Inactivation of α1-proteinase inhibitor by Candida albicans aspartic proteases favors the epithelial and endothelial cell colonization in the presence of neutrophil extracellular traps

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Candida albicans, a causative agent of opportunistic fungal infections in immunocompromised patients, uses ten secreted aspartic proteases (SAPS) to deregulate the homeostasis of the host organism on many levels. One of these deregulation mechanisms involves a SAP-dependent disturbance of the control over proteolytic enzymes of the host by a system of dedicated protease inhibitors, with one important example being the neutrophil elastase and alpha1-proteinase inhibitor (A1PI). In this study, we found that soluble SAPs 1-4 and the cell membrane-anchored SAP9 efficiently cleaved A1PI, with the major cleavage points located at the C-terminal part of A1PI in a close vicinity to the reactive-site loop that plays a critical role in the inhibition mechanism. Elastase is released by neutrophils to the environment during fungal infection through two major processes, a degranulation or formation of neutrophil extracellular traps (NET). Both, free and NET-embedded elastase forms, were found to be controlled by A1PI. A local acidosis, resulting from the neutrophil activity at the infection sites, favors A1PI degradation by SAPs. The deregulation of NET-connected elastase affected a NET-dependent damage of epithelial and endothelial cells, resulting in the increased susceptibility of these host cells to candidal colonization. Moreover, the SAP-catalyzed cleavage of A1PI was found to decrease its binding affinity to a pro-inflammatory cytokine, interleukin-8. The findings presented here suggest a novel strategy used by C. albicans for the colonization of host tissues and overcoming the host defense.

Key words: Candida albicans, aspartic proteases, α1-proteinase inhibitor, elastase, neutrophil extracellular traps, inflammation

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INTRODUCTION

Candida albicans is an opportunistic pathogen, harmlessly colonizing the human organism and detected in up to 70% of healthy individuals. However, weakening the host immune system and the frequent use of antibiotics and steroids increase a risk of fungal infections (Bakare et al., 2003). In severe conditions, the superficial infections may further develop into life-threatening disseminated candidiasis with a high (30–50%) mortality rate (Santos & Braga-Silva, 2013). To invade host mucosal surfaces, C. albicans uses many virulence factors that enable the fungal cells to survive in a diverse physiological niches such as oropharynx or urogenital or gastrointestinal tracts (Calderone & Fonzi, 2001; Mech et al., 2013). Among them, a crucial role is played by a family of secreted aspartic proteases (SAPS) whose expression differs between unicellular (yeast-like) and filamentous morphological forms of C. albicans (Naglik et al., 2003). A majority of SAPs are released to the surrounding environment (SAPI-SAPS) but SAP9 and SAP10 are attached to the cell wall/membrane through a glycosylphosphatidylinositol (GPI) anchor (Hube & Naglik, 2001). These proteases exhibit broad substrate specificities, promoting infection development by the degradation of host proteins that function in tissue barriers and in immune defense (Pietrella et al., 2010; Rapala-Kozik et al., 2015; Kozik et al., 2015).

Neutrophils are principal mediators of innate immunity against microbial invaders. Apart from the phagocytosis and extracellular degranulation, they are able to combat microbes by formation of neutrophil extracellular traps (NETs). These web-like structures are built of a decondensed chromatin and numerous proteins, mainly originating from neutrophil granules, with antimicrobial properties (Urban et al., 2009). However, the NET release poses a threat to the tissue, associated with the proteolytic activity of neutrophil proteases (such as elastase and proteinase 3) and the oxidative properties of myeloperoxidase, the enzymes that are important structural elements of NETs. Elastase (NE) plays pivotal roles in the pathogenesis of pulmonary fibrosis and in the development and progression of acute respiratory distress syndrome (Takemasa et al., 2012; Tagami et al., 2014). Initially, NE is stored in the azurophilic granules of neutrophils, but during NET formation it processes core histones to promote chromatin decondensation and contributes to NET-dependent tissue damage after NET release into the surrounding environment (Papayannopoulos et al., 2011). Therefore, processes such as the neutrophil migration, initiation of the trap formation and elastase activity must be strictly controlled. Regulation may occur by means of α1-protease inhibi-
tor (A1PI). This member of the serpin (serine protease inhibitor) family, synthesized mainly by hepatocytes, is the main NE inhibitor that circulates in the bloodstream from which it diffuses into the lower respiratory tract, providing >90% anti-elastase protection there (Greene et al., 2003). It is known that in addition to its inhibitory properties, A1PI also has a regulatory capacity in the inflammatory processes, weakening both neutrophil chemotaxis due to IL-8 binding (Bergin et al., 2010) and the production of free radicals, probably due to some ROS-scavenging properties (Taggart et al., 2000).

