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Triggering of Erythrocyte Cell Membrane Scrambling by Ursolic Acid

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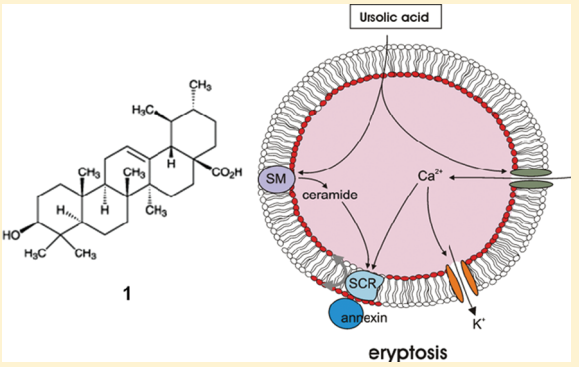
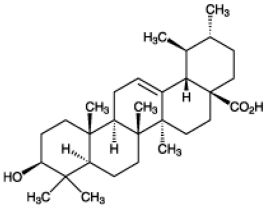
ABSTRACT: Ursolic acid (**1**), a triterpenoid with pleotropic effects including inhibition of tumor growth, is well known to trigger apoptosis of nucleated cells. The effect is at least partially due to altered gene expression and mitochondrial dysfunction. Erythrocytes lack nuclei and mitochondria but, similar to nucleated cells, may undergo suicidal cell death or eryptosis, which is characterized by cell shrinkage and phospholipid scrambling of the cell membrane. Triggers of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), ceramide formation and/or ATP depletion. The present study has investigated whether or not **1** induces eryptosis. $[Ca^{2+}]_i$ was estimated from Fluo-3 fluorescence, cell volume from forward scatter, phospholipid scrambling from annexin V binding, hemolysis from hemoglobin release, and cytosolic ATP concentration ($[ATP]_i$) utilizing a luciferase assay and ceramide-utilizing fluorescent antibodies in FACS analysis. As a result, exposure of erythrocytes for 48 h to **1** (≥ 5 μM) did not significantly modify $[ATP]_i$, but significantly increased $[Ca^{2+}]_i$, stimulated ceramide formation, decreased forward scatter, triggered annexin V binding, and elicited hemolysis. At 5 μM, **1** stimulated phospholipid scrambling in 10% and hemolysis in 2% of treated erythrocytes. Annexin V binding was blunted in the nominal absence of Ca^{2+}. In conclusion, the food component ursolic acid stimulates suicidal death of erythrocytes, i.e., cells devoid of nuclei and mitochondria.



Ursolic acid (**1**), a triterpenoid found in many foods with antiatherosclerotic, antidiabetic, anti-HIV, anti-inflammatory, antimicrobial, antineoplastic, antioxidant, antiulcer, antiwrinkle, cytotoxic, gastroprotective, hepatoprotective, hypolipidemic, and proinflamamtory activities,[1−3] has previously been shown to trigger apoptosis in a wide variety of cells[4] and sensitizes tumor cells to other apoptosis-inducing chemicals.[5] On the other hand, owing to its antioxidant activity, **1** may counteract apoptosis.[6] Ursolic acid triggers apoptosis partially due to its influence on gene expression[4,7−9] and mitochondrial function.[10]

Similar to nucleated cells, erythrocytes may undergo suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine (PS) exposure at the cell surface.[11] Triggers of eryptosis include activation of Ca^{2+}-permeable cation channels with subsequent increase of cytosolic Ca^{2+} concentration.[12−15] The Ca^{2+} concentration may increase following ATP depletion.[16] Cytosolic Ca^{2+} activates Ca^{2+}-sensitive K^+ channels,[17] leading to the exit of KCl, along with osmotically obliged water, and thus to cell shrinkage.[18] In addition, Ca^{2+} triggers phospholipid scrambling of the cell membrane with exposure of PS at the cell surface.[15] Cell membrane scrambling can be further stimulated by ceramide,[19] which is generated by a sphingomyelinase, an enzyme activated by platelet activating factor.[20] Erythrocyte cell membrane scrambling may then result from activation of caspases.[11]

The present study has investigated if ursolic acid (**1**) influences eryptosis. To this end, human erythrocytes were exposed to **1**, and the cytosolic Ca^{2+} concentration, cell volume, PS exposure, ceramide abundance, and ATP content were determined.



