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Original Paper

Patulin-Induced Suicidal Erythrocyte Death

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

Abstract
Background: Patulin, the most common mycotoxin in apples and apple-derived products, triggers apoptosis and has thus been considered for the treatment of cancer. Similar to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage and by cell membrane scrambling leading to phosphatidylserine-exposure at the erythrocyte surface. Stimulators of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$). The present study explored, whether exposure of human erythrocytes to patulin is followed by eryptosis. ***Methods:*** Forward scatter was measured to estimate cell volume, annexin V binding to detect phosphatidylserine-exposure, hemoglobin release to quantify hemolysis, and Fluo3-fluorescence to determine $[Ca^{2+}]_i$. ***Results:*** A 48 h exposure to patulin significantly increased $[Ca^{2+}]_I$ (5 μM), significantly decreased forward scatter (5 μM) and significantly increased annexin-V-binding (2.5 μM). Patulin (10 μM) induced annexin-V-binding was virtually abrogated by removal of extracellular Ca^{2+}. ***Conclusion:*** Patulin stimulates Ca^{2+} entry into erythrocytes, an effect triggering suicidal erythrocyte death or eryptosis.

Introduction

Patulin, a genotoxic mycotoxin produced by several species of *Aspergillus*, *Penicillium* and *Byssochlamys* [1, 2], is the most common mycotoxin in apples and apple-derived products [2]. It contaminates further fruits, such as grapes, oranges, pears and peaches [1, 3]. Cellular effects of patulin include formation of reactive oxygen species (ROS), cell cycle arrest, cytochrome c release from mitochondria, caspase-3 activation, PARP cleavage, ATF3 expression and subsequent apoptosis [1, 3-6]. Patulin causes DNA damage and is mutagenic, carcinogenic and teratogenic [1, 3]. On the other hand, in view of its ability to

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trigger apoptosis of tumor cells, patulin has been considered a candidate for the treatment of malignancy [4, 5].

Erythrocytes lack mitochondria and nuclei and cannot experience mitochondrial cytochrome c release and DNA fragmentation, hallmarks of apoptosis [7]. Nevertheless, erythrocytes may experience apoptosis-like suicidal death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage [7]. Eryptosis may be triggered by increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) following Ca^{2+} entry through Ca^{2+}-permeable cation channels [8, 9]. The channels are activated by oxidative stress [10]. Increased $[Ca^{2+}]_i$ activates Ca^{2+}-sensitive K^+ channels [11] leading to cell shrinkage due to K^+ exit, hyperpolarization, Cl^- exit and thus cellular KCl and water loss [12]. Increased $[Ca^{2+}]_i$ further triggers cell membrane scrambling with subsequent phosphatidylserine exposure at the erythrocyte surface [13]. The Ca^{2+} sensitivity of eryptosis is enhanced by ceramide [14]. Moreover, eryptosis is triggered by energy depletion [15], caspase activation [16-20], and deranged activity of distinct kinases, such as AMP activated kinase AMPK [9], cGMP-dependent protein kinase [21] or Janus-activated kinase JAK3 [22].

Eryptosis is stimulated by a wide variety of xenobiotics including several food contaminants [16, 22-25, 25-36]. Moreover, eryptosis is observed in several clinical disorders [7], such as diabetes [20, 37, 38], renal insufficiency [39], hemolytic uremic syndrome [40], sepsis [41], sickle cell disease [42], malaria [43-47], Wilson's disease [47], iron deficiency [48], phosphate depletion [49], and presumably metabolic syndrome [50].

The present study elucidated the effect of patulin on $[Ca^{2+}]_i$, cell volume and phosphatidylserine abundance at the erythrocyte surface. The results indicate that patulin is a powerful stimulator of eryptosis.

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 patulin (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.

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)

