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Original Paper

Stimulation of Suicidal Erythrocyte Death by Increased Extracellular Phosphate Concentrations

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Key Words
Phosphatidylserine • Uremia • Calcium • p38 kinase • Eryptosis • Phosphate

Abstract
Background/Aim: Anemia in renal insufficiency results in part from impaired erythrocyte formation due to erythropoietin and iron deficiency. Beyond that, renal insufficiency enhances eryptosis, the suicidal erythrocyte death characterized by phosphatidylserine-exposure at the erythrocyte surface. Eryptosis may be stimulated by increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$). Several uremic toxins have previously been shown to stimulate eryptosis. Renal insufficiency is further paralleled by increase of plasma phosphate concentration. The present study thus explored the effect of phosphate on erythrocyte death. ***Methods:*** Cell volume was estimated from forward scatter, phosphatidylserine-exposure from annexin V binding, and $[Ca^{2+}]_i$ from Fluo3-fluorescence. ***Results:*** Following a 48 hours incubation, the percentage of phosphatidylserine exposing erythrocytes markedly increased as a function of extracellular phosphate concentration (from 0-5 mM). The exposure to 2 mM or 5 mM phosphate was followed by slight but significant hemolysis. $[Ca^{2+}]_i$ did not change significantly up to 2 mM phosphate but significantly decreased at 5 mM phosphate. The effect of 2 mM phosphate on phosphatidylserine exposure was significantly augmented by increase of extracellular Ca^{2+} to 1.7 mM, and significantly blunted by nominal absence of extracellular Ca^{2+}, by additional presence of pyrophosphate as well as by presence of p38 inhibitor SB203580. ***Conclusion***: Increasing phosphate concentration stimulates erythrocyte membrane scrambling, an effect depending on extracellular but not intracellular Ca^{2+} concentration. It is hypothesized that suicidal erythrocyte death is triggered by complexed $CaHPO_4$.

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Introduction

A major complication of chronic kidney disease is the development of anemia [1, 2], resulting in part from decreased renal erythropoietin release and thus impairment of erythropoiesis [3, 4]. Moreover, erythropoiesis in uremic patients may be compromized by iron deficiency [5, 6]. The anemia in uremic patients is, however, at least in part the result of accelerated clearance of circulating erythrocytes [7], which could be caused by enhanced eryptosis, the suicidal death of erythrocytes [8, 9]. Eryptosis is characterized by cell shrinkage and by cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface [8, 9]. The percentage of phosphatidylserine exposing erythrocytes has been reported to be twice as high in patients on dialysis than in healthy individuals [10]. Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood *in vivo* [9] and the increased percentage of phosphatiylserine exposing erythrocytes in circulating blood is expected to be paralleled by the respective decrease of erythrocyte life span.

Eryptosis may be triggered by enhanced cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [8, 9] resulting from Ca^{2+} entry through Ca^{2+}-permeable cation channels [9]. An increase of $[Ca^{2+}]_i$ is followed by cell shrinkage due to activation of Ca^{2+}-sensitive K^+ channels [9], K^+ exit, hyperpolarization, Cl^- exit and thus cellular KCl and water loss [9]. Increased $[Ca^{2+}]_i$ further triggers cell membrane scrambling with phosphatidylserine translocation to the cell surface [9].

Further stimulators of eryptosis include ceramide [9], energy depletion [9], caspase activation [9, 11, 12] and deranged activity of kinases such as AMP activated kinase AMPK [9], casein kinase 1α [13, 14], cGMP-dependent protein kinase [9], Janus-activated kinase JAK3 [15], p38 kinase [16], protein kinase C [9], as well as sorafenib [17] and sunitinib [18] sensitive kinases. Eryptosis is further triggered by a wide variety of xenobiotics and is enhanced in a variety of clinical disorders [9, 19-43].