RESULTS AND DISCUSSION

In order to estimate cytosolic Ca^{2+} concentration, Fluo 3 fluorescence was determined by FACS analysis. As shown in Figure 1A and B, **1** increased cytosolic Ca^{2+} concentration. Following a 48 h exposure to Ringer, either in the absence or presence of **1** (0.5−10 μM), the cytosolic Ca^{2+} concentration was significantly higher in the presence of ≥ 5 μM **1** than in the absence of this triterpenoid.

An increase of cytosolic Ca^{2+} concentration is expected to activate Ca^{2+}-sensitive K^+ channels with subsequent exit of KCl and cell shrinkage. Thus, forward scatter was determined to estimate cell volume. As illustrated in Figure 2A and B, exposure of erythrocytes for 48 h to Ringer solution containing **1** (0.5−10 μM) decreased forward scatter, an effect reaching statistical

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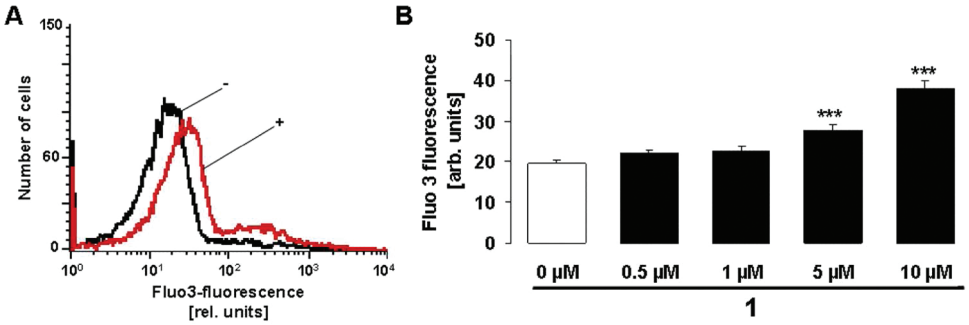

Figure 1. Effect of ursolic acid (**1**) on erythrocyte cytosolic Ca^{2+} concentration. (A) Original histogram of Fluo-3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, red line) the presence of 5 μM **1**. (B) Arithmetic means ± SEM (n = 10) of the 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) 0.5−10 μM **1**. *** indicates significant difference ($p < 0.001$) from the respective value in the absence of **1** (ANOVA).

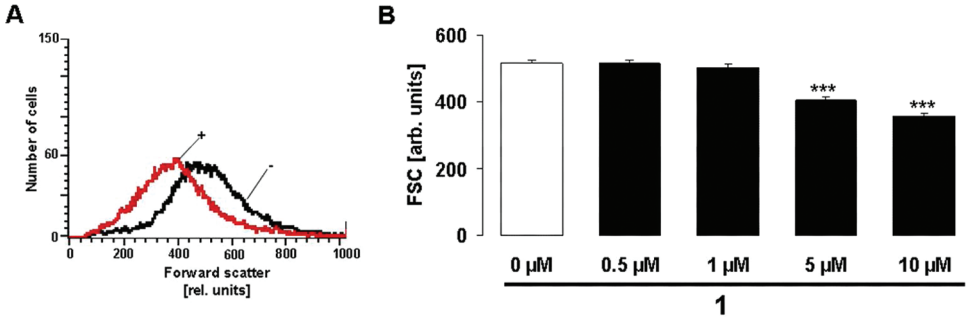

Figure 2. Effect of ursolic acid (**1**) on erythrocyte forward scatter. (A) Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, red line) the presence of 10 μM **1**. (B) Arithmetic means ± SEM (n = 10) of the erythrocyte forward scatter following incubation for 48 h in Ringer solution without (white bar) or with (black bars) 0.5−10 μM **1**. *** ($p < 0.001$) indicates significant difference from the absence of **1** (ANOVA).

significance at 5 μM **1**. Accordingly, exposure to ursolic acid resulted in cell shrinkage.

