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Original Paper

The Uremic Toxin Acrolein Promotes Suicidal Erythrocyte Death

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Key Words
Phosphatidylserine • Acrolein • Calcium • Cell volume • Eryptosis

Abstract
Background: Anemia is a major complication of end stage renal disease. The anemia is mainly the result of impaired formation of erythrocytes due to lack of erythropoietin and iron deficiency. Compelling evidence, however, points to the contribution of accelerated erythrocyte death, which decreases the life span of circulating erythrocytes. Erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage and by cell membrane scrambling with phosphatidylserine-exposure at the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$). Erythrocytes could be sensitized to cytosolic Ca^{2+} by ceramide. In end stage renal disease, eryptosis may possibly be stimulated by uremic toxins. The present study explored, whether the uremic toxin acrolein could trigger eryptosis. ***Methods:*** Cell volume was estimated from forward scatter, phosphatidylserine-exposure from annexin-V-binding, hemolysis from hemoglobin release, $[Ca^{2+}]_i$ from Fluo3-fluorescence, and ceramide from fluorescent antibodies. ***Results:*** A 48 h exposure to acrolein (30 – 50 μM) did not significantly modify $[Ca^{2+}]_i$ but significantly decreased forward scatter and increased annexin-V-binding. Acrolein further triggered slight, but significant hemolysis and increased ceramide formation in erythrocytes. Acrolein (50 μM) induced annexin-V-binding was significantly blunted in the nominal absence of extracellular Ca^{2+}. Acrolein augmented the annexin-V-binding following treatment with Ca^{2+} ionophore ionomycin (1 μM). ***Conclusion:*** Acrolein stimulates suicidal erythrocyte death or eryptosis, an effect at least in part due to stimulation of ceramide formation with subsequent sensitisation of the erythrocytes to cytosolic Ca^{2+}.

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Introduction

Chronic renal failure is complicated by severe anemia, which is at least in part explained by compromized renal erythropoietin formation and release with subsequent decrease of erythropoiesis [1-3]. Moreover, impaired formation of erythrocytes in chronic renal failure may result from iron deficiency [4, 5].

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At least in theory, the anemia of uremic patients could be worsened by accelerated death of circulating erythrocytes. Erythrocytes may undergo apoptosis-like suicidal death or eryptosis, which is characterized by cell membrane scrambling [6, 7]. Eryptosis may be triggered by enhanced cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). The increase of $[Ca^{2+}]_i$ may be caused by Ca^{2+} entry through Ca^{2+}-permeable cation channels [7, 8], which are activated by oxidative stress [7]. Increased $[Ca^{2+}]_i$ activates Ca^{2+}-sensitive K^+ channels [7] leading to cell shrinkage due to K^+ exit, hyperpolarization, Cl^- exit and thus cellular KCl and water loss [9]. Increased $[Ca^{2+}]_i$ further triggers cell membrane scrambling leading to phosphatidylserine exposure at the erythrocyte surface [7]. The Ca^{2+} sensitivity of cell membrane scrambling is enhanced by ceramide [7] . Eryptosis is further triggered by energy depletion [10] and activation of caspases [7, 11-14]. Eryptosis is modified by several kinases, such as AMP activated kinase AMPK [8], cGMP-dependent protein kinase [15], Janus-activated kinase JAK3 [16], casein kinase [17, 18], p38 kinase [19], as well as sorafenib [20] and sunifinib [21] sensitive kinases.

Accelerated suicidal erythrocyte death may contribute to the anemia of several clinical disorders and eryptosis may be triggered by a wide variety of xenobiotics [22-45].

The percentage of phosphatidylserine exposing erythrocytes is enhanced in chronic renal failure [46]. Little is known, however, about mechanisms stimulating eryptosis in end stage renal disease. Eryptosis is known to be stimulated by vanadate [47] and methylglyoxal [7], both substances increased in uremic plasma [7, 47]. Other uremic toxins have, to the best of our knowledge, not been tested.

