Original Paper

Sensitization of Erythrocytes to Suicidal Erythrocyte Death Following Water Deprivation

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Key Words

Eryptosis • Energy depletion • Hyperosmotic shock • Oxidative stress • Ionomycin

Abstract

Background/Aims: Klotho deficiency results in excessive formation of 1,25(OH)₂D₃, accelerated ageing and early death. Moreover, klotho deficiency enhances eryptosis, the suicidal erythrocyte death characterized by phosphatidylserine exposure at the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca²⁺-activity ([Ca²⁺]_i), glucose depletion, hyperosmotic shock and oxidative stress. Klotho expression is decreased and 1,25(OH)₂D₃-formation enhanced by dehydration. The present study thus explored whether dehydration influences eryptosis. **Methods:** Blood was drawn from hydrated or 36h dehydrated mice. Plasma osmolality was determined by vapour pressure method, plasma 1,25(OH)₂D₃ and aldosterone concentrations using ELISA, and plasma Ca²⁺-concentration utilizing photometry. Erythrocytes were exposed to Ca²⁺-ionophore ionomycin (1 μM, 30 min), energy depletion (12 h glucose removal), hyperosmotic shock (500 mM sucrose added, 2 h) and oxidative stress (100 μM tert-butyl-hydroperoxide, 30 min) and phosphatidylserine exposure at the erythrocyte surface estimated from annexin V binding. **Results:** Dehydration increased plasma osmolality and plasma 1,25(OH)₂D₃ and aldosterone concentrations. Dehydration did not significantly modify phosphatidylserine-exposure of freshly drawn erythrocytes but significantly enhanced the increase of phosphatidylserine-exposure under control conditions and following treatment with ionomycin, glucose-deprivation, hyperosmolarity or tert-butyl-hydroperoxide. **Conclusions:** Dehydration sensitizes the erythrocytes to spontaneous eryptosis and to the triggering of eryptosis by excessive Ca²⁺-entry, energy depletion, hyperosmotic shock and oxidative stress.

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Introduction

Hydration sensitive genes [1] include *klotho* [2], a coreceptor of the FGF23 receptor contributing to downregulation of $1,25(\text{OH})_2\text{D}_3$ formation [3, 4]. Mice with reduced *klotho* expression suffer from multiple age-related disorders with growth retardation, extensive soft tissue calcification and decreased life span paralleled by osteopenia/osteoporosis, endothelial dysfunction, impaired angiogenesis, sinoatrial node dysfunction with sudden cardiac arrest, pulmonary emphysema, skin atrophy, hypogonadotropic hypogonadism, infertility, muscle dystrophy, hearing loss, neuron degeneration, Parkinson's disease, cognition impairment, neoplasms, inflammation and tissue fibrosis [4-19]. *Klotho* deficiency is largely effective through excessive $1,25(\text{OH})_2\text{D}_3$ formation, stimulation of renal tubular phosphate transport and subsequent elevation of serum phosphate levels [20-22].

Klotho deficiency triggers eryptosis, the suicidal death of erythrocytes [8], an effect reversed by vitamin D deficient diet and thus requiring enhanced $1,25(\text{OH})_2\text{D}_3$ formation [8]. Eryptosis is characterized by erythrocyte shrinkage and cell membrane scrambling [23]. Eryptosis is triggered by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [24], which may result from Ca^{2+} entry through Ca^{2+} permeable cation channels [25, 26]. Increased $[\text{Ca}^{2+}]_i$ triggers cell shrinkage by activating Ca^{2+} sensitive K^+ channels [27, 28] with subsequent cellular K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular KCl loss together with osmotically obliged water [29]. Increased $[\text{Ca}^{2+}]_i$ further triggers cell membrane scrambling with translocation of phosphatidylserine to the erythrocyte surface [30]. Further stimulators of eryptosis include hyperosmotic shock [23], energy depletion [31], caspase activation [32-36] and oxidative stress [37]. Eryptosis is further influenced by several kinases including AMP activated kinase AMPK [26], cGMP dependent protein kinase [38], Janus activated kinase JAK3 [39], casein kinase [40, 41], p38 kinase [42], PAK2 kinase [43] as well as sorafenib [44] and sunitinib [45] sensitive kinases.