An increase of cytosolic Ca^{2+} concentration may be further expected to stimulate cell membrane scrambling with PS exposure at the cell surface. In order to identify erythrocytes exposing PS at the cell surface, annexin V binding was determined. As illustrated in Figure 3, annexin V-binding erythrocytes were more frequent in the presence than in the absence of **1**. To quantify PS exposure, annexin V-binding erythrocytes were counted by FACS analysis following a 48 h incubation of the erythrocytes in the absence or presence of **1** (0.5−10 μM). As illustrated in Figure 4A and B, the percentage of annexin V-binding erythrocytes was higher following exposure of erythrocytes for 48 h to Ringer solution containing **1** than was the case without this compound. The difference in the percentage of annexin V-binding erythrocytes between the presence and absence of **1** was statistically significant at ≥5 μM **1**.

Further experiments were conducted to determine if **1** triggers hemolysis. To this end, hemoglobin release was determined following exposure of the erythrocytes to Ringer solution for 48 h without or with 0.5−10 μM **1**. As shown in Figure 4B, the exposure to **1** was followed by hemolysis, an effect reaching statistical significance at 1 μM **1**. Following exposure to ursolic acid, the percentage of hemolyzed erythrocytes was 1 order of magnitude smaller than the percentage of PS-exposing erythrocytes. Thus, **1** triggers phospholipid scrambling and to a lesser extent hemolysis.

A further series of experiments explored whether the increase of cytosolic Ca^{2+} activity accounted for the stimulation of cell membrane scrambling following exposure to **1**. To this end, erythrocytes were treated with **1** in the presence and nominal absence of extracellular Ca^{2+}. As shown in Figure 5, the effect of ursolic acid on annexin V binding was reduced significantly but not fully abolished in the nominal absence of Ca^{2+}.

In a search for an additional mechanism accounting for the residual phospholipid scrambling, the effect of ursolic acid (**1**) on cytosolic ATP was determined following a 48 h exposure to Ringer in the absence and presence of **1** (10 μM). ATP depletion is known to trigger cell membrane scrambling. As shown in Figure 6, the exposure to **1** (10 μM) did not modify erythrocyte ATP concentration significantly. As a positive control, a 48 h exposure of erythrocytes to glucose-depleted Ringer was followed by the expected marked and significant decrease of intracellular ATP concentration (Figure 6).

A further series of experiments was designed to elucidate the effect of **1** on the formation of ceramide, which is well known to trigger phospholipid scrambling. As illustrated in Figure 7, following a 48 h exposure to Ringer in the absence and presence of **1** (10 μM) ceramide abundance was significantly higher in the presence of **1** (10 μM). Thus, ursolic acid does stimulate ceramide formation.

The present study discloses a novel effect of ursolic acid, i.e., triggering of Ca^{2+} entry, ceramide formation, cell shrinkage, and cell membrane scrambling in human erythrocytes. The concentrations of **1** required for such effects are only slightly higher than those achieved in vivo, which have been reported to approach 2 μM.[21]

The increase of Ca^{2+} concentration following exposure to ursolic acid depends apparently on Ca^{2+} entry across the cell membrane, so cell membrane scrambling is thus significantly blunted in the absence of extracellular Ca^{2+}. The Ca^{2+} entry is presumably accomplished by Ca^{2+}-permeable cation channels involving TRPC6.[22] An increase of cytosolic Ca^{2+} concentration is a powerful trigger of erythrocyte membrane scrambling and results in PS exposure at the cell surface.[23] The effect of **1** on cell membrane scrambling was not fully abolished in the nominal absence of extracellular Ca^{2+}, an observation pointing to some additional mechanism occurring. As a matter of fact, **1** stimulates the formation of ceramide, which is well known to trigger the cell membrane scrambling of erythrocytes.[19]

