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ARTICLE

Effect of Phytic Acid on Suicidal Erythrocyte Death

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Phytic acid, an anticarcinogenic food component, stimulates apoptosis of tumor cells. Similar to apoptosis, human erythrocytes may undergo suicidal death or eryptosis, characterized by cell membrane scrambling and cell shrinkage. Triggers of eryptosis include energy depletion. Phytate intake could cause anemia, an effect attributed to iron complexation. The present experiments explored whether phytic acid influences eryptosis. Supernatant hemoglobin concentration was determined to reveal hemolysis, annexin V-binding in FACS analysis was utilized to identify erythrocytes with scrambled cell membrane, forward scatter in FACS analysis was taken as a measure of cell volume, and a luciferin−luciferase assay was employed to determine erythrocyte ATP content. As a result, phytic acid ($\geq$1 mM) did not lead to significant hemolysis, but significantly increased the percentage of annexin V-binding erythrocytes, significantly decreased forward scatter, and significantly decreased cellular ATP content. In conclusion, phytic acid stimulates suicidal human erythrocyte death, an effect paralleling its proapoptotic effect on nucleated cells.

KEYWORDS: Cell volume; annexin; eryptosis; calcium; phosphatidylserine

INTRODUCTION

Phytic acid or inositol hexakisphosphate is an important food component of several nutrients, such as wheat bran, soybean, Canadian dry bean, and maize (*1−4*). Phytate is readily absorbed in the gastrointestinal tract and may thus enter the bloodstream (*5*).

Phytic acid has been shown to exert marked anticarcinogenic potency on a wide variety of tumors (*6*) including breast cancer (*7*), prostate cancer (*8−16*), pancreatic cancer (*17*, *18*), oral cavity and laryngeal carcinoma (*19*, *20*), Barrett's adenocarcinoma (*21*), colon cancer (*22*, *23*), skin tumors (*24*, *25*), and glioblastoma (*26*). The anticarcinogenic effect of phytic acid is considered to result from stimulation of suicidal death or apoptosis of the tumor cells (*6*, *22*, *27−31*). On the other hand, phytic acid may bind allergens (*32*), be neuroprotective (*33*), and counteract tumor necrosis factor α- and Fas-induced apoptosis (*34*).

In analogy to apoptosis of nucleated cells, human erythrocytes may similarly undergo suicidal death or eryptosis (*35*), which is characterized by cell shrinkage and exposure of phosphatidylserine (PS) at the erythrocyte surface (*36−40*). Phosphatidylserine-exposing erythrocytes are phagocytosed and thus rapidly cleared from circulating blood (*41−43*). The PS exposure results from phospholipid scrambling of the cell membrane (*44*, *45*), which is stimulated by increased cytosolic Ca^{2+} activity (*36*, *38*, *39*). Ca^{2+} further activates Ca^{2+}-sensitive K^+ channels, leading to exit of KCl together with osmotically obliged water and thus to cell shrinkage (*46*), which in turn augments the cell membrane scrambling (*47*). The cytosolic Ca^{2+} activity may be increased by Ca^{2+} entry through Ca^{2+}-permeable cation channels following osmotic shock, oxidative stress, and/or energy depletion (*48−51*). The erythrocytes are sensitized for the effects of cytosolic Ca^{2+} by ceramide (*52*). Enhanced eryptosis may cause or contribute to the anemia in several clinical conditions including iron deficiency, phosphate depletion, hemolytic uremic syndrome, sepsis, malaria, Wilson's disease, blood incompatibility, sickle cell disease, thalassemia, and glucose−phosphate dehydrogenase deficiency (*53−58*). Moreover, eryptosis is stimulated by a wide variety of xenobiotics known to induce anemia (*57*, *59−73*). It is inhibited by xanthohumol (*74*), nitroxide (*75*), erythropoietin (*57*), zidovudine (*76*), and caffeine (*77*).

Phytate has previously been shown to affect the morphology of erythrocytes (*78*). However, typical features of eryptosis, such as membrane phospholipid scrambling or increase in cytosolic Ca^{2+} activity, following treatment of erythrocytes with phytate have hitherto not been reported.

