Original Paper

Enhanced Apoptotic Death of Erythrocytes Induced by the Mycotoxin Ochratoxin A

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Key Words

Phosphatidylserine • Ochratoxin A • Calcium • Ceramide • Cell volume • Eryptosis

Abstract

Background: The mycotoxin ochratoxin A, an agent responsible for endemic Balkan nephropathy is known to trigger apoptosis and thus being toxic to several organs including the kidney. The mechanisms involved in ochratoxin A induced apoptosis include oxidative stress. Sequelae of ochratoxin intoxication include anemia. Similar to apoptosis of nucleated cells, erythrocytes may undergo suicidal cell death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling resulting in phosphatidylserine-exposure at the cell surface. Eryptosis could be triggered by Ca^{2+} -entry through oxidant sensitive unspecific cation channels increasing cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). The Ca^{2+} -sensitivity of cell membrane scrambling could be enhanced and eryptosis thus triggered by ceramide. The removal of suicidal erythrocytes may lead to anemia. Moreover, eryptotic erythrocytes could adhere to the vascular wall thus impeding microcirculation. The present study explored, whether ochratoxin A stimulates eryptosis. **Methods:** Fluo3-fluorescence was utilized to determine $[\text{Ca}^{2+}]_i$, forward scatter to estimate cell volume, annexin-V-binding to identify phosphatidylserine-exposing cells, fluorescent antibodies to detect ceramide formation and hemoglobin release to quantify hemolysis. Moreover, adhesion to human vascular endothelial cells (HUVEC) was determined utilizing a flow chamber. **Results:** A 48 h exposure to ochratoxin A was followed by significant increase of Fluo3-fluorescence ($\geq 2.5 \mu\text{M}$), increase of ceramide abundance ($10 \mu\text{M}$), decrease of forward scatter ($\geq 5 \mu\text{M}$) and increase of annexin-V-binding ($\geq 2.5 \mu\text{M}$). Ochratoxin A exposure slightly but significantly enhanced hemolysis ($10 \mu\text{M}$). Ochratoxin ($10 \mu\text{M}$) enhanced erythrocyte adhesion to HUVEC. Removal of extracellular Ca^{2+} significantly blunted, but did not abrogate ochratoxin A-induced annexin V binding. **Conclusions:** Ochratoxin A triggers suicidal erythrocyte death or eryptosis, an effect partially but not fully due to stimulation of Ca^{2+} -entry.

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Introduction

Ochratoxin A (OTA), one of the mycotoxins with greatest public health and agro-economic significance [1-3], has been reported to be toxic to stomach, intestine, liver, kidney, neurons, and immune cells [2-10]. Ochratoxin A further has teratogenic potency [11]. Most importantly, Ochratoxin A intoxication underlies the Balkan endemic nephropathy and may cause urinary tract tumors [11, 12]. Moreover, Ochratoxin intoxication may lead to anemia [13, 14] with hypochromic-microcytic erythrocytes [13]. Ochratoxin A is in part effective by triggering apoptosis [5, 6, 9, 10, 15-26]. Mechanisms involved in the stimulation of apoptosis by ochratoxin A include oxidative stress [18, 27, 28] and Ca^{2+} entry [29].

Similar to apoptosis of nucleated cells, erythrocytes may undergo suicidal death or eryptosis [30]. Hallmarks of eryptosis are cell membrane scrambling and cell shrinkage [30]. Eryptosis could be triggered by activation of oxidant sensitive Ca^{2+} -permeable cation channels [31, 32], which mediate Ca^{2+} -entry and subsequent increase of cytosolic Ca^{2+} -concentration [30]. Ca^{2+} activates Ca^{2+} -sensitive K^{+} -channels [33] leading to K^{+} -exit, hyperpolarization, Cl^{-} -exit, cellular KCl loss together with osmotically obliged water and thus cell shrinkage [34]. An increase of cytosolic Ca^{2+} -activity is further followed by cell membrane scrambling with phosphatidylserine exposure at the cell surface [35]. Cell membrane scrambling and thus suicidal erythrocyte death are in addition stimulated by ceramide [36], energy depletion [37] and caspase activation [38-42]. Eryptosis is further modified by AMP activated kinase AMPK [32], cGMP-dependent protein kinase [43], casein kinase CK1 [44] and Janus-activated kinase JAK3 [45].