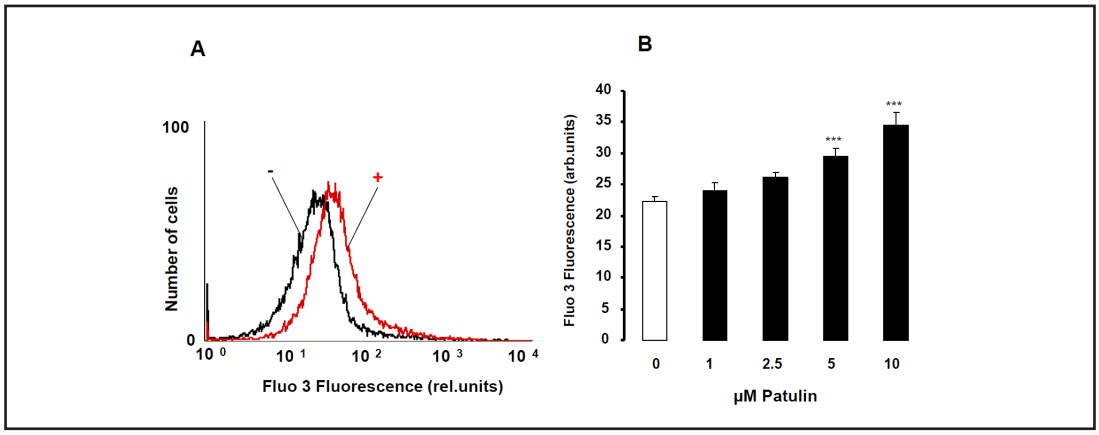

Fig. 1. Effect of patulin on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 10 µM patulin. B. Arithmetic means ± SEM (n = 12) of the Fluo3 fluorescence (arbitrary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

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

Following flow cytometry, the average of 10^4 cells has been calculated for each erythrocyte preparation. The arithmetic mean ± the standard error of the mean (SEM) has been calculated from the averaged values obtaind from the different erythrocyte preparations. 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 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 patulin exposure influences the cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) of human erythrocytes, the erythrocytes were loaded with Fluo3-AM and the Fluo3 fluorescence determined in FACS analysis. Prior to determination of Fluo3-fluorescence the erythrocytes were incubated in Ringer solution without or with patulin (1-10 µM). As shown in Fig. 1, a 48 hours exposure of human erythrocytes to patulin was followed by an increase of Fluo3 fluorescence, an effect reaching statistical significance at 5 µM patulin concentration. Accordingly, exposure to patulin resulted in an increase of cytosolic Ca^{2+} concentration.

An increase of $[Ca^{2+}]_i$ is known to activate Ca^{2+}-sensitive K^+ channels leading to cell shrinkage due to KCl exit paralleled by osmotically obliged water. In order to estimate cell volume, forward scatter was determined in FACS analysis. As shown in Fig. 2, a 48 hours exposure to patulin was followed by a decrease of forward scatter, an effect reaching statistical significance at 5 µM patulin. Accordingly, patulin exposure resulted in erythrocyte shrinkage.

An increase of $[Ca^{2+}]_i$ is further known to stimulate cell membrane scrambling with loss of lipid asymmetry and eventual phosphatidylserine exposure at the cell surface. To identify phosphatidylserine exposing erythrocytes annexin-V-binding was determined in FACS analysis. As illustrated in Fig. 3, a 48 h exposure to patulin increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 2.5 µM

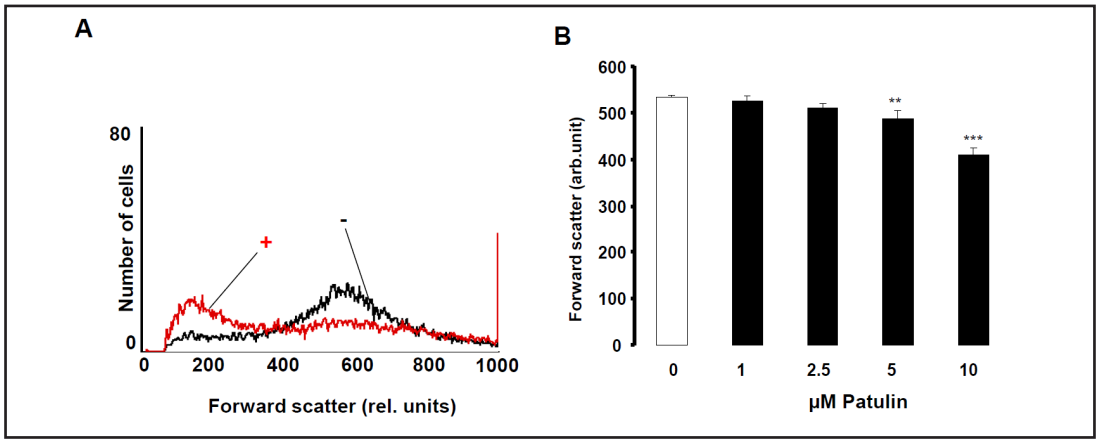

Fig. 2. Effect of patulin on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 10 µM patulin. B. Arithmetic means ± SEM (n = 12) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) patulin (1-10 µM). ** ($p<0.01$), *** ($p<0.001$) indicate significant difference from the absence of patulin (ANOVA).