Triggers of eryptosis in renal insufficiency are incompletely understood. Eryptosis has previously been shown to be stimulated by the uremic toxins acrolein [44], methylglyoxal [9], indoxyl sulfate [45] and vanadate [9]. A major complication of chronic kidney disease is the increase of plasma phosphate concentration leading to vascular calcification and increased cardiovascular mortality [46, 47]. The present study explored, whether eryptosis is modified by alterations of extracellular phosphate concentration. To this end, the sensitivity of $[Ca^{2+}]_i$, cell volume and phosphatidylserine abundance at the erythrocyte surface to alterations of extracellular phosphate concentration was determined.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes 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 (184/2003V). 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, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), $CaCl_2$ increased to 1.7 mM, or SB203580 (2 μM, Tocris, Bristol, UK) or pyrophosphate (10μM, Sigma-Aldrich, Steinheim, Germany) added. Erythrocytes were exposed to 0-5 mM phosphate by addition of sodium phosphate buffer (pH 7.4).

Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (10 min at 2000 RPM, 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.

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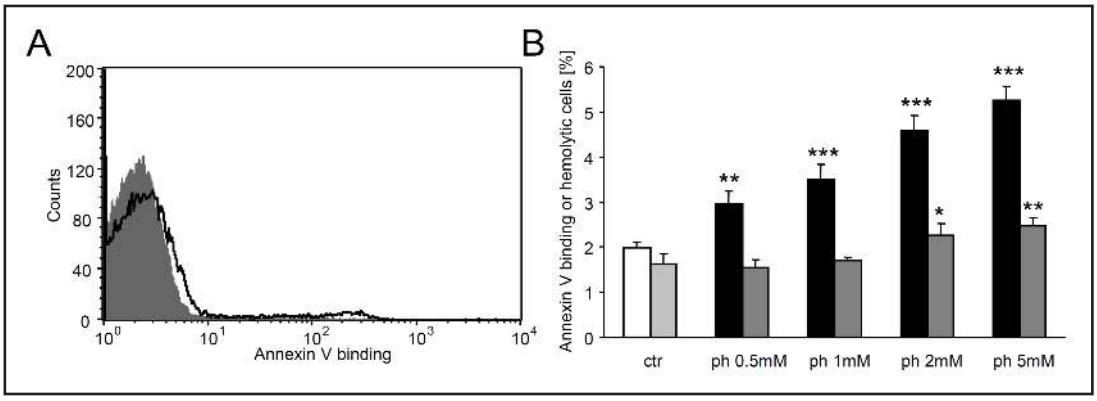

Fig. 1. Effect of extracellular phosphate concentration on phosphatidylserine exposure. A. Original histogram of annexin-V-binding erythrocytes following exposure for 48 hours to Ringer solution at 0 mM phosphate (grey area) and in the presence of 5 mM phosphate (black line). B. Arithmetic means ± SEM (n = 27) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution at 1 mM Ca^{2+} concentration and 0 (white bar) or 0.5-5 mM (black bars) phosphate concentration. For comparison, arithmetic means ± SEM (*n* = 9) of the percentage of hemolysis (grey bars) are shown. *,**,*** (p<0.05, 0.01, 0.001) indicates significant difference from the absence of phosphate (ANOVA),

FACS analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 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 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V fluorescence intensity was measured 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 erythrocytes were washed in Ringer solution and then 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 and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, 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.

Statistics

Data are expressed as arithmetic means ± SEM. Statistical analysis was made using repeated measures ANOVA (Tukey-test). 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

In order to explore whether an increase of phosphate concentration in renal insufficiency could participate in the triggering of suicidal erythrocyte death or eryptosis in uremia, erythrocytes were exposed for 48 hours to Ringer solution with phosphate concentrations ranging from 0 to 5 mM. Eryptosis was evidenced from cell membrane scrambling leading to phosphatidlyserine translocation to the cell membrane surface. Phosphatidylserine exposing erythrocytes were identified by annexin V binding. As illustrated in Fig. 1, the percentage of annexin V binding erythrocytes increased with increasing extracellular