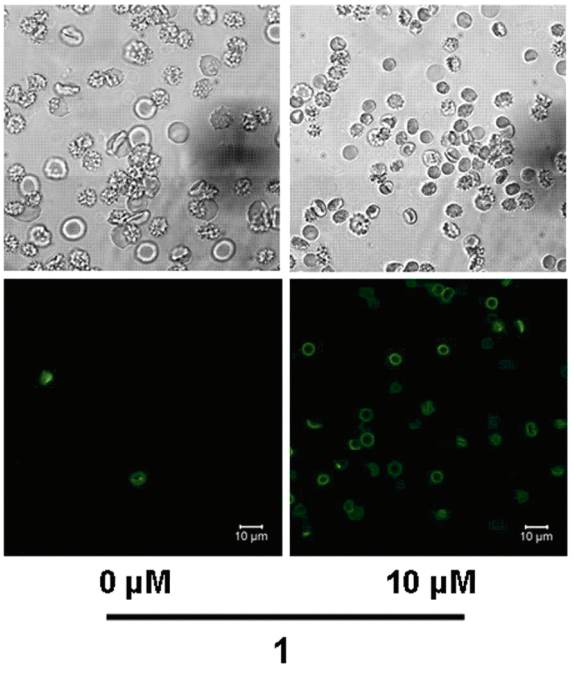

Figure 3. Confocal images of PS-exposing erythrocytes exposed to ursolic acid (**1**). Confocal microscopy of FITC-dependent fluorescence (lower panels) and light microscopy (upper panels) of human erythrocytes stained with FITC-conjugated annexin-V-Fluos following 48 h incubation in Ringer solution without (left panels) and with (right panels) 10 μM **1**.

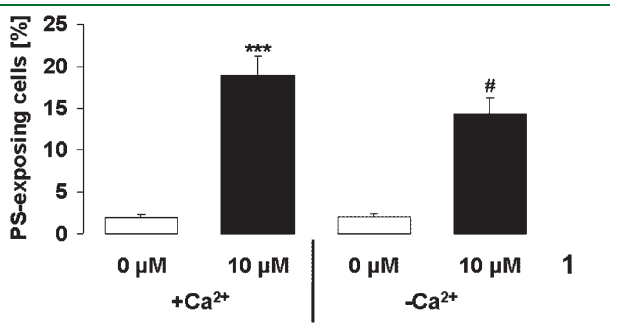

Figure 5. Effect of Ca^{2+} withdrawal on ursolic acid (**1**)-induced PS exposure. Arithmetic means ± SEM ($n = 4$) of the percentage of annexin V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 10 μM **1** in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of calcium. *** ($p < 0.001$) indicates significant difference from the absence of **1** (ANOVA); # indicates significant difference ($p < 0.05$) from the respective values in the presence of Ca^{2+}.

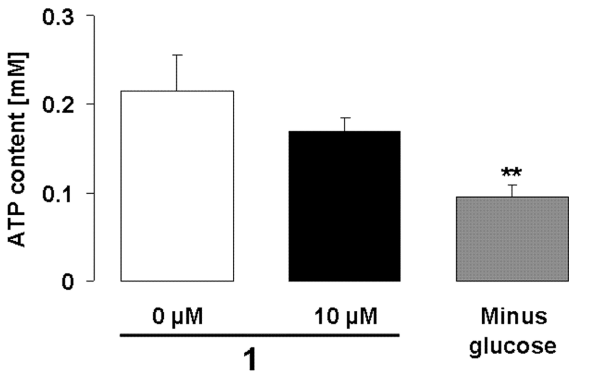

Figure 6. Effect of ursolic acid (**1**) on erythrocyte cytosolic ATP content. Arithmetic means ± SEM ($n = 4$) of the ATP concentration after a 48 h incubation in Ringer solution without (white bar) or with (black bar) **1** (10 μM) or in glucose-depleted Ringer solution (gray bar, minus glucose) ** ($p < 0.01$) indicates significant difference from control (absence of **1** and presence of glucose) (ANOVA).

