Original Paper

Aristolochic Acid Induced Suicidal Erythrocyte Death

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Key Words

Phosphatidylserine • Aristolochic Acid • Calcium • Ceramide • Cell volume • Eryptosis

Abstract

Background/Aims: Aristolochic Acid, a component of *Aristolochia* plants, has been shown to cause acute kidney injury, renal aristolochic acid nephropathy, Balkan endemic nephropathy, and urothelial carcinoma. Aristolochic acid nephropathy may be associated with severe anemia. The anemia could theoretically be due to stimulation of eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage and cell membrane scrambling with translocation of phosphatidylserine to the erythrocyte cell membrane surface. Signalling involved in the stimulation of eryptosis include increase of cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$) and formation of ceramide. **Methods:** Cell volume was estimated from forward scatter, phosphatidylserine-exposure from annexin V binding, $[\text{Ca}^{2+}]_i$ from Fluo3 fluorescence, and ceramide abundance from binding of fluorescent antibodies in flow cytometry. **Results:** A 48 hours exposure to Aristolochic Acid ($\geq 75 \mu\text{g/ml}$) was followed by a significant decrease of forward scatter and increase of annexin-V-binding. The effects were paralleled by a significant increase of $[\text{Ca}^{2+}]_i$ and significantly blunted, but not abrogated by removal of extracellular Ca^{2+} . Aristolochic Acid further significantly increased ceramide abundance. **Conclusions:** Aristolochic Acid triggers eryptosis, an effect at least in part due to entry of extracellular Ca^{2+} and ceramide formation.

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Introduction

Aristolochic Acid, a nephrotoxin and carcinogen [1] from plants of the genus *Aristolochia* [2], may lead to acute kidney injury [3], renal aristolochic acid nephropathy [4-10], Balkan endemic nephropathy [1, 7-9, 11, 12], and urothelial carcinoma [1, 2, 4, 6-10, 13-15]. Causes

of Aristolochic Acid intoxication include long-term intake of medicinal herbs containing Aristolochic Acid [16-19] or of flour obtained from wheat contaminated with seeds of *Aristolochia clematitis* [16]. Aristolochic Acid is considered to be at least partially effective by inducing DNA adduct formation and mutations [1, 4, 8, 9, 13, 14]. Aristolochic acid nephropathy is associated with anemia [20-31].

The anemia may result from eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface [32]. Phosphatidylserine exposing erythrocytes adhere to respective receptors of phagocytes leading to subsequent engulfment and degradation [32]. Accordingly, phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [32] and excessive eryptosis thus leads to anemia as soon as the loss of erythrocytes is not matched by a similar increase of erythrocyte formation. Signalling of eryptosis includes increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [33] and ceramide formation [32].

The present study explored, whether Aristolochic Acid triggers eryptosis, and if so, whether it influences $[\text{Ca}^{2+}]_i$ or ceramide abundance.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The blood was drawn from healthy individuals. The study is approved by the ethics committee of the University of Tübingen (184/2003V). The blood was centrifuged at 120 rcf for 20 minutes at 23°C and the platelets- and leukocytes-containing supernatant was disposed. Erythrocytes were washed 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). For the experiments, erythrocytes were incubated *in vitro* at a hematocrit of 0.4% at 37°C for 48 h. Erythrocytes were exposed to Aristolochic Acid (Sigma-Aldrich, Hamburg, Germany) in concentrations of 0, 25, 50, 75 and 100 $\mu\text{g}/\text{ml}$, respectively). 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 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.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 $\mu\text{g}/\text{ml}$ anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. 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

Fig. 1. Effect of Aristolochic Acid on phosphatidylserine exposure. A: Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 100 µg/ml Aristolochic Acid. B: Arithmetic means $\pm$ SEM ($n = 10$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of Aristolochic Acid (25-100 µg/ml) or, for comparison, DMSO (0.8%) alone. *** ($p < 0.001$) indicates significant difference from the absence of Aristolochic Acid (ANOVA).

PBS-BSA. The samples were then analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of hemolysis

For the determination of hemolysis, the samples were centrifuged (3 min at 1600 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.