Bacteria that cause pulmonary infections, such as Pseudomonas aeruginosa, Haemophilus influenzae and Staphylococcus aureus, express enzymes capable of inactivating A1PI, thus contributing to an impaired balance between NE and A1PI within the airways that can lead to tissue destruction and progression of the disease (Suter & Chevalier, 1991). It was also suggested that the common fungal pathogen — C. albicans yeast — can affect the integrity and function of A1PI (Kaminishi, 1999). Therefore, aim of the present study was to analyze the cleavage of A1PI by SAPs and the effects of this proteolytic processing on the NET-embedded NE activity and NET-dependent damage of epithelial and endothelial cells, with a possible further influence on the propagation of fungal infection.

MATERIALS AND METHODS

Cell culturing. Human microvascular endothelial cells (HMEC-1) and human epithelial carcinoma cell line A431 were obtained from ATCC. HMEC-1 were cultured in MCDB 131 medium (Sigma, St. Louis, MO, USA) and A431 cells in DMEM medium (Thermo Scientific, HyClone, Logan, USA). Media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin–streptomycin and 2 mM glutamine, and MCDB 131 medium was additionally supplemented with 1 μg/ml hydrocortisone and 10 ng/ml EGF. The cells were cultured in 5% CO₂ atmosphere at 37°C.

Yeast culturing. C. albicans strain ATCC 10231 was grown under conditions that favored either yeast-like or hyphal morphology. Yeast-like forms were cultured at 30°C in YPD medium (Serva, Heidelberg, Germany) buffered with 50 mM sodium-phosphate pH 6.5 and containing 0.2% yeast extract, 0.2% peptone and 2% glucose. In order to induce hyphae formation, C. albicans was grown in RPMI medium (Sigma) at 37°C. After 24 h, the yeast cells were harvested by centrifugation (3000 × g, 10 min) and SAP-containing supernatants were ten-fold concentrated using an Amicon ultrafiltration chamber (Danvers, MA) equipped with the ultrafiltration membrane with 10-kDa exclusion limit.

Isolation of neutrophils from human peripheral blood. Human neutrophils were isolated according to a previously described method (Rapala-Kozik et al., 2015) using EDTA-treated whole-blood samples collected from healthy donors. Neutrophil-containing fractions were separated by Pancoll gradient (PAN-Biotech, Aidenbach, Germany) and treated with 1% solution of polyvinyl alcohol (Sigma) in PBS. Contaminating erythrocytes were removed by hypotonic lysis, and neutrophils were resuspended in RPMI 1640 medium (phenol red-free). This preparation method yields a >95% pure population of neutrophils.

NET release and isolation. Neutrophils (1 × 10⁶ cells in 800 μl RPMI) were seeded for 30 min at 37°C and 5% CO₂ on a 12-well microplate (Greiner Bio-One, Germany), previously coated with 0.01% poly-L-ly}sine. Cells were then stimulated to produce NETs with 50 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) for 3 hours at 37°C and 5% CO₂. After gentle triple washing of each well with 1 ml of PBS, NETs were treated with 400 μl of Micrococcal nuclease (MNase, 1 U/ml) (Roche, Penzberg, Germany) for 20 min at 37°C. The reaction was stopped with EDTA. The supernatant was collected and centrifuged for 5 min at 300 × g at 4°C to remove the cell debris. The NET-rich supernatants were then analyzed for their DNA fragmentation using agarose gel electrophoresis. For protein determination in the samples, Micro-BCA protein assay reagent kit (Pierce, Germany) was used and DNA content was quantified using staining with SytoxGreen (Invitrogen Life Technologies, Germany) and fluorescence measurement (465/525nm) using a Biotek Synergy H1 microplate reader.

Degradation of A1PI in SAP-containing supernatants. A1PI (1 μg/30 μl) (Preparatol, Poland) was incubated with cell-free supernatants containing 5 μg/ml of secreted proteins at pH in the range of 4–7 (50 mM citrate pH 4-6 or 50 mM phosphate buffer pH 7) for 20 h at 37°C, in the presence of 10 μM pepstatin A or without this aspartic-protease inhibitor. The degradation of A1PI was examined using a colorimetric assay that monitors a decrease of inhibitory capacity against trypsin (Sigma). The reaction was conducted in 100 mM Tris-HCl pH 8, at an enzyme : inhibitor molar ratio of 1:1 at 37°C. Trypsin activity was detected using N-α-benzoyl-L-arginine 4-nitroanilide hydrochloride (Sigma) as the substrate, and determined after 30 min of incubation by absorbance measurement at 410 nm.