The present study explored the effect of phytic acid on eryptosis. It is shown that exposure of human erythrocytes to phytic acid stimulates PS exposure and decreases cell volume. The effect is paralleled by and at least partially due to a decrease of cytosolic adenosine triphosphate (ATP) concentration.

MATERIALS AND METHODS

Experiments were performed in leukocyte-depleted banked erythrocytes provided by the blood bank of the University of Tübingen. The study has been approved by the Ethical Commission of the University of Tübingen.

The erythrocytes (0.8 μL) were incubated at 37 °C in 200 μL of Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM

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$MgSO_4$, 32 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 mM glucose, and 1 mM $CaCl_2$ for 48 h. Where indicated, phytic acid (sodium salt from rice; cation traces $Ca^{2+} \leq 2\%$; Sigma, Schnelldorf, Germany) was added to the Ringer solution at the indicated concentrations.

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

To determine PS exposure and forward scatter, 50 μL of the cell suspension was utilized after incubation for staining. The erythrocytes were washed once in Ringer solution containing 5 mM $CaCl_2$. The cells were then stained with annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution for flow cytometry and at 1:80 dilution for fluorescence microscopy. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from BD; Heidelberg, Germany). Cells were analyzed by the forward scatter [automatically determined by Cell Quest software (BD)], 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.

For determination of intracellular ATP concentration, 90 μL of erythrocyte pellet was incubated for 48 h at 37 °C in Ringer solution with or without phytic acid at the indicated concentrations (final hematocrit = 5%). All manipulations were then performed at 4 °C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by the addition of $HClO_4$ (5%). After centrifugation, an aliquot of the supernatant (400 μL) was adjusted to pH 7.7 by the addition of saturated $KHCO_3$ solution. After dilution of the supernatant, the ATP concentration of the aliquots was 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 as millimoles per liter of packed cell volume.

Ionized Ca^{2+} was determined in the cell culture supernatant by the Central Laboratory of the University Hospital of Tübingen according to clinical standards.

Data are expressed as arithmetic means ± standard error of the mean (SEM), and statistical analysis was made by paired ANOVA; $p < 0.05$ was considered to be statistically significant. *n* denotes the number of different erythrocyte specimens tested.

RESULTS

Cell membrane scrambling, a key feature of eryptotic cells, involves exposure of PS at the cell surface, which was determined by identifying annexin V-binding cells in fluorescence-activated cell sorter (FACS) analysis. To this end, erythrocytes were incubated in Ringer solution in the absence and presence of phytic acid. As shown in **Figure 1**, phytic acid indeed increased the percentage of annexin V-binding erythrocytes, pointing to stimulation of PS exposure. The effect reached statistical significance at phytic acid concentrations of ≥1 mM.

Because phytic acid is known to be a strong chelator of Ca^{2+}, the ionized Ca^{2+} concentration was determined after a 48 h incubation in Ringer solution in the absence and presence of phytic acid (5 mM). As a result, the concentration of ionized Ca^{2+} in the cell culture supernatant was 1.11 ± 0.01 mM in the absence and 0.12 ± 0.00 mM in the presence of 5 mM phytic acid ($n = 4$).

A second hallmark of eryptosis is cell shrinkage. In FACS analysis, the signal of the forward scatter can be taken as a measure of cell volume. Therefore, the forward scatter of erythrocytes was measured after a 48 h incubation in Ringer solution in the absence and presence of phytic acid. As illustrated in **Figure 2**, phytic acid decreased the erythrocyte forward scatter, pointing to cell shrinkage.

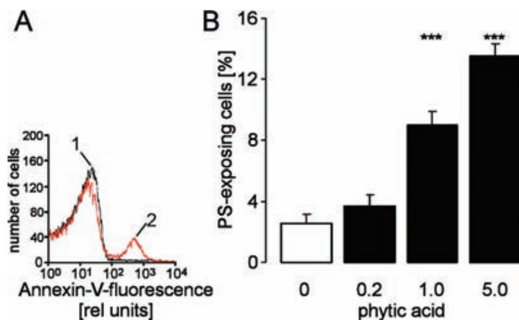

Figure 1. Stimulation of phosphatidylserine exposure at the erythrocyte surface by phytic acid: (**A**) graph of annexin V-binding in a representative FACS experiment of erythrocytes incubated for 48 h in Ringer solution (1, black line) or in Ringer solution containing 5 mM phytic acid (2, red line) (cells were labeled with annexin V Fluos); (**B**) bar diagram of the percentage of annexin V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or containing (black bars) the indicated concentration (all in mM) of phytic acid. *** indicates significant difference ($p < 0.001$) from control (absence of phytic acid)]. Data are means ± SEM ($n = 8$).