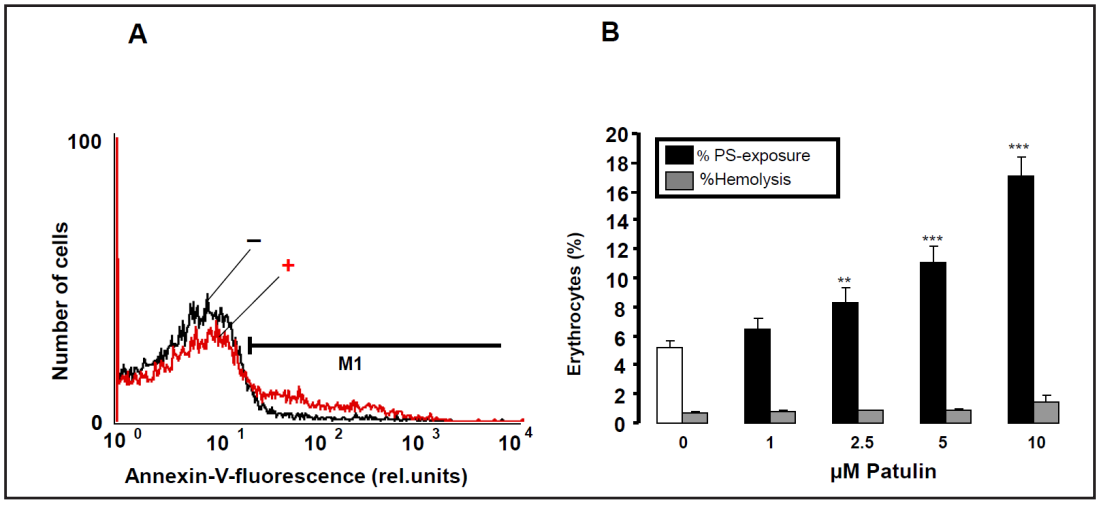

Fig. 3. Effect of patulin on phosphatidylserine exposure and hemolysis. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-) and with (+) presence of 10 µM patulin. B. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 12) following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

patulin. Accordingly, patulin triggered erythrocyte cell membrane scrambling with phosphatidylserine exposure at the cell surface.

In order to elucidate, whether patulin exposure causes hemolysis, the percentage of hemolysed erythrocytes was estimated from hemoglobin release into the supernatant. As shown in Fig. 3, exposure of erythrocytes for 48 h to patulin increased the hemoglobin concentration in the supernatant, an effect, however, not reaching statistical significance up to 10 µM patulin (Fig. 3 B)

In order to test, whether the patulin induced increase of $[Ca^{2+}]_i$ indeed contributed to or even accounted for the stimulation of patulin induced cell membrane scrambling, erythrocytes were exposed to 10 µM patulin for 48 hours in the presence and in the nominal

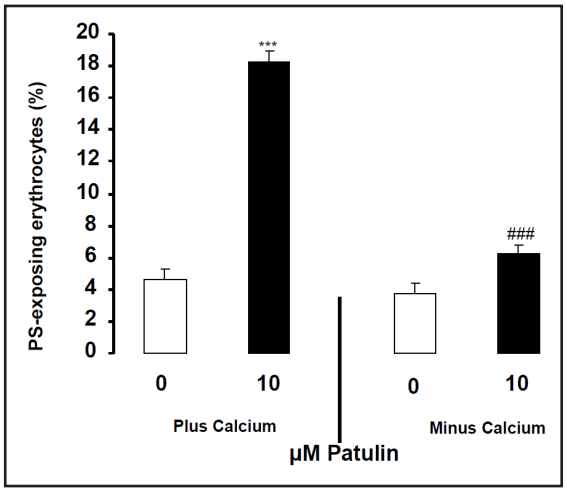

Fig. 4. Effect of Ca^{2+} withdrawal on patulin-induced annexin-V-binding. Arithmetic means ± SEM (n = 4) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 10 µM patulin in the presence (left bars, Plus Calcium) and absence (right bars, Minus Calcium) of calcium. *** ($p<0.001$) indicates significant difference from the absence of patulin (ANOVA) ### ($p<0.001$) indicates significant difference from the respective values in the presence of Ca^{2+}.

absence of extracellular Ca^{2+}. As illustrated in Fig. 4, the effect of patulin on annexin-V-binding was virtually abolished in the nominal absence of Ca^{2+}. Thus, the effect of patulin was mainly if not exclusively due to Ca^{2+} entry.