As eryptosis is triggered by *klotho* deficiency [8], and *klotho* expression is downregulated by dehydration [2], the present study explored, whether dehydration impacts on eryptosis. To this end, $[\text{Ca}^{2+}]_i$, cell volume and phosphatidylserine exposure at the cell surface were determined in erythrocytes drawn from hydrated and water deprived mice.

Materials and Methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Experiments were performed in male C57Bl6 mice ($n = 6$ each group) at the age of 7 weeks. The mice received control food (SSniff, Soest, Germany) throughout the study. The mice had either access to drinking water ad libitum or were water deprived for 36 hours [1].

Determination of plasma concentrations

To collect blood specimens, animals were lightly anaesthetized with isoflurane (Abott, Wiesbaden-Delkenheim, Germany) and about 50 - 200 μl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. Plasma osmolality was measured by the vapour pressure method, plasma $1,25(\text{OH})_2\text{D}_3$ concentrations (IDS, Boldon, UK) and plasma aldosterone concentrations (ADI, San Antonio, USA) were determined utilizing a commercial ELISA Kit, serum Ca^{2+} concentration was measured by a photometric method (FUJI FDC 3500i, Sysmex, Norsted, Germany).

Solutions

Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl_2 . Where indicated, 100 μM tert-butyl-hydroxyl-peroxide (tert-BOOH, Sigma, Taufkirchen, Germany) was used to induce oxidative stress, 1 μM Ca^{2+} ionophore ionomycin (Sigma, Taufkirchen, Germany) to increase $[\text{Ca}^{2+}]_i$,

Fig. 1. Plasma osmolarity, aldosterone, $1,25(\text{OH})_2\text{D}_3$ and calcium concentrations in hydrated and dehydrated mice. Arithmetic means $\pm$ SEM ($n = 5-6$) of (A) plasma osmolarity (B) plasma aldosterone concentration (C) plasma $1,25(\text{OH})_2\text{D}_3$ concentration and (D) plasma calcium concentration in hydrated (white bars) and 36 hours dehydrated (black bars) mice. * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) indicate significant differences from the respective hydrated animals (t-test).

glucose removal to cause energy depletion, or osmolarity increased (addition of 500 mM sucrose) to induce hyperosmotic shock.

Phosphatidylserine exposure

To determine annexin V binding, reflecting phosphatidylserine exposure at the cell surface, erythrocytes were washed once in Ringer solution + 4 mM CaCl_2 . The cells were then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany). After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson, Heidelberg, Germany). Annexin V-fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

Data are expressed as arithmetic means $\pm$ 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.

Results

The present study explored the effect of water deprivation on eryptosis, the suicidal erythrocyte death. To this end, mice were exposed to a 36 hours period of water deprivation and blood drawn for analysis of osmolarity, hormone concentrations and plasma calcium levels as well as phosphatidylserine exposure of erythrocytes. As illustrated in Fig. 1, a 36 hours water deprivation significantly increased plasma osmolarity, plasma aldosterone concentration, plasma $1,25(\text{OH})_2\text{D}_3$ concentration and plasma concentration of calcium.

Fig. 2 illustrates the effect of dehydration on annexin V binding, a measure of phosphatidylserine exposure at the erythrocyte surface. In freshly drawn blood the percentage of annexin V binding erythrocytes was not significantly different between dehydrated and hydrated animals. Following exposure to Ringer solution, the percentage of annexin V binding erythrocytes increased gradually. This increase was significantly steeper in erythrocytes from dehydrated animals than in erythrocytes from hydrated animals.

Fig. 2. Effect of dehydration on spontaneous phosphatidylserine exposure. A,B. Original histogram of annexin V binding of freshly drawn (A) or 48 hours incubated (B) erythrocytes from hydrated (grey shadows) and dehydrated (black lines) animals. C. Arithmetic means $\pm$ SEM (n = 6) demonstrating annexin V binding of erythrocytes drawn from hydrated (open circles) or dehydrated (closed circles) animals following incubation for 0, 24 or 48 hours in Ringer solution. * (p<0.05) indicate significant differences from erythrocytes of hydrated animals (ANOVA).

Accordingly, following both, a 24 hours and a 48 hours incubation in Ringer, the percentage of annexin V binding dehydrated erythrocytes was significantly higher than the percentage of annexin V binding hydrated erythrocytes. Thus, dehydration accelerated the spontaneous development of phosphatidylserine exposure.