The pathophysiology of anemia in renal failure has been considered to involve polyamines [48, 49]. The polyamine degradation product acrolein is strongly involved in the harmful effects of polyamines [50]. Acrolein is a highly reactive aldehyde with cytotoxic properties [51]. Human exposure to acrolein may result from food or most importantly cigarette smoking, but acrolein can also be generated endogenously by polyamine degradation [52]. Acrolein may be generated from spermine and spermidine by amine oxidase [50, 53]. In chronic renal failure the plasma amine oxidase activity and plasma concentration of acrolein are increased, whereas the plasma concentrations of spermidine and spermine are decreased [50, 53]. Cigarette smoking is particularly harmful in chronic kidney disease [54]. The present study therefore explored, whether eryptosis is triggered by the uremic toxin acrolein [55, 56]. In the present study the effect of acrolein on $[Ca^{2+}]_i$, cell volume and phosphatidylserine abundance at the erythrocyte surface were analysed. As a result, acrolein stimulates eryptosis, the suicidal death of erythrocytes.

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, erythrocytes were exposed to acrolein (Sigma-Aldrich, Steinheim, Germany) at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA).

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 in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

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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 2 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 in fluorescence channel FL-1 in FACS analysis.

Measurement of hemolysis

For the 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.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37°C with 1 μg/ml anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 minutes 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. The samples were then analyzed by flow cytometric analysis in FL-1.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA and *t* test as appropriate. 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

The present study explored the possibility that acrolein is capable to trigger eryptosis. A first series of experiments elucidated the potential effect of acrolein on cell volume. To this end, forward scatter as a measure of cell volume was determined utilizing flow cytometry of human erythrocytes. As illustrated in Fig. 1, a 48 hours exposure to acrolein was followed by a decrease of forward scatter, an effect reaching statistical significance at 30 μM acrolein concentration. Accordingly, acrolein decreased erythrocyte volume.

In a second series of experiments cell membrane scrambling was analysed by determination of phosphatidylserine abundance at the cell surface. Phosphatidylserine exposing erythrocytes were identified by annexin-V-binding in FACS analysis. As illustrated in Fig. 2, a 48 h exposure to acrolein dose dependently increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 30 μM acrolein concentration. Accordingly, acrolein exposure was followed by erythrocyte cell membrane scrambling with phosphatidylserine exposure at the cell surface. For comparison, effects of the precursors spermine and spermidine on annexin-V-binding have been tested. The administration of spermine (0, 1.5, 15 and 150 μM) did not significantly modify the percentage of annexin-V-binding erythrocytes (2.18 ± 0.36%, 2.49 ± 0.32%, 2.31 ± 0.49%, and 1.77 ± 0.40%, respectively, n = 5). Moreover, the administration of spermidine (0, 1.5, 15 and 150 μM) did not significantly modify the percentage of annexin-V-binding erythrocytes (2.78 ± 0.42%, 2.30 ± 0.41%, 2.31 ± 0.44%, and 2.05 ± 0.35%, respectively, n = 5).

Further experiments tested, whether acrolein exposure is followed by hemolysis. To this end, the percentage of hemolysed erythrocytes was quantified by determination of hemoglobin release into the supernatant. As illustrated in Fig. 2, exposure of erythrocytes

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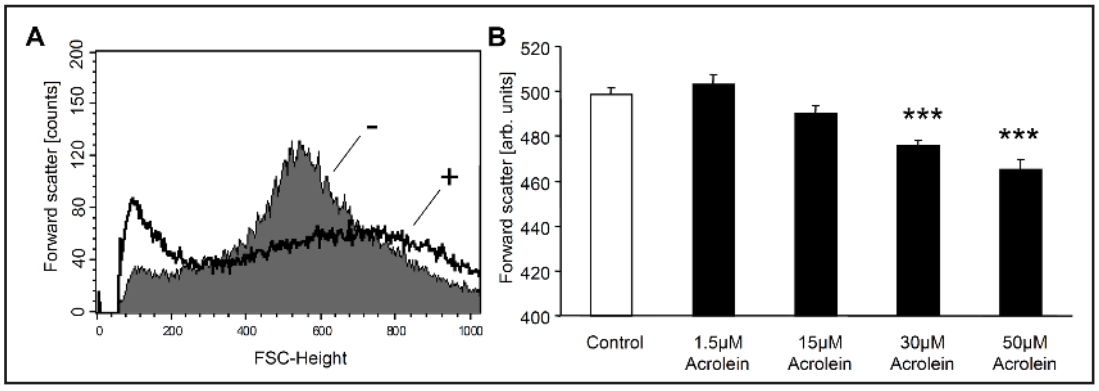