The present study explored, whether eryptosis is sensitive to ochratoxin A and to possibly disclose underlying mechanisms.

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 ochratoxin A (Enzo, Lörrach, 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).

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.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without ochratoxin A, 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.

Determination of intracellular ATP concentration

For determination of intracellular ATP, 90 µl of erythrocyte pellets were incubated for 48 hours at 37°C in Ringer solution (final hematocrit 5%) with or without ochratoxin A. All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of HClO₄ (5%). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated KHCO₃ solution. After dilution of the supernatant, the ATP concentrations of the aliquots were 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 in mmol/l cytosol of erythrocytes.

Cell culture and in vitro adhesion studies

Human umbilical vein endothelial cells (HUVEC) from early passage of culture were cultured in complete endothelial cell basal medium (PAA) containing growth factors and 10% fetal bovine serum and grown to confluency. HUVEC (5 × 10⁵) were attached on sterile coverslips coated with 0.2% gelatine (Sigma-Aldrich) by overnight incubation in complete endothelial cell basal medium under cell culture conditions. Erythrocytes prepared as indicated were perfused on a HUVEC monolayer in a flow chamber model (Oligene) at arterial shear rates (1,200 s⁻¹). The interaction events were recorded with a charge-coupled device camera (Carl Zeiss) with ×20 magnification, followed by analysis of the number of adherent erythrocytes per high powerfield.

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.

Statistics

Data are expressed as arithmetic means ± 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. Since batches of erythrocytes may differ in their susceptibility to triggers of eryptosis, the same erythrocyte batches have been used for control and experimental conditions.

Results

In order to determine, whether ochratoxin A stimulates Ca²⁺-entry into erythrocytes, cytosolic Ca²⁺-activity was estimated from Fluo3-fluorescence. Erythrocytes were loaded with Fluo3-AM and the Fluo3-fluorescence determined in FACS analysis. As illustrated in Fig. 1, a 48 hours exposure of human erythrocytes to ochratoxin A was followed by an increase of Fluo3-fluorescence, an effect reaching statistical significance at 2.5 µM ochratoxin A concentration.

An increased cytosolic Ca²⁺-concentration is expected to stimulate Ca²⁺-sensitive K⁺-channels with subsequent K⁺-exit, hyperpolarisation, Cl⁻-exit and loss of cell water due to cellular loss of KCl. To explore, whether ochratoxin A influences cell volume, forward scatter was determined in FACS analysis. As shown in Fig. 2, a 48 h treatment with ochratoxin A was

Fig. 1. Effect of ochratoxin A on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of ochratoxin A (10 μM). B. Arithmetic means $\pm$ SEM ($n = 12$) of the normalized geo means (geometric mean of the histogram in arbitrary units) of Fluo3-fluorescence in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) ochratoxin A (1-10 μM). * ($p < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of ochratoxin A (ANOVA).

Fig. 2. Effect of ochratoxin A on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of ochratoxin A (10 μM). B. Arithmetic means $\pm$ SEM ($n = 12$) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) ochratoxin A (1-10 μM). * ($p < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of ochratoxin A (ANOVA).

followed by a decrease of forward scatter, an effect reaching statistical significance at 5 μM ochratoxin A concentration.

Increased cytosolic Ca^{2+} is further expected to stimulate erythrocyte membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Accordingly, a further series of experiments explored whether ochratoxin A triggers cell membrane scrambling. Phosphatidylserine exposing erythrocytes were identified with binding of fluorescent annexin-V to the erythrocyte surface. As illustrated in Fig. 3, a 48 hours exposure to ochratoxin A resulted in an increase of annexin-V-binding, an effect reaching statistical significance at 2.5 μM ochratoxin A.

Further experiments addressed, whether ochratoxin A treatment triggers hemolysis, which was estimated from hemoglobin release into the supernatant. As shown in Fig. 3,

Fig. 3. Effect of ochratoxin A on phosphatidylserine exposure and hemolysis. A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of ochratoxin A (10 μM). B. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 12) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of ochratoxin A (1-10 μM). For comparison, arithmetic means ± SEM (n = 4) of the percentage of hemolysis is shown as grey bars. * (p<0.05), ** (p<0.01), *** (p<0.001) indicates significant difference from the absence of ochratoxin A (ANOVA).