Further experiments explored whether dehydration affects the sensitivity of erythrocytes to known triggers of eryptosis. In order to test the sensitivity to increased cytosolic Ca²⁺ activity, the erythrocytes were exposed for 30 min to the Ca²⁺ ionophore ionomycin (1 μ M). As illustrated in Fig. 3, ionomycin treatment was followed by a sharp increase of phosphatidylserine exposure in both, hydrated and dehydrated erythrocytes. The phosphatidylserine exposure following ionomycin treatment was, however, significantly higher in dehydrated than in hydrated erythrocytes.

In order to test whether hydration influences the sensitivity of erythrocytes to energy depletion, the erythrocytes were exposed for 12 hours to either glucose containing or glucose deprived Ringer solution. As shown in Fig. 4, glucose withdrawal was followed by a marked increase of phosphatidylserine exposure in both, hydrated and dehydrated erythrocytes. Again, the phosphatidylserine exposure following glucose deprivation was significantly higher in dehydrated than in hydrated erythrocytes.

The sensitivity of erythrocytes to hyperosmotic shock was tested by increasing the osmolarity of the Ringer solution by addition of 500 mM sucrose. The erythrocytes were exposed for 2 hours to either isotonic or hypertonic Ringer solution. As demonstrated in Fig. 5, hyperosmotic shock was followed by an increase of phosphatidylserine exposure in both, hydrated and dehydrated erythrocytes. Again, the phosphatidylserine exposure following osmotic shock was significantly higher in dehydrated than in hydrated erythrocytes.

Tert-butylhydroperoxide (t-BOOH) was utilized in order to test the sensitivity of erythrocytes against oxidative stress. The erythrocytes were exposed for 30 min to Ringer solution either without or with 100 μ M t-BOOH. As shown in Fig. 6, t-BOOH treatment was followed by an increase of phosphatidylserine abundance at the erythrocyte surface in both,

Fig. 3. Effect of Ca^{2+} ionophore ionomycin on phosphatidylserine exposure of erythrocytes drawn from hydrated and dehydrated animals. A,B. Original histograms demonstrating annexin V binding of erythrocytes drawn from hydrated (grey areas) or dehydrated (black lines) animals following exposure for 30 min to Ringer solution without (A) or with (B) presence of $1 \mu\text{M}$ Ca^{2+} ionophore ionomycin. C. Arithmetic means $\pm$ SEM ($n = 6$) of annexin V binding erythrocytes, drawn from hydrated (white bars) or dehydrated (black bars) animals, following incubation for 30 min to Ringer solution without (left bars, control) or with (right bars, ionomycin) presence of Ca^{2+} ionophore ionomycin ($1 \mu\text{M}$). * ($p < 0.05$) indicates significant difference to erythrocytes drawn from hydrated animals, ### ($p < 0.001$) indicates significant difference to absence of ionomycin (ANOVA).

Fig. 4. Effect of glucose deprivation on phosphatidylserine exposure of erythrocytes drawn from hydrated and dehydrated animals. A,B. Original histograms demonstrating annexin V binding of erythrocytes drawn from hydrated (grey areas) or dehydrated (black lines) animals following exposure for 12 h to Ringer solution with (A) or without (B) presence of glucose. C. Arithmetic means $\pm$ SEM ($n = 6$) of annexin V binding erythrocytes, drawn from hydrated (white bars) or dehydrated (black bars) animals, following incubation for 12 h to Ringer solution with (left bars, control) or without (right bars, without glucose) presence of glucose. * ($p < 0.05$) indicates significant difference to erythrocytes drawn from hydrated animals, ### ($p < 0.001$) indicates significant difference to presence of glucose (ANOVA).

hydrated and dehydrated erythrocytes. As already shown for the other triggers of eryptosis, the phosphatidylserine abundance following t-BOOH treatment was significantly higher in dehydrated than in hydrated erythrocytes.