Fig. 1. Effect of acrolein on erythrocyte forward scatter. A: Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-, grey) and with (+, black) presence of 50 µM acrolein. B: Arithmetic means ± SEM (n = 18 - 19) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) acrolein (1.5 - 50 µM). *** ($p<0.001$) indicate significant difference from the absence of acrolein (ANOVA).

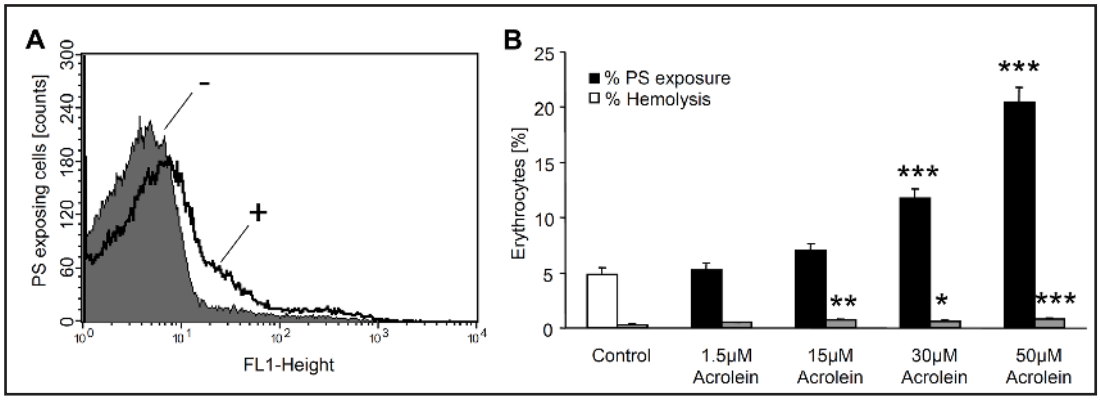

Fig. 2. Effect of acrolein on phosphatidylserine exposure and hemolysis. A: Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-, grey) and with (+, black) presence of 50 µM acrolein. B: Arithmetic means ± SEM (n = 18 - 19) of erythrocyte annexin-V-binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of acrolein (1.5 - 50 µM). For comparison, arithmetic means ± SEM (n = 6) of the percentage of hemolysis is shown as grey bars. *,**,*** ($p<0.05$, 0.01, 0.001 respectively) indicates significant difference from the absence of acrolein for the respective measurements (ANOVA).

for 48 h to acrolein significantly increased the hemoglobin concentration in the supernatant, an effect, however, affecting only a relatively small percentage of erythrocytes (Fig. 2).

Both, cell shrinkage and cell membrane scrambling could have resulted from an increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Thus, further experiments were performed to elucidate whether acrolein increases $[Ca^{2+}]_i$. To this end, erythrocytes were exposed to Ringer solution without or with added acrolein (1.5 - 50 µM). In the following the erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in FACS analysis. As illustrated in Fig. 3, following a 48 hours exposure of human erythrocytes up to 50 µM acrolein remained without significant effect on Fluo3 fluorescence. Accordingly, at the concentrations tested, acrolein did not significantly increase cytosolic Ca^{2+} concentration.

