluorescence was taken as a measure of cytosolic Ca\textsuperscript{2+} activity ([Ca\textsuperscript{2+}]). As shown in Fig. 2, a 48 hours exposure to quinine increased the Fluo3 fluorescence at all quinine concentrations tested.

A next series of experiments explored whether the quinine-induced translocation of phosphatidylserine required entry of extracellular Ca\textsuperscript{2+}. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 100 µM quinine in the presence or nominal absence of extracellular Ca\textsuperscript{2+}. As illustrated in Fig. 3, removal of extracellular Ca\textsuperscript{2+} significantly blunted the effect of quinine on annexin-V-binding. However, even in the absence of extracellular Ca\textsuperscript{2+}, quinine significantly increased the percentage of annexin-V-binding erythrocytes (Fig. 3). Thus, quinine-induced cell membrane scrambling was in large part but not fully triggered by entry of extracellular Ca\textsuperscript{2+}.

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. As illustrated in Fig. 4, a 48 hours exposure to 100 µM quinine significantly increased the DCF fluorescence, an observation pointing to induction of 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. 5, a 48 hours exposure to 100 µM quinine significantly increased the ceramide related fluorescence reflecting ceramide abundance at the erythrocyte surface.

To test for involvement of necroptosis, additional experiments were performed in the absence and presence of the necroptosis inhibitor necrostatin-1. As a result, the percentage of annexin-V-binding erythrocytes was 2.0 ± 0.9% in the absence and 1.8 ± 0.7% in the presence of 100 µM necrostatin-1 (n = 10), as well as 6.5 ± 1.7% following treatment with 100 µM quinine alone and 6.2 ± 1.5% in the presence of quinine and necrostatin-1 (n = 10). Thus, neither in the absence nor in the presence of quinine necrostatin-1 significantly modified cell membrane scrambling.

**Fig. 4.** Effect of quinine on reactive oxygen species. A. Original histograms of DCF fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 100 µM quinine. B. Arithmetic means ± SEM (n = 10) of DCF fluorescence in erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) quinine (100 µM). ***(p<0.001) indicates significant difference from the absence of quinine (ANOVA).**

**Fig. 5.** Effect of quinine on ceramide abundance. A. Original histograms of ceramide abundance at the erythrocyte surface following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 100 µM quinine. B. Arithmetic means ± SEM (n = 10) of ceramide abundance at the erythrocyte surface following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) quinine (100 µM). ***(p<0.001) indicates significant difference from the absence of quinine (ANOVA).**
To explore, whether the effects of quinine involved activity of casein kinase 1α, the influence of quinine on annexin-V-binding was tested in the absence and presence of casein kinase inhibitor D4476 (10 µM). As illustrated in Fig. 6, addition of D4476 significantly blunted the effect of quinine on annexin-V-binding. In the presence of D4476 quinine did not significantly increase the percentage of annexin-V-binding erythrocytes (Fig. 6). Thus, quinine-induced cell membrane scrambling was in large part sensitive to D4476.

Discussion

The present observations reveal that the anti-malarial drug quinine triggers erythrocyte cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The effect of quinine on cell membrane scrambling was in part dependent on Ca²⁺ entry from the extracellular space, as it was paralleled by a significant increase of cytosolic Ca²⁺ activity ([Ca²⁺]) and was significantly blunted by removal of extracellular Ca²⁺. However, quinine significantly triggered cell membrane scrambling even in the absence of extracellular Ca²⁺, an observation pointing to the involvement of additional cellular mechanisms. Along those lines quinine significantly increased oxidative stress, a known trigger of eryptosis [41]. Quinine further significantly increased the abundance of ceramide, which is known to sensitize erythrocytes to the scrambling effect of Ca²⁺ [41]. Moreover, the effect of quinine was significantly blunted in the presence of casein kinase 1α inhibitor D4476, and may thus require activation of casein kinase 1α. Needless to say that the present observations do not rule out the involvement of further signaling molecules.

Despite the increase of [Ca²⁺], quinine did not trigger cell shrinkage. An increase of [Ca²⁺] were expected to activate Ca²⁺ sensitive K⁺ channels leading to K⁺ exit, cell membrane hyperpolarization, Cl⁻ exit and thus cellular loss of KCl with water. The mechanisms accounting for the lack of cell shrinkage remained elusive. Potential mechanisms include inhibition of K⁺ channels, decreased Ca²⁺ sensitivity of the K⁺ channels, reduced activity of the Cl⁻ channels, as
well as impairment of Na⁺/K⁺ ATPase activity with decrease of cellular K⁺ concentration and thus of Ca²⁺ induced hyperpolarization.

Quinine did not trigger appreciable hemolysis. Quinine probably does trigger hemolytic uremic syndrome indirectly [30]. In any case, triggering of eryptosis fosters the clearance of defective erythrocytes from circulating blood prior to hemolysis [41]. Hemolysis otherwise leads 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 [41].

Eryptosis primes erythrocytes infected with the malaria pathogen *Plasmodium* for rapid clearance from circulating blood and thus counteracts development of parasitemia [41]. It is tempting to speculate that stimulation of eryptosis and subsequent removal of infected erythrocytes contributes to the antimarial effects of quinine.

The rapid clearance of phosphatidylserine exposing erythrocytes may, on the other hand, lead to anemia as soon as the loss of erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [41]. The effect of quinine treatment is presumably enhanced in clinical conditions with accelerated eryptosis, such as iron deficiency [41], dehydration [88], hyperphosphatemia [89], chronic kidney disease (CKD) [90-93], hemolytic-uremic syndrome [94], diabetes [95], hepatic failure [96], malignancy [41], sepsis [97], sickle-cell disease [41], beta-thalassemia [41], Hb-C and G6PD-deficiency [41], as well as Wilsons disease [98].

Phosphatidylserine exposing erythrocytes may further interfere with microcirculation [43, 99-103] due to adherance to the vascular wall [104], stimulation of blood clotting and triggering of thrombosis [99, 105, 106].

In conclusion, quinine triggers eryptosis with cell membrane scrambling, an effect at least partially due to Ca²⁺ entry, oxidative stress, ceramide and activation of casein kinase 1α.

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Disclosure Statement

None.

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