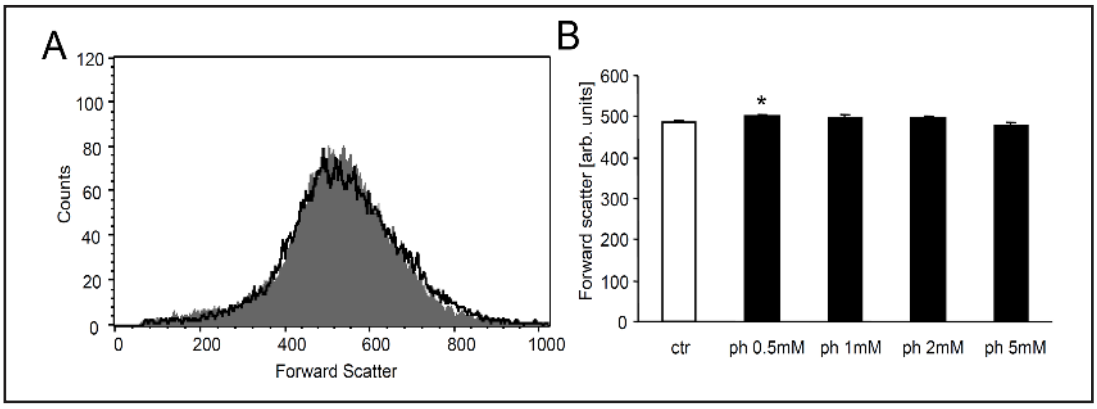

Fig. 2. Effect of extracellular phosphate concentration on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution at 0 mM phosphate (grey area) and in the presence of 5 mM phosphate (black line). B. Arithmetic means ± SEM (n = 27) of the normalized erythrocyte forward scatter following incubation for 48 hours to Ringer solution at 1 mM Ca^{2+} concentration and 0 (white bar) or 0.5-5 mM (black bars) phosphate concentration. * ($p<0.05$) indicates significant difference from the absence of phosphate (ANOVA).

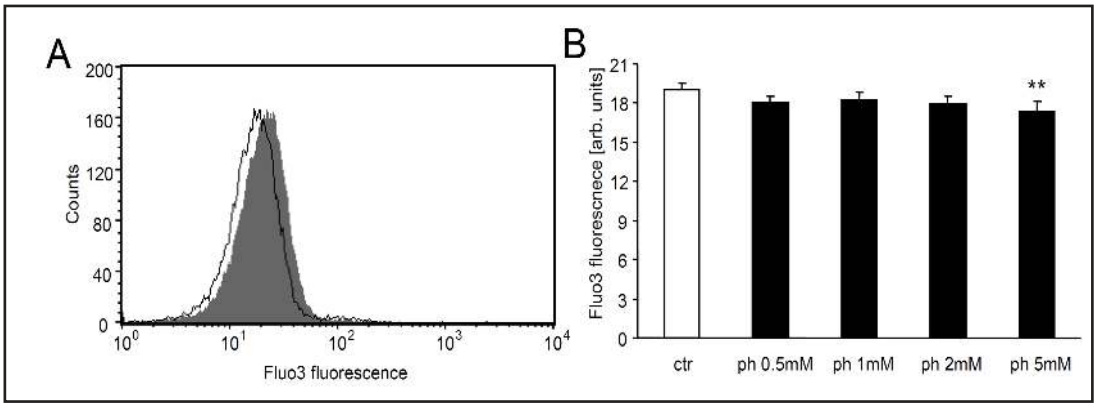

Fig. 3. Effect of extracellular phosphate concentration on Fluo3 fluorescence. A. Original histogram of Fluo3 fluorescence reflecting cytosolic Ca^{2+} concentration of erythrocytes following exposure for 48 hours to Ringer solution at 0 mM phosphate (grey area) and in the presence of 5 mM phosphate (black line). B. Arithmetic means ± SEM (n = 22) of erythrocyte Fluo3 fluorescence following incubation for 48 hours to Ringer solution at 1 mM Ca^{2+} concentration and 0 (white bar) or 0.5-5 mM (black bars) phosphate concentration. ** ($p<0.01$) indicates significant difference from the absence of phosphate (ANOVA).

phosphate concentration. To a smaller extent, increasing phosphate concentrations augmented hemolysis, an effect that reached statistical significance at 2 and 5 mM phosphate concentrations.

