Zinc-induced suicidal erythrocyte death1–3

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ABSTRACT
Background: Zn2+ stimulates secretory sphingomyelinase, which in turn produces ceramide, an important trigger of suicidal erythrocyte death or eryptosis. Eryptosis is characterized by exposure of phosphatidylserine (PS) at the erythrocyte surface and by cell shrinkage. As macrophages are equipped with PS receptors, they bind, engulf, and degrade PS-exposing cells.

Objective: We examined whether Zn2+ stimulates ceramide formation and PS exposure of erythrocytes and thus may be able to trigger suicidal erythrocyte death.

Design: In erythrocytes from healthy volunteers, PS exposure (Annexin V binding), cell volume (forward scatter), cytosolic Ca2+ activity (Fluo3 fluorescence), and ceramide formation (antiearimide antibody) were determined by fluorescence-assisted cell sorting.

Results: Exposure to Zn2+ (≥25 μmol/L Zn2+) significantly increased annexin binding. The effect was paralleled by increase of cytosolic Ca2+ activity (≥25 μmol/L Zn2+) and by ceramide formation (≥10 μmol/L Zn2+). Glucose depletion (24 h) similarly increased PS exposure, an effect significantly enhanced in the presence of Zn2+ (≥10 μmol/L Zn2+).

Conclusion: Zn2+ triggers suicidal erythrocyte death, an effect partially due to ceramide formation and an increase of cytosolic Ca2+ activity.

INTRODUCTION
Zinc, an essential nutrient, counteracts a variety of infectious diseases (1, 2) including malaria (3), diarrhea (3, 4), and respiratory infections (3, 5, 6). Zinc supplementation is considered particularly important in infants and children (7–10), pregnant women (11), and the elderly (12) and has proven beneficial in laboratory infections (3, 5, 6). Zinc supplementation is considered to be the authors) at the Blutspendezentrale Tu¨bingen (http://www.blutspendezentrale.de/), and erythrocyte concentrates were prepared with the use of leukocyte depletion filters as described (39). Aliquots of the individual erythrocyte concentrates were either used directly in the independent experiments or stored at 4 °C until usage. Experiments were performed at 37 °C in Ringer solution containing 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L Ca2+.

Enhanced eryptosis parallels several anemic conditions such as sickle cell disease, thalassemia, glucose-phosphate dehydrogenase deficiency, phosphate depletion, iron deficiency (27), hemolytic uremic syndrome, sepsis, malaria, and Wilson’s disease (16, 27, 28). Beyond that, eryptosis may be triggered by methylglyoxal (29), amyloid (30), listeriolysin (31), paclitaxel (32), chlorpromazine (33), cyclosporin (34), lead (35), and mercury (36). Eryptosis has similarities to, but may be distinct from, erythrocyte senescence (16).

Accelerated eryptosis has been shown to protect against a severe course of malaria (37, 38).

The present experiments were designed to test the hypothesis that Zn2+ ions could stimulate ceramide formation in erythrocytes and that the stimulation of ceramide formation may lead to stimulation of phosphatidylserine exposure. Thus, the present experiments were performed to explore whether exposure to Zn2+ ions triggers phosphatidylserine exposure and to elucidate the underlying mechanisms.

SUBJECTS AND METHODS
This study was approved by the local ethics committee of the University of Tübingen (project#: 184/2003V).

Erythrocytes and solutions
Blood was drawn from 9 healthy adult volunteers (not known to the authors) at the Blutspendenzentrale Tu¨bingen (http://www.blutspendenzentrale.de/), and erythrocyte concentrates were prepared with the use of leukocyte depletion filters as described (39). Aliquots of the individual erythrocyte concentrates were either used directly in the independent experiments or stored at 4 °C until usage. Experiments were performed at 37 °C in Ringer solution containing 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L Ca2+ and 25 mmol/L Zn2+. The concentration of Zn2+ used was based on results obtained in a previous study showing that Zn2+ ions stimulate ceramide formation in erythrocytes (14).

RESULTS
Exposure to Zn2+ (≥25 μmol/L Zn2+) significantly increased annexin binding. The effect was paralleled by increase of cytosolic Ca2+ activity (≥25 μmol/L Zn2+) and by ceramide formation (≥10 μmol/L Zn2+). Glucose depletion (24 h) similarly increased PS exposure, an effect significantly enhanced in the presence of Zn2+ (≥10 μmol/L Zn2+).

CONCLUSION
Zn2+ triggers suicidal erythrocyte death, an effect partially due to ceramide formation and an increase of cytosolic Ca2+ activity.


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MgSO₄, 32 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 mmol/L glucose, and 1 mmol/L CaCl₂. Zinc chloride (Sigma, Taufkirchen, Germany) was added to the Ringer solution.