To further elucidate the potential role of $[Ca^{2+}]_i$, erythrocytes were exposed to 50 µM acrolein 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 illustrated in Fig. 4, the

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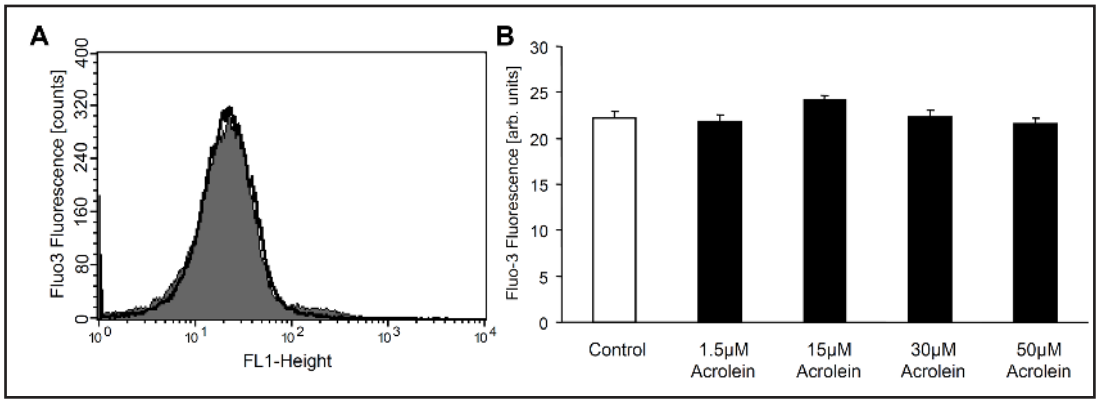

Fig. 3. Effect of acrolein on erythrocyte cytosolic Ca^{2+} concentration. A: Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey) and with (black) presence of 50 µM acrolein. B: Arithmetic means ± SEM (n = 18-19) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) acrolein (1.5 - 50 µM).

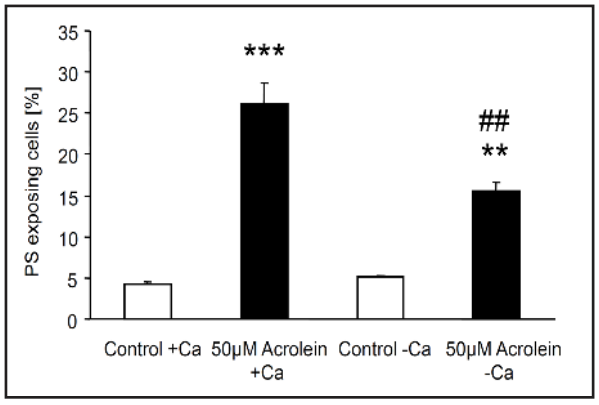

Fig. 4. Effect of Ca^{2+} withdrawal on acrolein-induced annexin-V-binding. Arithmetic means ± SEM (n = 4) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 50 µM acrolein in the presence (left bars, + Ca) and absence (right bars, - Ca) of calcium. **,*** ($p<0.01$, 0.001) indicates significant difference from respective control (absence of acrolein) (ANOVA) ## ($p<0.01$) indicates significant difference from the respective values in the presence of Ca^{2+}.

effect of acrolein on annexin-V-binding was significantly decreased in the nominal absence of extracellular Ca^{2+}. However, even in the absence of extracellular Ca^{2+}, acrolein still significantly increased the percentage of annexin-V-binding erythrocytes. Thus, the effect of acrolein was mainly, but not exclusively, dependent on Ca^{2+}.

In view of the Ca^{2+} sensitivity of acrolein-induced eryptosis and the absence of an increase of $[Ca^{2+}]_i$ following acrolein exposure, additional experiments were performed to test whether acrolein increases the Ca^{2+} sensitivity of cell membrane scrambling. To this end erythrocytes were exposed to the Ca^{2+} ionophore ionomycin (1 µM) in the absence and the presence of acrolein (50 µM). As illustrated in Fig. 5, exposure of erythrocytes to the Ca^{2+} ionophore ionomycin (1 µM) was followed by a marked increase of the percentage phosphatidylserine exposing erythrocytes, an effect significantly more pronounced in the presence than in the absence of acrolein (50 µM). Accordingly, acrolein augments the cell membrane scrambling effect of cytosolic Ca^{2+}.