Further experiments addressed the effect of phosphate on erythrocyte volume, which was estimated from forward scatter in flow cytometry. As shown in Fig. 2, the forward scatter of erythrocytes increased slightly but significantly following an increase of phosphate concentration from 0 to 0.5 mM. The forward scatter following exposure to any of the other phosphate concentrations was not significantly different from the forward scatter following exposure in the absence of extracellular phosphate.

Fluo3 fluorescence was employed to test, whether phosphate influences cytosolic Ca^{2+}

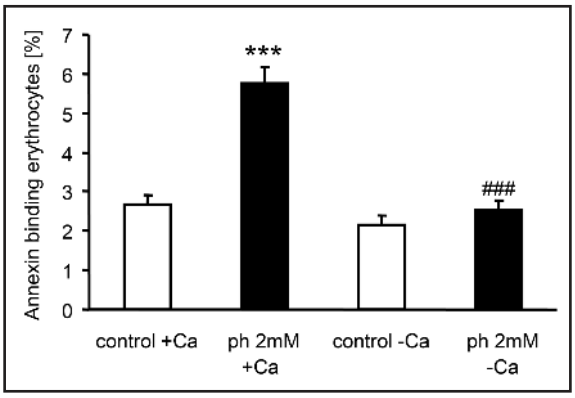

Fig. 4. Effect of Ca^{2+} withdrawal on phosphate-induced annexin-V-binding. Arithmetic means ± SEM (n = 9) of the percentage of annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) 2 mM phosphate in the presence (left bars, + Ca) and absence (right bars, - Ca) of 1 mM calcium. *** (p<0.001) indicates significant difference from the respective value in the absence of phosphate (ANOVA), ### (p<0.001) indicates significant difference from the respective values in the presence of Ca^{2+}.

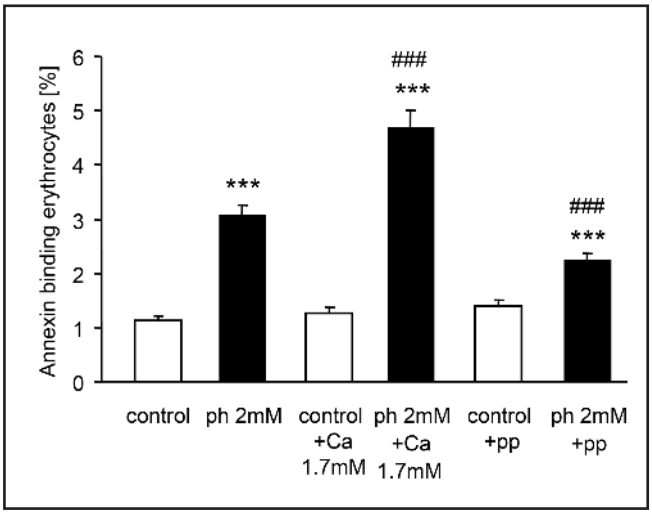

Fig. 5. Effect of Ca^{2+} or pyrophosphate addition on phosphate- induced annexin-V-binding. Arithmetic means ± SEM (n = 10) of the percentage of annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution containing 1 mM Ca^{2+}, (control), containing 1.7 mM Ca^{2+} (+Ca 1.7mM), or containing pyrophosphate 10μM (+pp) in the absence (white bars) and presence (black bars) of 2 mM phosphate. *** (<0.001) indicates significant difference from the respective value at 1 mM Ca^{2+} and 0 phosphate (ANOVA) ### (p<0.001) indicates significant difference from the respective value at 1 mM Ca^{2+} and 2 mM phosphate.

concentration. As illustrated in Fig. 3, the Fluo3 fluoresence did not change significantly up to 2 mM phosphate concentration and slightly but significantly decreased following exposure to 5 mM phosphate concentration.