Degradation of A1PI and IL-8 by individual purified SAPs. Ten SAPs were overproduced in Pichia pastoris GS115 (Invitrogen, USA) as was described previously (Aoki et al., 2011). A1PI and IL-8 were incubated with all SAPs at an enzyme:substrate molar ratio of 1:20 in 50 mM citrate buffer, pH 5 and 6, at 37°C for 20 h. The reaction was stopped by addition of pepstatin A at a final concentration of 10 μM. Products of degradation were separated and identified by SDS-PAGE in the Laemmli system (Laemmli, 1970) on a 12% gel, with band visualization by silver staining (Blum et al., 1987).

Analysis of the inhibitory properties of native and SAP-treated A1PI towards native or NET-associated neutrophil elastase. The analysis of inhibitory properties of native and SAP degraded A1PI (dA1PI) were performed in 100 mM Tris-HCl pH 8.0 for 30 min at 37°C in a 96-well microtiter plate. Both forms of the inhibitor were incubated with native or NET-associated neutrophil elastase at an enzyme : inhibitor molar ratio of 0:1, 0.25:1, 0.5:1, 1:1, 2:1 and 3:1. The amount of NET-associated NE in total pool of isolated NET-associated protein was estimated according to data presented by Urban et al. (2009). The residual enzyme activity was determined using a chromogenic substrate, MeOSuc-Ala-Ala-Val-pNA (Sigma). Progress of the reaction was measured spectrophotometrically at 410 nm.

Mass spectrometry analysis of A1PI degradation products generated by SAP-catalyzed proteolysis. The products obtained after degradation of A1PI by those SAPs which exhibited the highest hydrolytic activity toward this inhibitor were analyzed by liquid chromatography (LC)-coupled tandem mass spectrometry (MS/MS). The low molecular mass degradation products were analyzed directly using the LC-MS/MS system. After SDS-PAGE, the bands of the main degradation products were in-gel digested with trypsin. Briefly, after the visualization of the protein fragments with the collei-
dal Coomassie Brilliant Blue dye, the excised pieces were washed twice with 50 mM (NH₄)₂HCO₃ in 25% and 50% acetonitrile (ACN, high-performance liquid chromatography (HPLC)-gradient grade, Merck, Darmstadt, Germany) to remove the dye from the gel. After reduction with 10 mM dithiothreitol (DTT) for 60 min at 37°C, samples were alkylated with 55 mM iodoacetamide for 2 hours in the dark and then washed with 100 mM (NH₄)₂HCO₃. After dehydration with ACN, samples were dried and subsequently re-swelled with a digestion buffer, containing 12.5 ng/μl of the sequencing-grade modified trypsin (Promega, Madison, WI, USA), 50 mM (NH₄)₂HCO₃, and 4 mM CaCl₂. The gel pieces were incubated overnight at 37°C. The extractions of acidic and basic peptides were conducted by addition of 50 mM (NH₄)₂HCO₃ and 5% formic acid in 50% ACN, respectively. Combined supernatants were diluted with water and subjected to the LC-MS/MS analysis using ultra-high-performance liquid chromatography (UHPLC) Dionex Ultimate 3000 system (Thermo-Scientific, Waltham, MA, USA) coupled to HTHCultra ETDII ion-trap mass spectrometer equipped with electrospray (ESI) ion source and electron transfer dissociation (ETD II) fragmentation module (Bruker, Bremen, Germany). The A1PI fragments were separated on an Accucore C-18 column (2.6 μm, 2.1 × 100 mm, ThermoScientific) in a binary linear gradient formed between solvent A (0.1% formic acid (Sigma)) and solvent B (0.1% formic acid in 80% ACN), from 10 to 55% of B in 38 min, at a flow rate of 0.2 ml/min.

The mass spectrometry measurements were performed with the following instrument setup: a positive ion mode, capillary voltage of 3.5 kV, the nebulizing gas (nitrogen) pressure of 4 psi, drying gas (nitrogen) flow of 12 l/min, sheath gas (nitrogen) flow of 9 l/min, and a temperature of 350°C. Spectra were acquired in MS/MS mode in the range of 100–3000 m/z with CID (collision-induced dissociation) and ETD ion fragmentation, and analyzed using Data AnalysisTM 4.0 and BiotoolsTM 3.2 software (Bruker, Germany). The identification of peptide sequences was performed by submission of the data to an in-house Mascot server ver. 2.3.0 (Matrix Science, UK) and based on searching against Swiss-Prot database.