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. The number of different blood samples studied is given as n . 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 Aristolochic Acid triggers eryptosis, the suicidal death of erythrocytes. The major hallmark of eryptosis is cell membrane scrambling with translocation of phosphatidylserine from the cell interior to the cell surface. Phosphatidylserine exposing erythrocytes were identified by binding of annexin-V and detected by flow cytometry. As shown in Fig. 1, a 48 hours exposure to Aristolochic Acid was followed by an increase of annexin-V-binding erythrocytes, an effect reaching statistical significance at 75 µg/ml Aristolochic Acid concentration.

Hemoglobin concentration in the supernatant was determined in order to estimate the effect of Aristolochic Acid exposure on hemolysis. According to hemoglobin concentration in the supernatant, a 48 hours incubation with 0, 25, 50, 75 and 100 µg/ml Aristolochic Acid resulted in hemolysis of 1.84 ± 0.24 %, 6.20 ± 3.01 %, 13.53 ± 2.80 %, 16.29 ± 2.64 and 18.36 ± 3.34 % ($n = 7$), respectively.

Eryptosis is further typically associated with cell shrinkage. Accordingly, cell volume was estimated from forward scatter in flow cytometry. As illustrated in Fig. 2, a 48 hours

Fig. 2. Effect of Aristolochic Acid on erythrocyte forward scatter. A: Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 100 µg/ml Aristolochic Acid. B: Arithmetic means $\pm$ SEM ($n = 10$) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Aristolochic Acid (25-100 µg/ml) or, for comparison, DMSO (0.8 %) alone. *** ($p < 0.001$) indicates significant difference from the absence of Aristolochic Acid (ANOVA).

Fig. 3. Effect of Aristolochic Acid on erythrocyte cytosolic Ca^{2+} concentration. A: Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 100 µg/ml Aristolochic Acid. B: Arithmetic means $\pm$ SEM ($n = 10$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) Aristolochic Acid (25-100 µg/ml) or, for comparison, DMSO (0.8 %) alone. C: Arithmetic means $\pm$ SEM ($n = 6$) of the percentage of annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) 100 µg/ml Aristolochic Acid in the presence (left bars, Plus Calcium) and absence (right bars, Minus Calcium) of calcium. *** ($p < 0.001$) indicates significant difference from the respective values in the absence of Aristolochic Acid, ### ($p < 0.001$) indicates significant difference from the respective values in the presence of calcium (ANOVA).

exposure to Aristolochic Acid decreased forward scatter, an effect reaching statistical significance at 75 µg/ml Aristolochic Acid concentration.

Cell membrane scrambling and cell shrinkage could both have resulted from an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Accordingly, Fluo3 fluorescence was employed to estimate alterations of $[\text{Ca}^{2+}]_i$. To this end, erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined by flow cytometry. As shown in Fig. 3 (A, B), a 48 hours exposure of human erythrocytes to Aristolochic Acid increased Fluo3 fluorescence, an effect reaching statistical significance at 100 µg/ml Aristolochic Acid. Further experiments were designed to test, whether the cell membrane scrambling following Aristolochic Acid treatment required

Fig. 4. Effect of Aristolochic Acid on ceramide abundance. A: Original histogram of anti-ceramide FITC fluorescence in erythrocytes after exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 100 µg/ml Aristolochic Acid. B: Arithmetic means $\pm$ SEM (n = 4) of ceramide abundance at the erythrocyte surface following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) presence of Aristolochic Acid (100 µg/ml). * (p<0.05) indicates significant difference from the absence of Aristolochic Acid (ANOVA).

entry of extracellular Ca^{2+} . To this end, erythrocytes were exposed for 48 hours to 100 µg/ml Aristolochic Acid in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 3C, the effect of Aristolochic Acid on annexin-V-binding was significantly blunted in the nominal absence of Ca^{2+} . However, Aristolochic Acid still significantly increased the percentage of annexin V binding erythrocytes in the nominal absence of extracellular Ca^{2+} . Thus, the effect of Aristolochic Acid on cell membrane scrambling did not fully depend on Ca^{2+} entry.

Since cell membrane scrambling may be triggered even at constant $[\text{Ca}^{2+}]_i$ by ceramide, further experiments were performed to explore, whether Aristolochic Acid increases ceramide formation. The abundance of ceramide at the erythrocyte surface was determined utilizing an anti-ceramide antibody. As illustrated in Fig. 4, a 48 hours exposure to 100 µg/ml Aristolochic Acid significantly increased the ceramide abundance at the erythrocyte surface.

