Stimulation of Erythrocyte Cell Membrane Scrambling by Nystatin

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Abstract: The antifungal ionophore nystatin dissipates the Na⁺ and K⁺ gradients across the cell membrane, leading to cellular gain of Na⁺ and cellular loss of K⁺. The increase of cellular Na⁺ concentration may result in Ca²⁺ accumulation in exchange for Na⁺. Increase of cytosolic Ca²⁺ activity ([Ca²⁺]) and loss of cellular K⁺ foster apoptosis-like suicidal erythrocyte death or eryptosis, which is characterised by cell shrinkage and cell membrane scrambling leading to phosphatidylserine exposure at the erythrocyte surface. The present study explored whether nystatin stimulates eryptosis. Cell volume was estimated from forward scatter (FSC), phosphatidylserine exposure from annexin V binding and [Ca²⁺], from Fluo3-fluorescence in flow cytometry. A 48-hr concentration may result in Ca²⁺ concentrations.

Partial replacement of extracellular Na⁺ with extracellular K⁺ blunted the nystatin-induced erythrocyte shrinkage but increased [Ca²⁺], and annexin V binding. Nystatin triggers cell membrane scrambling, an effect at least partially due to entry of extracellular Ca²⁺.

The polyene antibiotic nystatin, an ionophore allowing the passage of several ions including Na⁺, K⁺, H⁺ and Cl⁻ across the cell membrane [1], has antifungal potency and is thus used in the prophylaxis or treatment of fungal infections [2,3]. The present study explored whether nystatin influences [Ca²⁺], cell volume and phosphatidylserine translocation to the erythrocyte surface, and whether the effects are sensitive to extracellular K⁺ concentrations.

Materials and Methods

Erythrocytes, solutions and chemicals. Leucocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study was approved by the ethics committee of the University of Tübingen (184/2003V). Erythrocytes were incubated in vitro at a haematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl₂; pH 7.4 at 37°C for 48 hr. Where indicated, erythrocytes were exposed to nystatin (Sigma–Aldrich, Germany) at the indicated concentrations and/or extracellular K⁺ concentration increased to 10, 20, 40 and 80 mM at the expense of Na⁺. In Ca²⁺-free Ringer solution, 1 mM CaCl₂ was substituted by 1 mM glycol-bis(2-aminoethyl ether)-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 CaCl₂ and then stained with Annexin-V-FITC (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 Ca²⁺. After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluor-3/AM (Biotium, Hayward, CA, USA) in Ringer solution containing 5 mM CaCl₂ and 5 μM Fluor-3/AM. The cells were incubated at 37°C for 30 min. and washed twice in Ringer solution containing 5 mM CaCl₂. The Fluor-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca²⁺-dependent fluorescence intensity was measured.
Measurement of haemolysis. For the determination of haemolysis, the samples were centrifuged (3 min. at 400 × g, room temperature) after incubation, and the supernatants were harvested. As a measure of haemolysis, the haemoglobin (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% haemolysis.

Statistics. Data are expressed as arithmetic means ± S.E.M. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey’s test as post-test or with t-test as appropriate. n denotes the number of different erythrocyte specimens studied. As 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 whether exposure of human erythrocytes to nystatin triggers eryptosis, which is characterised by breakdown of phosphatidylserine asymmetry of the erythrocyte cell membrane with phosphatidylserine translocation to the erythrocyte surface. Phosphatidylserine exposing erythrocytes were identified by annexin V binding in FACS analysis. As illustrated in fig. 1, a 48-hr exposure to nystatin increased the percentage of annexin V binding erythrocytes, an effect reaching statistical significance at 10 μg/ml nystatin concentration.

Cell volume was estimated utilising FSC, which was determined by flow cytometry. As shown in fig. 2, a 48-hr exposure to nystatin significantly decreased erythrocyte FSC, an effect reaching statistical significance at 5 μg/ml nystatin concentration.

Known triggers of cell membrane scrambling include increase of cytosolic Ca²⁺ activity ([Ca²⁺]ᵢ). In order to determine [Ca²⁺]ᵢ, erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in flow cytometry after prior incubation in Ringer solution without or with nystatin. As illustrated in fig. 3, Fluo3 fluorescence reflecting [Ca²⁺]ᵢ was increased after a 48-hr exposure of human erythrocytes to nystatin, an effect reaching statistical significance at 15 μg/ml nystatin concentration. In order to determine the effect of nystatin exposure on haemolysis, the percentage of haemolysed erythrocytes was quantified from haemoglobin concentration in the supernatant. According to haemoglobin concentration in the supernatant, a 48-hr incubation with 0, 5, 10 and 15 μg/ml nystatin resulted in haemolysis of 1.2 ± 0.3%, 2.8 ± 0.5%, 8.5 ± 4.7% and 9.3 ± 3.1% (n = 5), respectively.

