Original Paper

Stimulation of Eryptosis by Caspofungin

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Key Words

Phosphatidylserine • Caspofungin • Cell volume • Eryptosis • Hemolysis • Casein kinase

Abstract

Background/Aims: The echinocandin antifungal agent caspofungin has been shown to trigger apoptosis of fungal cells. Beyond that, caspofungin is toxic for host mitochondria. Even though lacking mitochondria, erythrocytes may enter apoptosis-like suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling involved in triggering of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress, ceramide, caspase activation and/ or activation of p38 kinase, protein kinase C, and casein kinase. The present study explored, whether caspofungin induces eryptosis and, if so, to shed some light on the cellular mechanisms involved. **Methods:** Flow cytometry was employed to determine phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, ROS formation from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. Hemolysis was quantified from hemoglobin concentration in the supernatant. **Results:** A 48 hours exposure of human erythrocytes to caspofungin ($\geq 30 \mu\text{g/ml}$) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter, significantly enhanced hemolysis, but did not significantly increase Fluo3-fluorescence, DCFDA fluorescence or ceramide abundance. The effect of caspofungin on annexin-V-binding was not significantly blunted by removal of extracellular Ca^{2+} , by inhibition of caspases with pancaspase inhibitor zVAD ($10 \mu\text{M}$), or by addition of the antioxidant N-acetyl-cysteine (1 mM), p38 kinase inhibitor SB203580 ($2 \mu\text{M}$) or protein kinase C inhibitor staurosporine ($1 \mu\text{M}$). The effect of caspofungin on annexin-V-binding was, however, significantly blunted in the presence of casein kinase inhibitor D4476 ($10 \mu\text{M}$). **Conclusions:** Caspofungin triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect possibly involving activation of casein kinase.

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Introduction

Caspofungin, an echinocandin antifungal agent [1], is utilized for the treatment of diverse fungal infections [1-27]. Its antifungal action is accomplished by inhibition of β -1,3-d-glucan polymer synthesis [1]. Moreover, caspofungin has been shown to trigger apoptosis

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of *Candida albicans* [28]. The substance may be toxic as well for host cells and for host mitochondria [29].

Erythrocytes lack mitochondria but could nevertheless enter apoptosis-like suicidal death or eryptosis [30], which is characterized by cell shrinkage [31] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [30]. A wide variety of apoptosis inducing substances similarly trigger eryptosis [30, 32-75]. Triggering of eryptosis may involve increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [30], ceramide [76], oxidative stress [30], energy depletion [30], activated caspases [30, 77, 78], and stimulation of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and/or p38 kinase [30]. Eryptosis is inhibited following activation of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [30].

The present study explored, whether caspofungin is capable to trigger eryptosis. Erythrocytes from healthy volunteers were treated with caspofungin and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, ROS formation, and ceramide abundance determined by flow cytometry.

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 study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 x g for 20 min at 21 °C and the platelets and leukocytes-containing supernatant was disposed. 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; pH 7.4), 5 glucose, 1 CaCl_2 , at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to caspofungin (MedChem Express, Princeton, USA). In order to explore, whether eryptosis required entry of extracellular Ca^{2+} , experiments were performed in the nominal absence of extracellular Ca^{2+} and addition of 1 mM EDTA (Sigma Aldrich, Hamburg, Germany). To test for an involvement of kinases, erythrocytes were exposed for 48 hours to a combination of caspofungin and casein kinase inhibitor D4476 (Sigma Aldrich, Hamburg, Germany), p38 kinase inhibitor SB203580 (Tocris bioscience, Bristol, UK) or protein kinase C inhibitor staurosporine (Sigma Aldrich, Hamburg, Germany). The involvement of oxidative stress was tested by addition of the antioxidant N-acetyl-cysteine (Sigma Aldrich, Hamburg, Germany), the involvement of caspases by addition of the pancaspase inhibitor zVAD (Sigma Aldrich, Hamburg, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 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 15 min under protection from light. The annexin-V-abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and caspofungin treated erythrocytes. Alterations of cell volume were estimated by determination of forward scatter (FSC). The threshold of forward scatter was set at the default value of "52".

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 once in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer solution. Then, Ca^{2+} -dependent fluorescence intensity was measured in FL-1 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Afterwards, the geomean of the Ca^{2+} dependent fluorescence was determined.

Reactive oxygen species (ROS)

The abundance of ROS was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μ l suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnellendorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μ l Ringer solution and ROS-dependent fluorescence intensity was measured in FL-1 channel at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD). Subsequently, the geomean of the DCFDA dependent fluorescence was determined.

Ceramide abundance

To determine the ceramide abundance at the erythrocyte surface, a monoclonal antibody was used. After incubation, cells were stained for 1 h at 37°C with 1 μ g/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) at a dilution of 1:10. After two washing steps with PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG/IgM (concentration 0.5 mg/ml) specific antibody (BD Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analyzed by flow cytometric analysis in FL-1 channel at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Finally, the geomean of the ceramide dependent fluorescence was determined.