Discussion

The present study discloses a novel effect of Aristolochic Acid, i.e. the triggering of eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage and cell membrane scrambling with translocation of phosphatidylserine to the erythrocyte surface. Aristolochic Acid concentrations similar to those required for the triggering of eryptosis have previously been reported to occur *in vivo* [34]. However, nephrotoxicity may be observed at lower plasma concentrations [35]. It must be kept in mind that the anemia following Aristolochic Acid intoxication could at least partially result from impaired formation of new erythrocytes and/or indirect stimulation of erythrocyte death, e.g. due to chronic kidney disease [36, 37].

Aristolochic Acid was at least partially effective through stimulation of Ca^{2+} entry with subsequent increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Ca^{2+} is a major trigger of eryptosis [32]. Ca^{2+} stimulates cell membrane scrambling by some illdefined mechanism and triggers cell shrinkage presumably by activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged

water [33]. Moreover, Aristolochic Acid is effective in part by increasing the abundance of ceramide, another well known trigger of eryptosis [32]. Stimulators of ceramide formation in erythrocytes include platelet activating factor (PAF), which is generated by a phospholipase A2 [38]. In other cell types Aristolochic Acid has been shown to inhibit phospholipase A2 [39, 40]. It remains to be shown whether Aristolochic Acid stimulates ceramide formation in other cell types. Possibly, Ca^{2+} entry and ceramide production contribute to the known stimulation of kidney cell apoptosis [4, 25, 41-70]. In nucleated cells, Aristolochic Acid is, however, at least partially effective by further mechanisms, such as formation of DNA adducts [71]. Erythrocytes are devoid of nuclei and thus would not be affected by DNA adduct forming activities.

The present observations do not rule out the involvement of further signalling pathways in the triggering of eryptosis by Aristolochic Acid. Signalling possibly contributing to stimulation of eryptosis include caspase activation [72-76], lack of AMP activated kinase AMPK [77] or cGMP-dependent protein kinase [78], inhibition of p21 activated kinase PAK2 [79] or sorafenib [80] and sunitinib [81] sensitive kinases as well as stimulation of casein kinase 1 α [82, 83], Janus-activated kinase JAK3 [84], protein kinase C [85] or p38 kinase [86].

Eryptosis is a physiological mechanism removing defective erythrocytes prior to hemolysis [32]. Eryptotic cell shrinkage [33] counteracts swelling of the defective cells thus minimizing hemolysis with release of cellular hemoglobin. Released hemoglobin may otherwise undergo glomerular filtration and subsequent precipitation and occlusion of renal tubules [87].

Excessive eryptosis may, however lead to anemia, a known side effect of Aristolochic Acid [20-31]. Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [32]. To the extent that the clearance of eryptotic erythrocytes is not compensated by similar increase of erythrocyte formation, anemia develops [32]. Moreover, the adherence of phosphatidylserine exposing erythrocytes to endothelial receptors [88] compromises microcirculation [88-93]. In addition, phosphatidylserine exposing erythrocytes foster blood clotting and thrombosis [89, 94, 95]. Derangement of renal microcirculation may contribute to the toxic effect of Aristolochic Acid on the kidney.

The stimulation of eryptosis by Aristolochic Acid may be enhanced in patients suffering from disorders facilitating eryptosis [32], such as diabetes [76, 96, 97], renal insufficiency [36, 37], hemolytic uremic syndrome [98], dehydration [99], sepsis [100], malaria [101], sickle cell disease [101], Wilson's disease [102], iron deficiency [103], malignancy [104], phosphate depletion [105], and metabolic syndrome [106]. Moreover, Aristolochic Acid induced eryptosis may be augmented by additional exposure to other eryptosis triggering xenobiotics [32, 81, 86, 106-130]. Those substances include several uremic toxins [32, 36, 113, 128, 131] and phosphate [132]. Thus, Aristolochic Acid induced uremia could sensitize erythrocytes to the proeryptotic effect of Aristolochic Acid.

Conclusion

Aristolochic Acid is a powerful stimulator of eryptosis, the suicidal erythrocyte death. Signalling involved in the stimulation of eryptosis by Aristolochic Acid include stimulation of Ca^{2+} entry and ceramide formation.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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