Fig. 4. Effect of Ca²⁺ withdrawal on ochratoxin A-induced annexin-V-binding. Arithmetic means ± SEM (n = 4) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) ochratoxin A (10 μM) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. * (p<0.05), *** (p<0.001) indicates significant difference from the absence of ochratoxin A (ANOVA) # (p<0.05) indicates significant difference from the respective value in the presence of Ca²⁺.

exposure of erythrocytes to ochratoxin A for 48 hours led to a slight increase of hemoglobin concentration in the supernatant, an effect reaching statistical significance at 10 μM.

An additional series of experiments explored, whether the ochratoxin A induced cell membrane scrambling resulted from Ca²⁺ entry. To this end, erythrocytes were treated with 10 μM ochratoxin A in the presence or in the nominal absence of extracellular Ca²⁺. As shown in Fig. 4, Ca²⁺-removal indeed blunted significantly the increase of annexin-V-binding following ochratoxin A treatment. However, the percentage of phosphatidylserine exposing erythrocytes still increased significantly following ochratoxin A treatment in the nominal absence of Ca²⁺. Thus, Ca²⁺-removal significantly blunted, but did not fully abrogate the ochratoxin A induced erythrocyte membrane scrambling.

Additional experiments were performed to explore, whether ochratoxin A stimulates the formation of ceramide, which is known to enhance the Ca²⁺-sensitivity of cell membrane

Fig. 5. Effect of ochratoxin A on ceramide formation. A. Original histogram of anti-ceramide FITC-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) presence of ochratoxin A (10 μ M). B. Arithmetic means $\pm$ SEM ($n = 4$) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) ochratoxin A (10 μ M). *** ($p < 0.001$) indicates significant difference from control (absence of ochratoxin A) (t test).

Fig. 6. Effect of ochratoxin A on adhesion of erythrocytes under flow conditions. Arithmetic means $\pm$ SEM ($n = 4$) of erythrocytes binding to human umbilical vein endothelial cells (HUVEC) under flow conditions following exposure for 48 hours to Ringer solution without (white bar), or with ochratoxin A (10 μ M) (black bar). *** ($p < 0.001$) indicates statistically significant difference from the absence of ochratoxin A (10 μ M).

scrambling and thus to trigger eryptosis. Ceramide abundance at the erythrocyte surface was quantified utilizing FITC-labeled anti-ceramide antibodies. As shown in Fig. 5, ochratoxin A (10 μ M) significantly increased ceramide formation. Additional experiments explored the effect of the sphingomyelinase inhibitor amitriptyline. Treatment with ochratoxin A significantly enhanced annexin V binding from $2.9 \pm 0.1\%$ ($n = 4$) to $26.4 \pm 1.5\%$ ($n = 4$) in the absence and to $31.8 \pm 0.2\%$ ($n = 4$) in the presence of amitriptyline (10 μ M). Thus, amitriptyline did not decrease annexin binding following ochratoxin A treatment.

Further experiments explored whether or not ochratoxin A influences the energy status of erythrocytes. To this end, cytosolic ATP concentrations were determined. As a result, the ATP concentrations were virtually identical in the presence (2.7 ± 0.1 mM; $n = 4$) and absence (2.7 ± 0.1 mM; $n = 4$) of ochratoxin A. Thus, ochratoxin induced suicidal death of erythrocytes did not result from ATP depletion of erythrocytes.

Phosphatidylserine exposing erythrocytes are expected to adhere to the vascular wall. Thus, additional experiments were performed to test for an effect of ochratoxin A

on adherence of erythrocytes to human umbilical vein endothelial cells (HUVEC) under in vitro flow conditions at arterial shear rates of 1200^s. As illustrated in Fig. 6, treatment of erythrocytes with ochratoxin A (10 μ M) was followed by a marked increase of the number of erythrocytes adhering to HUVEC.

Discussion

The present study reveals a novel effect ochratoxin A, i.e. the stimulation of erythrocyte shrinkage and plasma membrane scrambling. Thus, ochratoxin A triggers suicidal erythrocyte death or eryptosis. The concentration required for the stimulation of cell membrane scrambling is 2.5 μ M, which is well in the range of the concentrations encountered in vivo [46]. A plasma concentration of 2 μ M, for instance, has been reported following treatment of Wistar rats with 450 μ g/kg body weight [46]. Thus, the present observations may be relevant for the in vivo toxicity of ochratoxin A. On the other hand, effects of ochratoxin A in nanomolar concentrations have been described [47].