Hemolysis

Following incubation, the erythrocyte suspension was centrifuged for 3 min at 1600 rpm, 4°C, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants 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. *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 whether caspofungin influences eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Erythrocyte volume was estimated from forward scatter, which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with caspofungin (30 – 60 μ g/ml). As illustrated in Fig. 1A,B, caspofungin significantly decreased erythrocyte forward scatter at each caspofungin concentration tested. Moreover, caspofungin ($\geq$ 45 μ g/ml) significantly increased the percentage of shrunken erythrocytes (Fig. 1C) and significantly decreased the percentage of swollen erythrocytes (Fig. 1D) at each caspofungin concentration tested.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with caspofungin (30 – 60 μ g/ml). As shown in Fig. 2A,B, a 48 hours exposure to caspofungin increased the percentage of phosphatidylserine exposing erythrocytes at each caspofungin concentration tested.

Hemoglobin concentration in the supernatant was taken as a measure of hemolysis. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with caspofungin (30 - 60 µg/ml). As shown in Fig. 3, a 48 hours exposure to caspofungin increased the percentage of hemolytic erythrocytes at each caspofungin concentration tested.

Fluo3 fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). As a result, following a 48 hours incubation, the Fluo3 fluorescence was similar in the absence of caspofungin (24.77 ± 1.02 a.u., n = 21) and in the presence of 30 µg/ml caspofungin

Fig. 3. Effect of caspofungin on hemolysis. Arithmetic means $\pm$ SEM ($n = 21$) of the percentage of hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) caspofungin (30 - 60 $\mu\text{g/ml}$). ***($p < 0.001$) indicates significant difference from the absence of caspofungin (ANOVA).

Fig. 4. D4476 sensitivity of caspofungin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) caspofungin (60 $\mu\text{g/ml}$) in the absence (A) and presence (B) of casein kinase inhibitor D4476 (10 μM). C. Arithmetic means $\pm$ SEM ($n = 16$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) caspofungin (60 $\mu\text{g/ml}$) in the absence (left bars, -D4476) and presence (right bars, +D4476) of casein kinase inhibitor D4476 (10 μM). ***($p < 0.001$) indicates significant difference from the absence of caspofungin, ###($p < 0.001$) indicates significant difference from the absence of D4476 (ANOVA).

(23.87 ± 1.05 a.u., $n = 21$), of 45 $\mu\text{g/ml}$ caspofungin (24.61 ± 1.04 a.u., $n = 21$) and of 60 $\mu\text{g/ml}$ caspofungin (27.20 ± 1.23 a.u., $n = 21$). Further experiments were performed in order to elucidate whether the caspofungin-induced translocation of phosphatidylserine required entry of extracellular Ca^{2+} . To this end, erythrocytes were incubated for 48 hours in the absence or presence of 60 $\mu\text{g/ml}$ caspofungin in the presence or nominal absence of extracellular Ca^{2+} . As a result, exposure to caspofungin significantly ($p < 0.001$) increased the percentage of annexin-V-binding cells to a similar extent in the presence (from 1.36 ± 0.13 % to 9.61 ± 0.63 %, $n = 16$) and in the absence (from 1.19 ± 0.08 % to 9.37 ± 0.57 %, $n = 16$) of extracellular Ca^{2+} . Thus, the caspofungin-induced cell membrane scrambling was in large part triggered by mechanisms other than entry of extracellular Ca^{2+} .

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As a result, the DCFDA fluorescence was even significantly ($p < 0.001$) lower following exposure to 45 $\mu\text{g}/\text{ml}$ caspofungin (10.04 ± 0.30 a.u., $n = 8$) than in the absence of caspofungin (15.41 ± 0.26 a.u., $n = 8$). Thus, caspofungin did not appreciably induce oxidative stress. To further test for an impact of oxidative stress, erythrocytes were incubated for 48 hours in the absence or presence of 60 $\mu\text{g}/\text{ml}$ caspofungin in the presence or nominal absence of the antioxidant N-acetyl-cysteine (1 mM). As a result, exposure to caspofungin significantly ($p < 0.001$) increased the percentage of annexin-V-binding cells to a similar extent in the absence (from 1.50 ± 0.19 % to 12.27 ± 0.84 %, $n = 8$) and in the presence (from 2.08 ± 0.47 % to 12.84 ± 0.86 %, $n = 8$) of N-acetyl-cysteine. Thus, caspofungin-induced cell membrane scrambling was not dependent on oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As a result, the ceramide abundance was similar following exposure to 45 $\mu\text{g}/\text{ml}$ caspofungin (13.64 ± 0.45 a.u., $n = 5$) and in the absence of caspofungin (15.42 ± 0.22 a.u., $n = 5$). Thus, caspofungin did not appreciably induce ceramide abundance.