As ceramide is known to enhance the sensitivity of cell membrane scrambling to cytosolic Ca^{2+}, a further series of experiments was performed to define the effect of acrolein on formation of ceramide. Ceramide abundance at the cell surface was elucidated utilizing FITC-labeled anti-ceramide antibodies. As shown in Fig. 6, acrolein significantly increased ceramide-dependent fluorescence.

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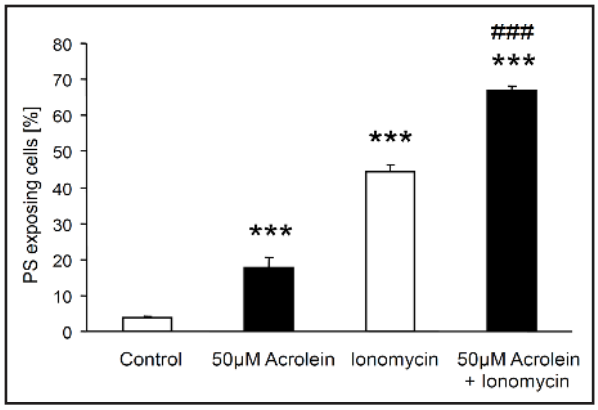

Fig. 5. Effect of Ca^{2+} ionophore ionomycin on annexin-V-binding in absence and presence of acrolein. Arithmetic means ± SEM (n = 5) of the percentage of annexin-V-binding erythrocytes after a a 48 h pretreatment with Ringer solution without (white bar) or with (black bars) 50 µM acrolein followed by a 30 minutes treatment in the absence (left bars, -Iono) and presence (right bars, +Iono) of the Ca^{2+} ionophore ionomycin (1 µM). *** ($p<0.001$) indicates significant difference from control (absence of acrolein and ionomycin) (ANOVA), ### ($p<0.001$) indicates significant difference from the ionomycin treated erythrocytes in the absence of acrolein.

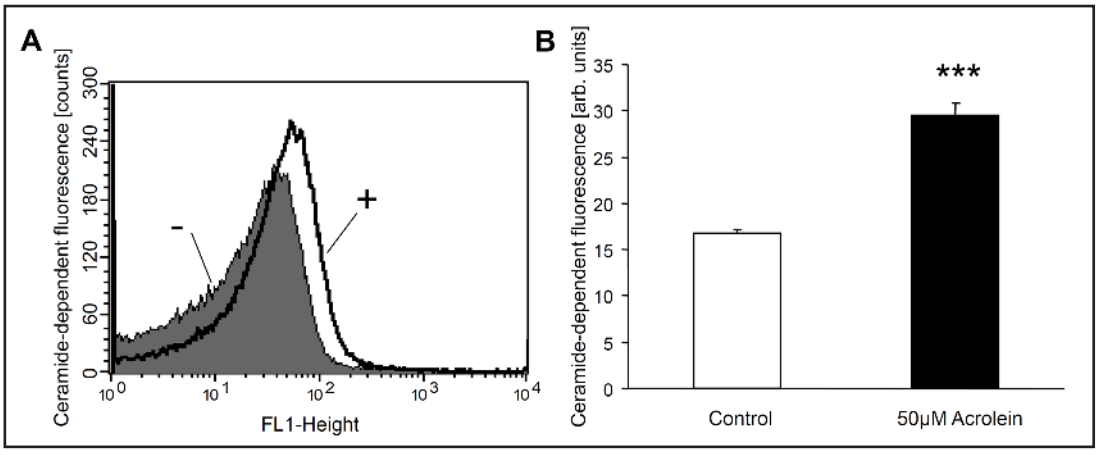

Fig. 6. Effect of acrolein on ceramide formation. A: Original histogram of anti-ceramide FITC-fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, grey) and with (+, black) presence of 50 µM acrolein. B: Arithmetic means ± SEM (n = 6) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) acrolein (50 µM). *** (p <0.001) indicates significant difference from control (absence of acrolein) (t test).