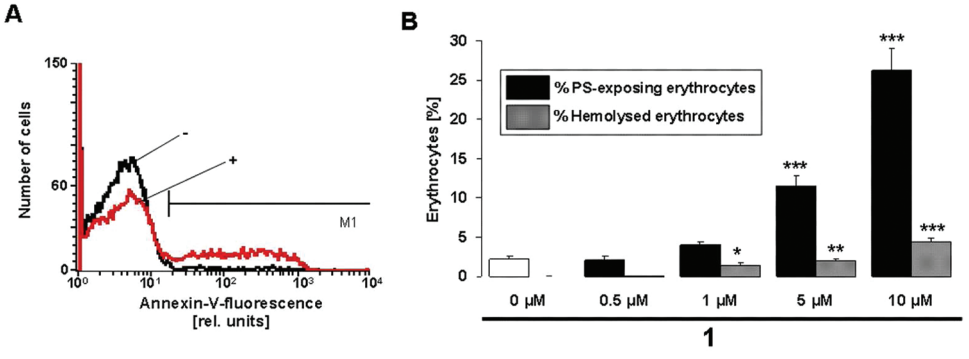

Figure 4. Effect of ursolic acid (**1**) on PS exposure and erythrocyte membrane integrity. (A) Original histogram of annexin V binding of erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, red line) the presence of 10 μM **1**. (B) Arithmetic means ± SEM ($n = 10$) of erythrocyte annexin V binding following incubation for 48 h in Ringer solution without (white bar) or with (black bars) the presence of 0.5−10 μM **1**. For comparison, arithmetic means ± SEM ($n = 4$) of the percentage of hemolysis are shown as gray bars. *, **, *** ($p < 0.05$, $p < 001$, $p < 0.001$) indicate significant difference from the absence of **1** (ANOVA).

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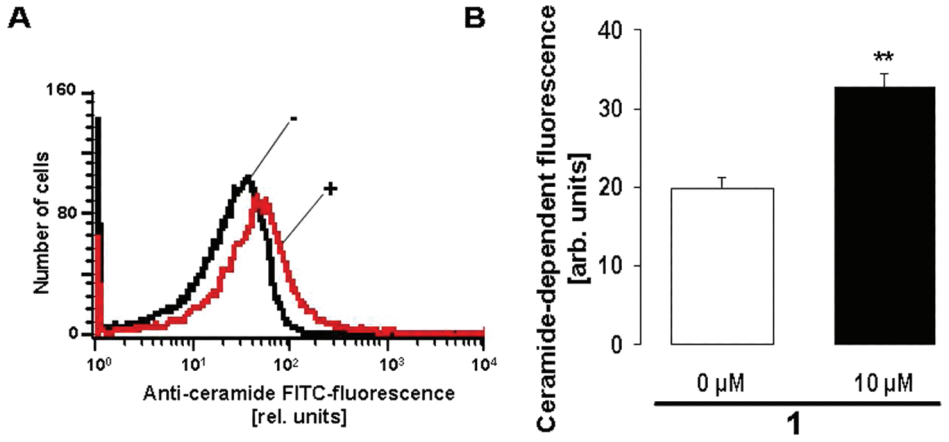

Figure 7. Effect of ursolic acid (**1**) on ceramide formation. Arithmetic means ± SEM (n = 4) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) **1** (10 μM). ** ($p < 0.01$) indicates significant difference from control (absence of **1**) (t test).

An increase of erythrocyte Ca^{2+} concentration is further expected to activate Ca^{2+}-sensitive K^+ channels,[17] resulting in cell shrinkage due to the exit of K^+, hyperpolarization of the cell membrane, exit of Cl^-, and parallel cellular loss of osmotically obliged water.[18] The exposure of erythrocytes to **1** indeed decreased the forward scatter, reflecting cell shrinkage. Cell membrane scrambling and cell shrinkage are the hallmarks of eryptosis.[11]

The effect of **1** on eryptosis may be compounded by a variety of clinical conditions known to enhance erythrocyte cell membrane scrambling, such as iron deficiency,[24] phosphate depletion,[25] hemolytic uremic syndrome,[26] sepsis,[27] sickle cell disease,[28] malaria,[28−32] Wilson's disease,[33] and possibly metabolic syndrome.[34] Erythrocyte cell membrane scrambling is further stimulated by a myriad of xenobiotics and endogenous substances.[35−41] Thus, the sensitivity to the scrambling effect of **1** may be exaggerated by any of these clinical conditions or chemicals.