To explore, whether the effect of caspofungin on phosphatidylserine translocation required caspase activation, the influence of caspofungin on annexin-V-binding was tested in the presence or absence of pancaspase inhibitor zVAD. As a result, exposure to caspofungin significantly ($p < 0.001$) increased the percentage of annexin-V-binding cells to a similar extent in the absence (from 1.36 ± 0.10 % to 12.18 ± 0.81 %, $n = 8$) and in the presence (from 1.59 ± 0.11 % to 11.32 ± 0.78 %, $n = 8$) of zVAD (10 μM). Thus, the caspofungin-induced cell membrane scrambling did not require caspase activity.

To explore, whether the effects of caspofungin involved kinase activity, the influence of caspofungin on annexin-V-binding was tested in the presence or absence of kinase inhibitors. As a result, exposure to caspofungin significantly ($p < 0.001$) increased the percentage of annexin-V-binding cells to a similar extent in the absence (from 2.18 ± 0.54 % to 11.04 ± 0.90 %, $n = 8$) and in the presence (from 2.25 ± 0.53 % to 10.32 ± 0.72 %, $n = 8$) of p38 kinase inhibitor SB203580 (2 μM). Thus, the caspofungin-induced cell membrane scrambling was not dependent on p38 kinase activity. Exposure to caspofungin further significantly ($p < 0.001$) increased the percentage of annexin-V-binding cells even to a significantly ($p < 0.05$) higher extent in the presence (from 1.61 ± 0.16 % to 15.90 ± 0.65 %, $n = 16$) than in the absence (from 1.60 ± 0.13 % to 11.12 ± 0.69 %, $n = 16$) of protein kinase C inhibitor staurosporine (1 μM). Thus, the caspofungin-induced cell membrane scrambling was not dependent on protein kinase C activity.

As illustrated in Fig. 4A-C, the caspofungin (60 $\mu\text{g}/\text{ml}$) increased phosphatidylserine exposure was significantly blunted in the presence of casein kinase inhibitor D4476. However, even in the presence of D4476, caspofungin significantly increased phosphatidylserine exposure, an observation pointing to the involvement of additional mechanisms.

Discussion

The present study reveals a novel effect of caspofungin, i.e. the stimulation of eryptosis, the suicidal erythrocyte death. Treatment of human erythrocytes to caspofungin is followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentration of caspofungin required for this effect is well in the range of caspofungin concentrations encountered *in vivo* [79]. It must be kept in mind that the susceptibility to stimulators of eryptosis may be enhanced in several clinical conditions, such as dehydration [80], hyperphosphatemia [81], chronic kidney disease (CKD) [82-85], hemolytic-uremic syndrome [86], diabetes [87], hepatic failure [88], malignancy [30], sepsis [89], sickle-cell disease [30], beta-thalassemia [30], Hb-C and G6PD-deficiency [30], as well as Wilsons disease [90].

The effect of caspofungin on cell membrane scrambling was not paralleled by increase of cytosolic Ca^{2+} activity and not sensitive to Ca^{2+} removal from the extracellular space. Moreover, caspofungin triggered phosphatidylserine translocation to the erythrocyte surface without enhancing the ceramide abundance. Caspofungin triggered eryptosis without inducing oxidative stress and the effect of caspofungin on cell membrane scrambling was not significantly modified by addition of the antioxidant N-acetyl-cysteine. The effect of caspofungin did further not require caspase activation, the activity of p38 kinase or of staurosporine sensitive kinases such as protein kinase C. The caspofungin induced cell membrane scrambling was thus independent from increase of cytosolic Ca^{2+} activity, oxidative stress, ceramide formation or activity of p38 kinase and staurosporine sensitive kinases, all major triggers of eryptosis [30]. Instead, the effect of caspofungin on cell membrane scrambling was blunted in the presence of casein kinase inhibitor D4476. Thus, the effect of caspofungin on cell membrane scrambling could involve casein kinase activation. However, effects of D4476 other than casein kinase inhibition cannot be ruled out. Moreover, even in the presence of D4476, caspofungin significantly enhanced phosphatidylserine translocation pointing to the involvement of further mechanisms.

Besides its effect on eryptosis, caspofungin triggered hemolysis. The physiological function of eryptosis is actually the clearance of defective erythrocytes from circulating blood prior to hemolysis [30]. If defective erythrocytes fail to enter timely eryptosis, hemolysis leads to release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and may thus cause renal failure [91].

The clearance of phosphatidylserine exposing erythrocytes may, on the other hand, lead to anemia if the loss of erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [30]. Adherence of phosphatidylserine exposing erythrocytes to the vascular wall [92, 93] may further stimulate blood clotting and trigger thrombosis [94-96], thus compromising microcirculation [76, 94, 97-100].

Conclusion

Caspofungin triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect apparently independent from Ca^{2+} entry, oxidative stress, ceramide, caspases, p38 kinase, and protein kinase C but presumably involving activation of casein kinase.

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Disclosure Statement

The authors have declared that no competing interests exist.

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