Discussion

The present observations disclose a novel effect of acrolein, i.e. the stimulation of erythrocyte cell membrane scrambling, a typical feature of suicidal death or eryptosis. The concentrations of acrolein required for statistically significant stimulation of cell membrane scrambling (30 µM) are similar to those (25 -100 µM) previously shown to trigger death of nucleated cells [57, 58].

In nucleated cells acrolein can induce both, necrotic and apoptotic cell death [52, 57-59]. According to the present observations the extent of hemolysis is clearly smaller than the percentage of cell membrane scrambling suggesting that the erythrocytes die from eryptosis rather than hemolysis. Moreover, according to forward scatter, in average the erythrocytes do not swell following acrolein exposure [7, 42].

Despite the weak effect of acrolein on cytosolic Ca2+ activity, acrolein exposure leads to decrease of forward scatter reflecting a decrease of cell volume. Eryptotic erythrocyte shrinkage may result from activation of Ca^{2+} sensitive K^+ channels [7] with subsequent K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [9]. Whether or not those channels are activated following exposure to acrolein, remains to be shown.

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The acrolein induced cell membrane scrambling is similarly not the result of increased cytosolic Ca^{2+} activity. Nevertheless, the presence of extracellular Ca^{2+} is required for full stimulation of cell membrane scrambling. Accordingly, acrolein is effective by increasing the Ca^{2+}sensitivity of cell membrane scrambling. Along those lines, acrolein increases the scrambling effect of the Ca^{2+} ionophore ionomycin. As observed earlier [7], the Ca^{2+} sensitivity of the erythrocyte cell membrane scrambling is enhanced by ceramide. Acrolein treatment indeed resulted in an increase of ceramide formation. Thus, acrolein triggers cell membrane scrambling at least in part by increasing the formation of ceramide, which in turn increases the Ca^{2+} sensitivity of cell membrane scrambling. Besides its effect on erythrocyte cell membrane scrambling [7], ceramide is known to stimulate apoptosis of nucleated cells [60]. To the best of our knowledge, an effect of acrolein on ceramide formation has never been shown.

At least in theory, the stimulation of eryptosis by acrolein could contribute to the accelerated erythrocyte death in chronic renal failure. Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [7, 42]. In renal insufficiency, the accelerated loss of erythrocytes cannot be fully compensated by enhanced formation of new erythrocytes and anemia develops [7]. Acrolein is formed in renal failure due to increased spermine degradation by serum amine oxidase [53]. Acrolein is increased in end stage renal disease and partially removed by hemodialysis [61]. Increased acrolein levels are also observed in nephropathy models [62]. In a rat model of CKD, acrolein was lowered by an oral charcoal absorbent, a treatment associated with beneficial cardiovascular effects [63].

Acrolein is not the only substance stimulating eryptosis in renal insufficiency. Further substances reported to stimulate eryptosis and to be enhanced in renal insufficiency are vanadate [47] and methylglyoxal [7] . Along those lines, iron deficiency, which contributes to anemia in chronic renal failure [4, 5] is a known trigger of eryptosis [64]. It is expected that additional uremic toxins will be identified, which stimulate eryptosis and thus contribute to the anemia of patients with end stage renal disease.

Phosphatidylserine exposing erythrocytes may adhere to endothelial CXCL16/SR-PSO of the vascular wall [65] thus compromising microcirculation. Accordingly, the adhering erythrocytes may interfere with blood flow [65-70]. Moreover, phosphatidylserine exposing erythrocytes may stimulate blood clotting and thus trigger thrombosis [66, 71, 72]. The attempt to fully compensate the accelerated loss of erythrocytes by eryptosis may result in a high turnover of erythrocytes with increased numbers of phosphatidylserine exposing erythrocytes in circulating blood. Accordingly, at least in theory, uncritical use of erythropoietin or other erythropoiesis stimulating agents [73] may jeopardize microcirculation.

Conclusion

Acrolein triggers cell membrane scrambling and cell shrinkage and thus suicidal death of human erythrocytes. The effect is at least in part due to enhanced formation of ceramide with subsequent sensitization of cell membrane scrambling to cytosolic Ca^{2+}.

Conflict of Interests

The authors state that they have not any conflict of interests.

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