The consequences of erythrocyte phospholipid scrambling include the clearance of affected erythrocytes from circulating blood and thus the development of anemia.[11] The anemia is counteracted by release of erythropoietin and subsequent hormonal stimulation of erythrocyte formation with increase of the reticulocyte number.[42] Erythrocyte phospholipid scrambling may further impede microcirculation due to adherence of PS-exposing erythrocytes to the vascular wall.[43] Moreover, eryptotic erythrocytes may stimulate blood clotting.[43−45]

The present observations not only may be relevant for erythrocytes but might shed novel light on mechanisms involved in the suicidal death of nucleated cells induced by ursolic acid. To the extent that **1** similarly stimulates Ca^{2+} entry into nucleated cells, the effect may well contribute to the stimulation of apoptosis. Excessive Ca^{2+} entry is a well-known trigger of apoptosis.[46−48] Moreover, ceramide formation is well known to foster apoptosis in a wide variety of cells.[33,49] Thus, the ability of **1** to generate ceramide formation may contribute to its ability to stimulate apoptosis.

In conclusion, the present observations disclose that ursolic acid (**1**) stimulates eryptosis characterized by cell shrinkage and cell membrane scrambling. The effect is due at least partially to Ca^{2+} entry and ceramide formation.

EXPERIMENTAL SECTION

Erythrocytes, Solutions, and Chemicals. Leukocyte-depleted erythrocytes (aged 1−2 weeks) were kindly provided by the blood bank of the University of Tübingen. Viability of erythrocytes may depend on the donor and the storage time, thus causing some interindividual variability. To avoid any bias potentially introduced from the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch. The study was approved by the ethics committee of the University of Tübingen (184/2003 V).

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, extracellular glucose was removed or ursolic acid (**1**) added at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid. Following pretreatment with the respective Ca^{2+}-containing or Ca^{2+}-free solutions for the indicated time periods, the erythrocytes were washed with Ringer solution containing 5 mM $CaCl_2$ and resuspended for 20 min with the same solution containing annexin V−Fluos. The time period of 20 min is considered too short for the triggering of eryptosis.

Ursolic acid (**1**) used in the present study was procured from Enzo (Lörrach, Germany). This compound was isolated from the leaves of *Eriobotrya japonica* (Thunb.) Lindl. (Rosaceae). Its purity was estimated to be 90% (TLC).

FACS Analysis of Annexin V Binding and Forward Scatter. After incubation under the respective experimental conditions, 50 μL of cell suspension was washed in Ringer solution containing 5 mM $CaCl_2$ and then stained for 20 min with annexin V−Fluos (1:500 dilution; Roche, Mannheim, Germany), under protection from light.[50] The forward scatter 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).

Measurement of Intracellular Ca^{2+}. After incubation of a 50 μL suspension, 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 of Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Determination of Ceramide Formation. To determine ceramide abundance at the cell surface, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37 °C with 1 μg/mL anticeramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. 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 (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 in FL-1.

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

Determination of Intracellular ATP Concentration. For the determination of intracellular ATP, 90 μL of erythrocyte pellets was incubated for 48 h at 37 °C in Ringer solution with or without **1** (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose-depleted Ringer solution as a positive control. All manipulations were then performed at 4 °C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of $HClO_4$ (5%). 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.

Confocal Microscopy and Immunofluorescence. For the visualization of eryptotic erythrocytes, 4 μL of erythrocytes, incubated in the respective experimental conditions, was stained with FITC-conjugated annexin V−Fluos (1:250 dilution; Roche, Mannheim, Germany) in 200 μL of Ringer solution containing 5 mM $CaCl_2$. Then the erythrocytes were washed twice and finally resuspended in 50 μL of Ringer solution containing 5 mM $CaCl_2$. A 20 μL aliquot was smeared onto a glass slide and covered with a coverslip, and images were subsequently taken on a Zeiss LSM 5 Exciter confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany), with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Statistics. Data are expressed as arithmetic means ± SEM. Statistical analysis was made using *t* test or paired ANOVA with Tukey's test as post-test, as indicated in the figure legends; *n* denotes the number of different erythrocyte specimens studied. The batches of erythrocytes differed moderately in their susceptibility to eryptosis. Thus, the control values were not identical in all series of experiments. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch.

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