Ochratoxin A is at least in part effective by increase of cytosolic Ca^{2+} -activity, which presumably results from Ca^{2+} -entry from extracellular space. Thus, ochratoxin A most likely activates Ca^{2+} -permeable cation channels. The channels have earlier been shown to involve the transient receptor potential channel TRPC6 [31]. The channels are known to be activated by oxidative stress [48].

Increase of cytosolic Ca^{2+} -activity results in activation of Ca^{2+} -sensitive K^{+} -channels [33, 49] leading to K^{+} -exit following its chemical gradient. The resulting cell membrane hyperpolarisation drives Cl^{-} -exit, the cellular KCl-loss is paralleled by exit of osmotically obliged water and thus by cell shrinkage [34]. The decrease of erythrocyte volume is reflected by a decrease of forward scatter.

Increased cytosolic Ca^{2+} -activity further triggers cell membrane scrambling with subsequent phosphatidylserine exposure at the erythrocyte surface [34, 35, 50]. The ochratoxin A induced cell membrane scrambling is significantly blunted by removal of extracellular Ca^{2+} , indicating that ochratoxin A is at least partially effective by stimulating Ca^{2+} -entry from extracellular space. Removal of extracellular Ca^{2+} does, however, not fully abrogate the ochratoxin A induced cell membrane scrambling, indicating that one or more additional mechanisms may be involved in the stimulation of cell membrane scrambling by ochratoxin A.

Such an additional mechanism contributing to the ochratoxin A induced cell membrane scrambling is the stimulation of ceramide formation. Ceramide sensitizes the scrambling machinery for Ca^{2+} and may by itself stimulate cell membrane scrambling in erythrocytes [36, 51]. Moreover, ceramide is a powerful stimulator of apoptosis [52]. Ceramide participates in the pathophysiology of several diseases, including diabetes [53-55], Wilson's disease [56], multiple sclerosis [57], major depression [52], Parkinson's disease [58], Alzheimer's disease [52, 59, 60], cardiovascular disease [61, 62], cystic fibrosis [63], lung inflammation, fibrosis and infection [64]. However, inhibition of sphingomyelinase with amitriptylin did not prevent the stimulation of cell membrane scrambling by ochratoxin A. Thus, activation of sphingomyelinase may contribute to but does not account for the stimulation of cell membrane scrambling by ochratoxin A.

Eryptosis is further stimulated by a wide variety of xenobiotics [38, 45, 65-77]. Moreover, enhanced eryptosis occurs in several clinical disorders [30], such as diabetes [42, 78, 79], renal insufficiency [80], hemolytic uremic syndrome [81], sepsis [82], sickle cell disease [83], glucose 6-phosphate dehydrogenase deficiency [84], malaria [56, 85-88], Wilson's disease [56], iron deficiency [89], malignancy [90] phosphate depletion [91] and metabolic syndrome [92].

Eryptosis may be pathophysiologically relevant. Phosphatidylserine exposing erythrocytes adhere to endothelial CXCL16/SR-PSO [93]. The erythrocyte adhesion to the vascular wall is expected to compromise microcirculation [93-98]. Phosphatidylserine exposing erythrocytes are further known to foster blood clotting [94, 99, 100] and thus to

increase the risk of thrombosis. Eventually, phosphatidylserine exposing erythrocytes are engulfed and thus cleared from circulating blood [30]. To the extent that the accelerated loss of erythrocytes is not compensated by a similar increase of erythropoiesis, enhanced eryptosis may result in anemia [30]. Thus, the present observations could well explain the ochratoxin induced anemia known since several decades [13, 14].

The present observations may be relevant not only for the ochratoxin A induced erythrocyte death, but may, in addition, shed additional light on the mechanisms underlying ochratoxin A induced apoptosis of nucleated cells. Evidence for the involvement of Ca^{2+} in the stimulation of apoptosis has been provided before [29]. To the best of our knowledge, the involvement of ceramide formation in ochratoxin A toxicity has never been reported. In view of the present observations, ceramide may be involved in ochratoxin A induced apoptosis of nucleated cells.

Conclusion

The exposure of erythrocytes to ochratoxin A leads to Ca^{2+} entry and ceramide formation with subsequent triggering of cell membrane scrambling and cell shrinkage. Thus, ochratoxin A stimulates eryptosis, the suicidal death of erythrocytes.

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