Discussion

The present study reveals an influence of dehydration on the phosphatidylserine abundance at the erythrocyte cell membrane surface. Accordingly, dehydration fosters the development

Fig. 5. Effect of hyperosmotic shock on phosphatidylserine exposure of erythrocytes drawn from hydrated and dehydrated animals. A,B. Original histograms demonstrating annexin V binding of erythrocytes drawn from hydrated (grey areas) or dehydrated (black lines) animals following exposure for 2 h to isotonic (A) or hypertonic (B, 500 mM sucrose added) Ringer solution. C. Arithmetic means $\pm$ SEM (n = 6) of annexin V binding erythrocytes, drawn from hydrated (white bars) or dehydrated (black bars) animals, following incubation for 2 h to isotonic (left bars, control) or hypertonic (right bars, 500 mM sucrose added) Ringer solution. * (p<0.05) indicates significant difference to erythrocytes drawn from hydrated animals, ### (p<0.001) indicates significant difference to isotonic Ringer (ANOVA).

Fig. 6. Effect of oxidative stress on phosphatidylserine exposure of erythrocytes drawn from hydrated and dehydrated animals. A,B. Original histograms demonstrating annexin V binding of erythrocytes drawn from hydrated (grey areas) or dehydrated (black lines) animals following exposure for 30 min to Ringer solution without (A) or with (B) presence of 100 μ M tert-butyl-hydroperoxide. C. Arithmetic means $\pm$ SEM (n = 6) of annexin V binding erythrocytes, drawn from hydrated (white bars) or dehydrated (black bars) animals, following incubation for 30 min to Ringer solution without (left bars, control) or with (right bars, + t-BOOH) presence of tert-butyl-hydroperoxide (100 μ M). ** (p<0.01) indicates significant difference to erythrocytes drawn from hydrated animals, ### (p<0.001) indicates significant difference to absence of tert-butyl-hydroperoxide (ANOVA).

of eryptosis, the suicidal death of erythrocytes. The difference of phosphatidylserine exposure in freshly drawn erythrocytes is not significantly different between hydrated and dehydrated animals. It must be kept in mind that phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [23]. Thus, appreciable effects on the percentage phosphatidylserine exposing erythrocytes could only be expected following dramatic stimulation of eryptosis. This is apparently not the case following dehydration. The *in vitro* incubation of the erythrocytes discloses the slight but statistically significant difference between erythrocytes from dehydrated and erythrocytes from hydrated animals.

The present study did not elucidate the mechanisms accounting for the enhanced susceptibility of erythrocytes to triggers of eryptosis following dehydration of the animal. Notably, erythrocytes isolated from hydrated and dehydrated animals have subsequently been incubated in the same Ringer solution. Thus, the trigger of eryptosis remained effective even following removal of plasma. Thus, plasma osmolarity and hormone levels are not immediately involved in the observed stimulation of erythrocyte cell membrane scrambling following dehydration. Hormones, plasma osmolarity, or other hydration sensitive plasma components could, however, lead to lasting changes of erythrocyte properties rendering them more susceptible to triggers of eryptosis.

The sensitization of erythrocytes to stimulators of eryptosis by dehydration may be important in the presence of other stimulators of eryptosis or in clinical conditions associated with enhanced eryptosis. Eryptosis is stimulated by a wide variety of xenobiotics [45-76] and eryptosis is increased in several clinical disorders [23], including diabetes [36, 77, 78], renal insufficiency [79], hemolytic uremic syndrome [80], sepsis [81], malaria [82-86], sickle cell disease [87], Wilson's disease [85], iron deficiency [88], malignancy [89], phosphate depletion [90], and metabolic syndrome [72]. According to the present observations, dehydration may augment the effect of eryptosis inducing drugs or disorders.

Phosphatidylserine exposure fosters the adhesion of erythrocytes to endothelial CXCL16/SR PSO [91], which may, at least in theory, compromise microcirculation and thus interfere with blood flow [91-96]. Phosphatidylserine exposure may further foster blood clotting with subsequent triggering of thrombosis [92, 97, 98]. It is thus tempting to speculate that phosphatidylserine exposure of erythrocytes contributes to the well known stimulation of thrombosis by dehydration [99-101].

Conclusions

Dehydration has a subtle stimulating effect on phosphatidylserine scrambling in the erythrocyte cell membrane and may augment the phosphatidylserine scrambling following excess Ca^{2+} entry, energy depletion, hyperosmotic shock and oxidative stress.

Conflict of Interests

Competing interests: the authors have no competing interests.

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