Discussion

The present study reveals a novel effect of patulin, i.e. its ability to trigger eryptosis, the suicidal death of erythrocytes. Specifically, patulin exposure was followed by erythrocyte shrinkage and erythrocyte membrane scrambling. The concentrations required (2.5-5 µM) were similar to those (2.5 - 10 µM) effective in tumor cells [4]. In several countries the tolerance level of patulin contamination amounts to 50 µg/Kg (320 µM) [51] Following oral ingestion of radiolabelled patulin, some 36% of the label appeared in urine pointing to intestinal absorption [52]. Peritoneal administration of 1-3.75 mg/kg has been reported [53]. From all tissues, erythrocytes displayed the stongest retention of the label [52].

Patulin stimulates cell membrane scrambling mainly by increasing cytosolic Ca^{2+} activity. Patulin presumably activates the Ca^{2+} permeable non-selective cation channels in erythrocytes. Those channels have not yet been defined at the molecular level but apparently involve the transient receptor potential channel TRPC6 [8]. Patulin is known to cause oxidative stress [5, 6], which in turn is known to activate the Ca^{2+} permeable unspecific cation channels in erythrocytes [10]. How increase of cytosolic Ca^{2+} activity triggers cell membrane scrambling is still incompletely understood. The scrambling has been considered to be a function of phospholipid scramblases [54]. Ca^{2+} has been claimed to stimulate phospholipid scramblase 1 (PLSCR1), a view, however, challenged later [54]. Instead, PLSCR1 may be involved in protein phosphorylation [54]. Thus, the molecular mechanisms underlying Ca^{2+} sensitive cell membrane scrambling remained incompletely understood.

Activation of the Ca^{2+} permeable unspecific cation channels most likely accounts in addition for the patulin induced erythrocyte shrinkage. Increase of cytosolic Ca^{2+} activity results in activation of Ca^{2+} sensitive K^+ channels [11, 55], which is followed by K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [12]. Closer inspection of Fig. 2A reveals that a subpopulation of erythrocytes underwent dramatic cell shrinkage, whereas the cell volume of another subpopulation remained almost constant. As a matter of fact, erythrocytes are not uniformly sensitive to triggers of eryptosis. The sensitivity increases for instance with erythrocyte age [56].

As apparent from Fig. 4, removal of Ca^{2+} virtually abolishes the effect of patulin on cell membrane scrampling. Thus, patulin is indeed largely effective by a Ca^{2+} dependent mechansim. Further mechanisms involved in the stimulation of eryptosis include decline of cytosolic ATP with subsequent activation of protein kinase C [15], activation of caspases [16-

20], inhibition or lack of AMP activated kinase AMPK [9] or cGMP-dependent protein kinase [21] and activation of Janus-activated kinase JAK3 [22], casein kinase [57, 58], p38 kinase [24], PAK2 kinase [59] as well as sorafenib- [60]) and sunifinib- [61] sensitive kinases.

Consequences of eryptosis include derangement of microcirculation, as phosphatidylserine exposing erythrocytes adhere to endothelial CXCL16/SR-PSO of the vascular wall [62], which presumably interferes with blood flow [62-67]. Phosphatidylserine exposure may further foster blood clotting thus predisposing to thrombosis [63, 68, 69].

Eryptosis further leads to the clearance of eryptotic erythrocytes from circulating blood [7]. To the extent that the accelerated loss of erythrocytes is not compensated by a similar increase of erythrocyte formation, enhanced eryptosis leads to anemia [7].

Triggering of apoptosis-like eryptosis may, on the other hand, precede and thus protect against hemolysis, the necrosis like death of erythrocytes. Erythrocytes face the risk to undergo hemolysis following impaired Na^+ extrusion by the Na^+/K^+-ATPase, e.g. under energy depletion, or following enhanced net entry of Na^+ and Cl^-, e.g. following leakiness of the cell membrane [70]. In both cases the net gain of Na^+ and Cl^- leads to entry of osmotically obliged water and thus to cell swelling [70]. Excessive erythrocyte swelling may eventually result in cell membrane rupture with release of cellular hemoglobin [70], which may be filtered in renal glomerula and subsequently occlude renal tubules [71]. The activation of K^+ channels following triggering of eryptosis counteracts cell swelling by hyperpolarization of the cell membrane and thus increase of the driving force for Cl^- exit [12]. The exposure of phosphatidylserine at the surface of eryptotic cells leads to subsequent engulfment by phagocytosing cells thus leading to clearance of affected erythrocytes prior to hemolysis [70].

In conclusion, patulin stimulates Ca^{2+} entry into human erythrocytes with subsequent cell membrane scrambling and cell shrinkage, hallmarks of suicidal death of erythrocytes.

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