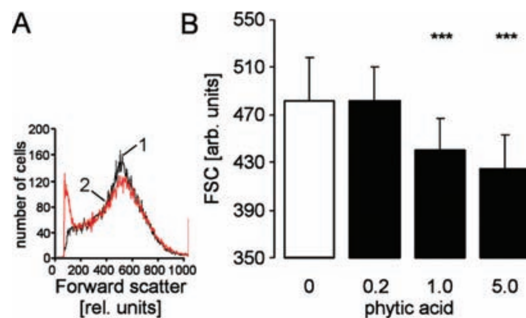

Figure 2. Erythrocyte forward scatter following exposure to phytic acid: (**A**) graph of forward scatter as a measure of cell size in a representative FACS experiment of erythrocytes incubated for 48 h in Ringer solution (1, black line) or in Ringer solution containing 5 mM phytic acid (2, red line); (**B**) bar diagram of the geo means of the forward scatter of erythrocytes after a 48 h treatment with Ringer solution without (white bar) or containing (black bars) the indicated concentration (all in mM) of phytic acid [*** indicates significant difference ($p < 0.001$) from control (absence of phytic acid)]. Data are means ± SEM ($n = 6$).

A further series of experiments aimed to possibly identify a hemolytic action of phytic acid. To that end, the hemoglobin concentration was determined in the supernatant of erythrocytes following a 48 h exposure to Ringer solution with or without phytic acid. As shown in **Figure 3B**, phytic acid up to a concentration of 5 mM did not appreciably induce hemolysis. Thus, phytic acid stimulated cell membrane scrambling and decreased cell volume without disrupting the integrity of the cell membrane. The maintenance of the cell membrane integrity following treatment with phytic acid was further apparent from microscopy (**Figure 3A**).

Additional experimental efforts aimed to identify the cause for phytic acid-induced eryptosis. As energy depletion is a known trigger of eryptosis (*79*), the effect of phytic acid on cytosolic ATP concentration was analyzed. As shown in **Figure 4**, the exposure to phytic acid for 48 h was indeed followed by a significant decline of the cytosolic ATP concentration. For comparison, erythrocytes were exposed to glucose-depleted Ringer, which, similar to phytic acid, decreased the erythrocyte ATP concentration (**Figure 4**, gray bar).

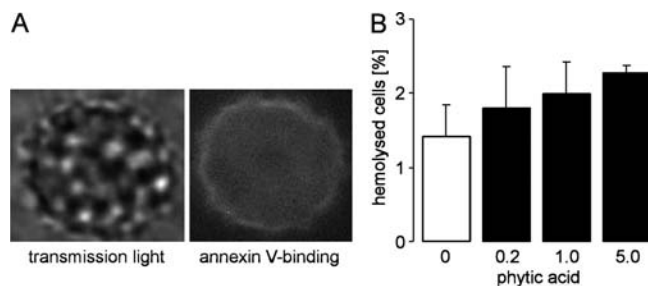

Figure 3. Analysis of the integrity of the erythrocyte membrane under the influence of phytic acid: (**A**) transmission microphotograph (left panel) and fluorescence microphotograph (right panel) of an erythrocyte stained with fluorescent annexin V (prior to microscopy, the erythrocyte was exposed for 48 h to 5 mM phytic acid in Ringer solution); (**B**) bar diagram of the percentage of hemolyzed erythrocytes exposed for 48 h to Ringer solution without (white bar) or containing (black bars) the indicated concentration (all in mM) of phytic acid. Data are means ± SEM ($n = 4$).

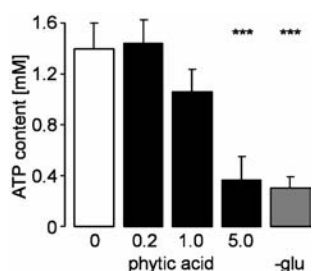

Figure 4. Effect of phytic acid on erythrocyte ATP concentration: bar diagram of the ATP concentration in erythrocytes from healthy volunteers incubated for 48 h in Ringer solution without (open bar) or with (black bars) phytic acid at the indicated concentrations. A 48 h exposure to glucose-free Ringer solution served as a positive control (gray bar). *** ($p < 0.001$) indicates significant difference from control (absence of phytic acid and presence of glucose). Data are means ± SEM ($n = 4$).