**Fluorescence-assisted cell sorting**

Fluorescence-assisted cell sorting (FACS) analysis was performed as described (39). After incubation in the presence or absence of Zn²⁺ ions, cells were washed in annexin-binding buffer containing 125 mmol/L NaCl, 10 mmol/L HEPES/NaOH (pH 7.4), and 5 mmol/L CaCl₂. Erythrocytes were suspended in a solution composed of Annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin-binding buffer (dilution of 1:5) as described recently (39). After 3 washes with PBS/1% FCS, cells were stained with polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig-specific antibody.

**Measurement of intracellular Ca²⁺**

Intracellular Ca²⁺ measurements were performed as described (23). Briefly, erythrocytes were washed in sodium-chloride Ringer solution and then loaded with Fluo-3/AM (Clariochrome; Bad Soden, Germany) in 2 mmol/L CaCl₂ and 2 µmol/L Fluo-3/AM-containing sodium-chloride Ringer solution. The cells were incubated at 37 °C for 20 min under shaking condition and washed twice in 2 mmol/L CaCl₂-containing sodium-chloride Ringer solution. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µL Ringer solution in the presence or absence of Zn²⁺. Then, Ca²⁺-dependent fluorescence intensity was measured in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As an exposure time of 24 h would result in considerable loss of fluorescent dye, exposure time was restricted to 6 h.

**Determination of ceramide formation**

Cells were stained for 1 h at 4 °C with anticeramide antibody or isotype-matched pure mouse antibody in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) at a dilution of 1:5 as described recently (39). After 3 washes with PBS/1% FCS, cells were stained with polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig-specific antibody.

**FIGURE 1.** Stimulation of phosphatidylserine exposure at the erythrocyte surface by Zn²⁺. A) Histograms of annexin binding in a representative experiment of erythrocytes incubated for 24 h in Ringer solution (left panel), or in Ringer solution containing 25 µmol/L Zn²⁺ ions (right panel). The numbers indicate the percentage of annexin-binding erythrocytes of the respective cell population as confined by the horizontal line. B) Arithmetic means ± SEM (n = 9) of annexin binding erythrocytes after 24-h treatment with Ringer solution as a function of the Zn²⁺ concentration. *Significant difference (ANOVA using Dunnett’s test as post hoc test, P < 0.05) from absence of Zn²⁺ ions (Ringer solution, white bar).

**FIGURE 2.** Erythrocyte shrinkage following exposure to Zn²⁺. A) Histograms of forward scatter in a representative experiment of erythrocytes incubated for 24 h in Ringer solution (left panel), or in Ringer solution containing 25 µmol/L Zn²⁺ ions (right panel). The numbers indicate the GeoMean of the forward scatter of the respective cell population. B) Arithmetic means ± SEM, (n = 9) of forward scatter of erythrocytes after a 24-h treatment with Ringer solution as a function of the Zn²⁺ concentration. *Significant difference (ANOVA using Dunnett’s test as post hoc test, P < 0.05) from absence of Zn²⁺ ions (Ringer solution, white bar).
exposure with subsequent annexin binding in 4.1 ± 0.5% (n = 9) of the cells. Addition of Zn²⁺ (Zn²⁺ concentrations varying from 1 to 50 μmol/L) to the Ringer solution significantly increased the percentage of annexin-binding cells in a dose-dependent manner (Figure 1, A and B).

The effect of Zn²⁺ on annexin binding was paralleled by a decrease of the forward scatter reflecting erythrocyte shrinkage (Figure 2, A and B). The effect of Zn²⁺ was dependent on the Zn²⁺ concentration and achieved statistical significance at 25 μmol/L Zn²⁺ (Figure 2B).

As illustrated in Figure 3, exposure to Zn²⁺ for 6 h increased cytosolic Ca²⁺. The effect was statistically significant at 25 μmol/L Zn²⁺.

As illustrated in Figure 4, exposure of erythrocytes to Zn²⁺ (≥10 μmol/L) led within 24 h to a significant increase of ceramide formation. Bacterial sphingomyelinase, utilized as positive control, similarly increased ceramide formation (Figure 4, A and B).

Exposure of erythrocytes to glucose depletion resulted in the expected (40) increase of phosphatidylserine exposure (Figure 5A; compare the upper left histogram with the lower left histogram). The increase of phosphatidylserine exposure after glucose removal was significantly enhanced in the presence of Zn²⁺ (≥10 μmol/L) (Figure 5B). Thus, Zn²⁺ and energy depletion mutually enhance the susceptibility of erythrocytes to undergo eryptosis.

**RESULTS**

Incubation of freshly drawn erythrocytes in Ringer solution for 24 h resulted in low but appreciable phosphatidylserine

**FIGURE 3.** Increase of cytosolic Ca²⁺ activity in erythrocytes following exposure to Zn²⁺. A) Histograms of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 6 h to Ringer solution without (left panel) and with (right panel) 50 μmol/L Zn²⁺. The numbers indicate the mean fluorescence of erythrocytes of the respective cell population. B) Arithmetic means ± SEM (n = 4) of Fluo3 fluorescence in erythrocytes exposed for 6 h to Ringer solution without Zn²⁺ (white bar) and with 10 μmol/L Zn²⁺ (light gray bar), 25 μmol/L Zn²⁺ (dark gray bar), or 50 μmol/L Zn²⁺ (black bar). *Significant difference to value in absence of Zn²⁺ (ANOVA using Dunnett’s test as post hoc test, P < 0.05).