Further experiments were performed to test, whether the effect of phosphate was sensitive to extracellular Ca^{2+} concentration. To this end, erythrocytes were exposed to 2 mM phosphate for 48 hours either in the presence of extracellular Ca^{2+} (1 mM) or in the nominal absence of Ca^{2+} and presence of the Ca^{2+} chelator EGTA (1 mM). As shown in Fig. 4, the effect of phosphate on annexin-V-binding was virtually abolished in the nominal absence of extracellular Ca^{2+}. Further experiments were performed elucidating the effect of phosphate at enhanced extracellular Ca^{2+} concentration. To this end, erythrocytes were exposed to 2 mM phosphate for 48 hours either at 1 mM Ca^{2+} concentration or at 1.7 mM extracellular Ca^{2+} concentration As illustrated in Fig. 5, the effect of enhanced phosphate concentration was augmented by the additional increase of extracellular Ca^{2+} concentration. Those observations pointed to a role of $CaHPO_4$ precipitations. Since those precipitations could be inhibited by pyrophosphate, additional experiments were made in the presence of pyrophosphate. As illustrated in Fig 5, addition of pyrophosphate significantly blunted the annexin-V-binding following exposure to 2 mM phosphate.

Further experiments explored the involvement of p38 kinase in the triggering of cell membrane scrambling by phosphate. To this end, erythrocytes were treated with 2 mM phosphate in the presence or absence of p38 kinase inhibitor SB203580. As shown in Fig. 6, the effect of phosphate on annexin-V-binding was significantly blunted by addition of 2 μM SB203580.

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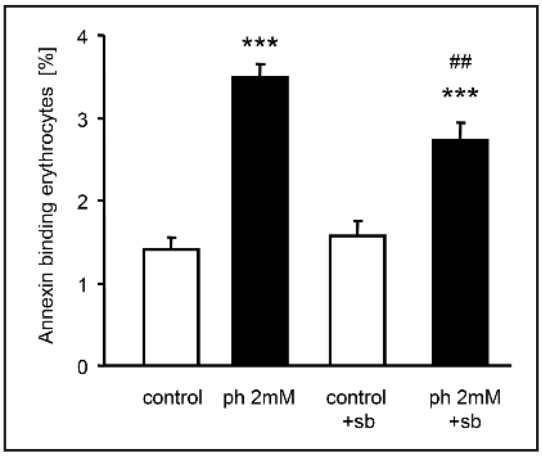

Fig. 6. Effect of phosphate on phosphatidylserine exposure in the presence or absence of p38 kinase inhibitor SB203580. Arithmetic means ± SEM (*n* = 9) of erythrocyte annexin-V-binding following incubation for 48 h to Ringer solution without (white bars, control) or with presence of 2 mM phosphate (black bars) in the absence (left bars) or presence (sb, right bars) of p38 kinase inhibitor SB203580 (2 μM). *** (<0.001) indicates significant difference from the respective value in control erythrocytes (ANOVA) ## (p<0.01) indicates significant difference from the respective value in the absence of SB203580.

Discussion

The present study reveals a novel effect of phosphate, i.e. an influence on erythrocyte cell membrane scrambling, a hallmark of suicidal erythrocyte death or eryptosis. The effect of phosphate was dependent on the presence of extracellular Ca^{2+} and was enhanced following an increase of extracellular Ca^{2+} concentration.

An increase of phosphate concentration from 0 to 0.5 mM was followed by a slight increase of cell volume which, however, remained virtually constant following further increases of extracellular phosphate concentration. Along those lines intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was rather decreased following increases of extracellular phosphate concentration. Other triggers of eryptosis shrink erythrocytes by increase of cytosolic Ca^{2+} concentration with subsequent activation of Ca^{2+} sensitive K^+ channels [9, 48], K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [9].