DISCUSSION

The present study reveals that exposure of human erythrocytes to phytic acid is followed by cell shrinkage and stimulation of cell membrane scrambling, both typical features of suicidal cells. The effect of phytic acid was at least partially due to a decrease of cytosolic ATP concentration, which is known to stimulate eryptosis, presumably through activation of protein kinase C (*79*). As phytate may rapidly enter cells (*5*), it is apparently transported across the cell membrane and may exert its effects within the cells. We do not, however, know which cytosolic concentrations are reached following extracellular application of phytate.

In view of the present observations the effect of phytic acid on nucleated cells may similarly involve a decrease of the ATP concentration. On the other hand, additional mechanisms may contribute to the stimulation of suicidal erythrocyte death by phytic acid. In nucleated cells, phytic acid may be effective through a variety of signaling pathways (*6*, *7*, *26*, *29*), including p21/Cip1 and p27/Kip1 (*13*); p53, Bcl-2, and caspases (*31*); induction of insulin-like growth factor binding protein-3 and inhibition of vascular endothelial growth factor (*16*); and inhibition of the Akt/NFkB pathway (*8*, *28*), as well as CDKI, CDK-cyclin, and pRb-related protein-E2F complexes (*15*). Several of those signaling pathways affect cell survival by modifying mitochondrial function or gene expression and thus cannot be effective in erythrocytes.

Phosphatidylserine-exposing erythrocytes are taken up by macrophages (*80*, *81*) and are thus rapidly eliminated from circulating blood (*43*). Accordingly, the stimulation of PS exposure by phytic acid may cause anemia. Phytic acid has indeed been shown to trigger anemia, an effect, however, attributed to its iron-chelating ability, leading to iron deficiency (*82*−*88*). Iron-deficient erythrocytes are particularly prone to eryptosis (*43*), which thus contributes to the shortening of the erythrocyte life span in iron deficiency anemia. The accelerated clearance of eryptotic erythrocytes may be partially or fully compensated by enhanced formation of new erythrocytes, thus ameliorating or even preventing the development of overt anemia. The enhanced erythrocyte formation would be apparent from an increase in the reticulocyte numbers in blood.

Eryptotic erythrocytes may further adhere to the vascular wall (*89*−*95*). Accordingly, excessive eryptosis may interfere with microcirculation. Moreover, eryptosis may trigger the release of pro-inflammatory cytokines, which may activate a hormonal stress response (*96*).

In view of the present observations, phytate-containing nutrients may have the potential to trigger suicidal erythrocyte death. Phytate is an important component of several widely consumed nutrients, such as wheat bran, soybean, Canadian dry bean, and maize (*1*−*4*), and consumption of those nutrients may predispose the consumer to the development of anemia. Most importantly, phytate consumption may influence eryptosis in particularly susceptible individuals, such as patients with iron deficiency, sickle cell anemia, thalassemia and glucose−phosphate dehydrogenase deficiency, phosphate depletion, and/or Wilson's disease (*53*−*58*). As pointed out above, phytate-containing cereals may impair the intestinal absorption of iron and thus predispose the consumer to the development of iron deficiency anemia (*97*). Moreover, phytate impairs the intestinal absorption of zinc (*97*), which protects erythrocytes from oxidation (*96*) and has previously been shown to inhibit eryptosis (*98*). Thus, phytate may modify erythrocyte survival and cause anemia not just by direct stimulation of eryptosis.

In conclusion, exposure of erythrocytes to phytic acid triggers cell membrane scrambling and cell shrinkage, typical features of suicidal erythrocyte death. The eryptotic effect of phytic acid may accelerate the clearance of erythrocytes from circulating blood and thus predispose the consumer to the development of anemia.

ABBREVIATIONS USED

FACS, fluorescence-activated cell sorter; PS, phosphatidylserine; Hb, hemoglobin; ATP, adenosine triphosphate; SEM, standard error of the mean.

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