Further experiments explored whether nystatin-induced alterations of cell volume were sensitive to partial replacement of extracellular Na⁺ by extracellular K⁺. Erythrocytes were exposed to 15 μg/ml nystatin for 48 hr in the presence of 5, 10, 20, 40 and 80 mM extracellular K⁺ concentration. As shown in fig. 4, the nystatin (15 μg/ml)-induced decrease of FSC was blunted by increasing extracellular K⁺ concentration, an effect reaching statistical significance at 20 mM extracellular K⁺ concentration.

Additional experiments explored whether the cell membrane scrambling was similarly sensitive to extracellular K⁺ concentrations. As illustrated in fig. 5, an increase of extracellular K⁺ concentration at the expense of extracellular of Na⁺ did not appreciably influence the effect of nystatin on annexin V binding from 5 to 40 mM extracellular K⁺. Exposure to 80-mM extracellular K⁺ concentration, however, significantly augmented the nystatin-induced increase of annexin V binding.

As illustrated in fig. 6, partial replacement of extracellular Na⁺ by extracellular K⁺ augmented the nystatin (15 μg/ml)-induced increase of Fluo3 fluorescence reflecting [Ca²⁺]ᵢ, an effect reaching statistical significance at 80-mM extracellular K⁺ concentration.

A further series of experiments explored whether extracellular Ca²⁺ entry was required for the effect of nystatin on cell membrane scrambling. Erythrocytes were exposed to 15 μg/ml nystatin for 48 hr either in the presence of 1 mM Ca²⁺ or in the absence of Ca²⁺. As shown in fig. 7, the effect of nystatin on annexin V binding was significantly decreased in the nominal absence of Ca²⁺.
Discussion

The present study reveals that permeabilisation of the erythrocytes to monovalent ions by the ionophore nystatin is followed by stimulation of cell membrane scrambling leading to phosphatidylserine translocation to the erythrocyte surface.

Treatment of human erythrocytes with 15 µg/ml nystatin further increased cytosolic Ca²⁺ activity ([Ca²⁺]). The increase of [Ca²⁺] should activate Ca²⁺-sensitive K⁺ channels [6] with subsequent K⁺ exit, cell membrane hyperpolarisation, Cl⁻ exit and thus cellular loss of KCl with osmotically obliged water [5]. Accordingly, the observed decrease of FSC was blunted by increasing extracellular K⁺ concentration.

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erythrocytes after a 48-hr treatment with Ringer solution without gates Cl vents hyperpolarisation of the cell membrane and thus abro-

$K^+$ loss is an important enhancer of apoptosis in a wide vari-

ation rather augments cell membrane scrambling. Cellular $Na^+$ rather amplifies cell membrane scrambling, an effect cates significant difference from the respective values in the presence of 5 mM $K^+$.

which decreases the chemical driving force for exit of $K^+$, prevents hyperpolarisation of the cell membrane and thus abro-

gates Cl$^-$ exit. The activation of $Ca^{2+}$-sensitive $K^+$ channels counteracts erythrocyte swelling with eventual rupture of the cell membrane and release of cellular haemoglobin, which is filtered in renal glomeruli and may thus occlude renal tubules [57].

Somewhat surprisingly, an increase of extracellular $K^+$ concentration rather augments cell membrane scrambling. Cellular $K^+$ loss is an important enhancer of apoptosis in a wide variety of cells [58–69]. However, in nystatin-treated erythrocytes, an increase of extracellular $K^+$ concentration at the expense of $Na^+$ rather amplifies cell membrane scrambling, an effect obviously due to increased Ca$^{2+}$ entry. In view of the present observations, the use of nystatin along with high $K^+$ concentrations in order to minimize cellular $K^+$ loss and shrinkage after storage [4] may lead to enhanced eryptosis.

In theory, stimulation of eryptosis may add to nystatin toxicity. Eryptotic erythrocytes are removed from circulating blood as phosphatidylserine at the surface of eryptotic cells binds to the respective receptors of phagocytosing cells [6]. The clearance of eryptotic erythrocytes from circulating blood may lead to anaemia [6]. However, common side effects of nystatin do not include anaemia [70–73]. Possibly, the nystatin concentrations required for eryptosis are higher than those during nystatin treatment. It must further be kept in mind that accelerated eryptosis does not lead to eryptosis as long as it is matched by similarly enhanced formation of new erythrocytes [6].

Phosphatidylserine exposing erythrocytes further adhere to endothelial CXCL16/SR-PSO, [74] leading to interference with microcirculation [74–79]. In addition, phosphatidylserine exposing erythrocytes may trigger blood clotting and thus foster thrombosis [75,80,81].

In conclusion, nystatin stimulates $Ca^{2+}$ entry and cell membrane scrambling of erythrocytes, effects sensitive to extracel-

lular $K^+$ concentration.

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References