The effect of increasing phosphate concentration on cell membrane scrambling require the presence of extracellular Ca^{2+}. Other Ca^{2+}-sensitive triggers of eryptosis are effective by increasing $[Ca^{2+}]_i$ [9] with subsequent Ca^{2+} dependent stimulation of cell membrane scrambling. The effect of high phosphate concentration on erythrocyte cell membrane scrambling is, however, paralleled by decreasing $[Ca^{2+}]_i$ and obviously not due to enhanced entry of extracellular Ca^{2+}. Instead, the effect of phosphate requires extracellular Ca^{2+}. Furthermore, the effect was blunted by addition of pyrophosphate. Pyrophosphate inhibits hydroxyapatite formation and tissue calcification [49, 50]. Calcium-phosphate crystals induce cell death in vascular smooth muscle cells [51]. In view of the present observations, it is tempting to speculate that the effect of phosphate is at least in part due to calcium phosphate supersaturation and precipitation. In osteoarthritic synovial fibroblasts, basic calcium phosphate crystals stimulate p38 kinase [52]. Along those lines, addition of the p38 inhibitor SB203580 blunted the effects of phosphate treatment on suicidal erythrocyte cell death.

The present paper did not elucidate the p38 kinase dependent mechanisms mediating calcium phosphate induced eryptosis. It is noteworthy, though, that p38 kinase targets include phospholipase 2 [53], which plays a dual role in the stimulation of eryptosis [9]. Phospholipase A2 has been shown to generate platelet activating factor, which activates sphingomyelinase and thus ceramide formation [9]. Ceramide sensitizes erythrocytes to the scrambling effect of Ca^{2+} [9]. Whether or not phosphate stimulates ceramide formation, however, remains to be shown. Phospholipase A2 further generates arachidonic acid, which is converted by cycloxygenase to prostaglandin E_2, a stimulator of the Ca^{2+} permeable cation channels [9]. Since cytosolic Ca^{2+} activity did not change, this pathway is apparently not activated by phosphate. At least in theory, phosphate could in addition be effective by modifying further regulators of eryptosis, such as ATP [9], AMP activated kinase AMPK [9], casein kinase 1α [13, 14], cGMP-dependent protein kinase [9], Janus-activated kinase JAK3 [15], protein kinase C [9] and caspases [9, 11, 12].

In healthy individuals, extraosseus hydroxyapatite formation is prevented by calcification inhibitors, most notably pyrophosphate but also Fetuin-A and matrix Gla protein [54]. In chronic kidney disease (CKD), the inhibitory mechanisms are depleted or overridden by marked hyperphosphatemia resulting in hydroxyapatite formation [49, 54]. The serum calcification propensity is a predictor of mortality in CKD [55]. The stimulation of eryptosis by hyperphosphatemia or calcium phosphate supersaturation could therefore well contribute to the decreased life span of circulating erythrocytes in uremic patients. Chronic kidney disease is associated with increased levels of phosphatidylserine exposing erythrocytes [10, 56]. Phosphatidylserine exposing erythrocytes adhere to phagocytosing cells and are thus rapidly cleared from circulating blood [9]. In renal insufficiency, anemia develops in part due to accelerated loss of erythrocytes, and in part due to impaired formation of new erythrocytes [57]. According ot the present observations the effect of hyperphosphatemia contributes to the eryptotic effects of uremic toxins. Further substances or disorders presumably contribute to the triggering of eryptosis and development of anemia in uremic patients.

As phosphatidylserine exposing erythrocytes adhere to the vascular wall [58] eryptosis could interfere with blood flow [9, 58]. Phosphatidylserine exposing erythrocytes are further known to stimulate blood clotting [9, 59, 60]. Uncritical use of erythropoietin or other erythropoiesis stimulating agents [61-63] may thus foster the turnover of erythrocytes thus increasing the concentration of eryptotic erythrocytes with subequent impairment of microcirculation.

Conclusion

Increasing extracellular phosphate concentration fosters erythrocyte cell membrane scrambling and thus eryptosis, the suicidal death of erythrocytes. Phosphate thus shares the ability of some organic uremic toxins to trigger eryptosis.

Conflict of Interests

All authors of this manuscript declare that they have no competing interests.

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