**FIGURE 4.** Stimulation of ceramide formation in erythrocytes following exposure to Zn²⁺. A) One-dimensional flow cytometry histograms of anti-ceramide fluorescence isothiocyanate (FITC)-coupled antibody binding (x axis) in a representative experiment of erythrocytes incubated for 24 h in Ringer solution without (control, left panel), or with 25 μmol/L Zn²⁺ (Zn²⁺, middle panel) or for 5 min in Ringer solution containing 0.1 U/mL sphingomyelinase (SMase, right panel). The numbers indicate the GeoMean of anticeramide FITC binding of the respective cell population. B) Anticeramide FITC-coupled antibody binding (arithmetic means ± SEM; n = 6) of erythrocytes after incubation for 24 h in Ringer solution without Zn²⁺ (left, open bar), with 10 μmol/L Zn²⁺ (left, gray bar) or with 25 μmol/L Zn²⁺ (left, black bar), or for 5 min in the absence (right, white bar) or presence (right, black bar) of 0.1 U/mL SMase in Ringer solution. *Significant difference to the value in absence of Zn²⁺ (ANOVA using Dunnett’s test as post hoc test, P < 0.05).
DISCUSSION

The present experiments disclose a novel action of Zn\(^{2+}\) on erythrocytes. The trace element triggers erythrocyte cell membrane phospholipid scrambling leading to phosphatidylserine exposure at the cell membrane surface. The effect of Zn\(^{2+}\) is paralleled by erythrocyte shrinkage, another typical feature of eryptosis (16). Similarly, cell shrinkage is a hallmark of apoptosis of nucleated cells (41).

The effects are at least partially due to increased cytosolic Ca\(^{2+}\) activity, which is known to stimulate phospholipid scrambling of the cell membrane (17, 18) and to activate Ca\(^{2+}\)-sensitive K\(^{+}\) channels (24, 25). The K\(^{+}\) exit through those channels leads to hyperpolarization of the cell membrane, which drives Cl\(^{-}\) toward the extracellular space. The combined exit of K\(^{+}\), Cl\(^{-}\), and osmotically obliged water then leads to cell shrinkage.

The effect is, further, the result of ceramide formation. Previous studies revealed the ability of Zn\(^{2+}\) to activate a ceramide-producing secretory sphingomyelinase (14). Ceramide, in turn, sensitizes erythrocytes for the scrambling effect of Ca\(^{2+}\) (15).

Exposure to Zn\(^{2+}\) further sensitizes the cell for the scrambling effect of energy depletion. Previous studies (40) have shown that energy depletion leads to activation of protein kinase C, which in turn triggers cell membrane scrambling.

The effect of Zn\(^{2+}\) requires Zn\(^{2+}\) concentrations in the range of those encountered in human plasma (42). Thus, excessive intake of Zn\(^{2+}\) could well trigger suicidal death of circulating erythrocytes. Thus, the observed effects of Zn\(^{2+}\) could indeed be relevant for in vivo conditions.

Phosphatidylserine-exposing cells are bound to respective receptors on macrophages (19), leading to engulfment and subsequent degradation (20). Accordingly, phosphatidylserine-exposing erythrocytes are rapidly cleared from circulating blood (27). Thus, at least in theory, the stimulation of suicidal death of circulating erythrocytes could cause anemia. Side effects of Zn\(^{2+}\) excess indeed include anemia (43, 44), an effect, however, in large part attributed to copper deficiency (45). During malaria, on the other hand, enhanced susceptibility to eryptosis could foster the elimination of infected erythrocytes and thus protect against a severe course of the disease (37, 38). Additional studies are required, however, to test whether the beneficial effect of zinc supplementation in malaria (1–3) is indeed related to accelerated eryptosis.

Zn\(^{2+}\) has previously been shown to induce (46–48) and to counteract (49) apoptosis of nucleated cells. The mechanisms effective in erythrocytes may similarly participate in the regulation of nucleated cell death.

In conclusion, exposure to Zn\(^{2+}\) leads to phosphatidylserine exposure of erythrocytes. The effect is at least partially due to increased cytosolic Ca\(^{2+}\) activity and ceramide formation. In vivo, the effects could lead to accelerated clearance of phosphatidylserine-exposing erythrocytes and thus to development of anemia.

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The authors’ responsibilities were as follows—VK: designed the study, and analyzed and interpreted the data; FL: wrote the manuscript; FL and TW: contributed to the analysis and interpretation of data; TW: contributed to writing the manuscript; and VK, AA and OMN: contributed to the collection of data. All authors reviewed the manuscript. None of the authors had a conflict of interest.

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