**ELISA method for the detection of the IL-8 binding by native and degraded A1PI.** Native A1PI and its degraded forms obtained by treatment with representative SAPs (dA1PI) were used to detect their binding capacity to IL-8 (Novoprotein Scientific). The formation of the A1PI- and dA1PI-IL-8 complex was analyzed by a sandwich ELISA method. The mixtures, containing constant amount of IL-8 and native or degraded A1PI at a molar ratio of 0.05:1, 0.1:1, 1:1, 2:1 were incubated at room temperature for 1 h. The samples were then added to 96-well microtiter plates coated with anti-IL-8 antibody and incubated for 1.5 h at room temperature. The amount of free IL-8 versus A1PI/dA1PI-bound IL-8 was determined using HUMAN IL-8 ELISA KIT II (BD OptEIA) according to the supplier’s instructions.

**Treatment of human cells with NETs and the viability tests.** HMEC-1 and A431 cells were seeded on 96-well plates (2 × 10⁴ cells/well in 200 μl medium) and cultured to 90% of confluency. The cells were washed with PBS, and then the NET suspension with total protein concentrations of 0, 10, 20, 30 μg/ml in the medium were added. After 22 h of incubation at 37°C, the wells were washed with PBS. Viability of the cells was estimated with the MTT assay (Sigma).

To determine the influence of A1PI or dA1PI inhibition on the activity of NE-accompanying NETs, the NET samples (10 μg/ml) were first incubated with A1PI or dA1PI for 1 h as above, and then applied to the cells. The cells treated with pure medium were used as a control. Special references for possible influence of SAPs used for A1PI treatment at corresponding concentrations were applied instead of NET samples during the determination of cell viability.

**Treatment of C. albicans with NETs and the yeast survival tests.** C. albicans cells were harvested at the stationary phase by centrifugation at 1500 × g for 10 min, then were washed twice with PBS and diluted to a concentration of 5 × 10⁴ cells/ml in PBS. In order to evaluate the impact of NETs on the viability of C. albicans cells, the yeast cell suspensions (200 μl/well) were incubated with NETs as described above at 0–30 μg/ml concentration of NET protein components, and at various time intervals. After incubation, the cell viability analysis was performed using MTT test, as well as the number of viable C. albicans was determined by plating the serial dilutions of cell suspensions on Sabouraud dextrose agar plates. The CFU (colony forming units) were counted after 24 h of incubation at 30°C.

**Analysis of NET-dependent human cell destruction effects on C. albicans propagation.** To analyze the effect of human cell death by NETs on C. albicans propagation two models of co-cultures were used. A one-step co-culture. The human cell monolayers covered with C. albicans cells (5 × 10⁴ cells/well of 96-well microplate) were treated with NETs (0, 10, 20, 30 μg/ml) for 2–4 h at 37°C. After incubation, the supernatants were removed and the cells were gently washed with PBS. Yeasts attached to the human cell monolayers were scraped gently with pipet tips and washed with 200 μl of distilled water. The resulting suspensions were diluted 10-fold, spread on Sabouraud agar plates and handled further as described above.

A two-step co-culture. The human cell monolayers were first treated with NET samples (0, 10, 20, 30 μg/ml of protein concentration) for 2–4 h at 37°C. Then the supernatant was removed and the stability of monolayer was checked by phase contrast microscopy before addition of C. albicans cells (5 × 10⁶ cells/well of 96-well plate) for the next 2 h of incubation under the same conditions. The C. albicans cells attached to the human cell monolayer were quantified as described above.

**Statistical analysis.** The unpaired one-tailed Student’s t-test with a Bonferroni correction where necessary was used. A p value < 0.05 was considered significant.

**RESULTS**

**Several aspartic proteases of C. albicans efficiently degrade A1PI**

In order to demonstrate a potential ability of aspartic proteases secreted by C. albicans to degrade A1PI at the infection foci, A1PI samples were treated with SAP-containing supernatants from C. albicans cultures propagated under conditions favoring either yeast-like (YPD supplemented with 0.2% BSA) or hyphal (RPMP) morphology of the fungus. Influence of the degradation process on the inhibitory properties of A1PI towards trypsin was tested (Fig. 1A). We found that a more efficient inactivation of A1PI occurred in the presence of supernatants obtained from conditions that favor yeast-like morphology, at the pH range from 4 to 6. Using pepstatin A, a selective aspartic protease inhibitor, we confirmed SAP involvement in the presented A1PI inactivation.
To select among all SAPs for those enzymes that mainly participate in the process, A1PI samples were treated with purified individual SAPs at enzyme:substrate ratios of 1:20 for 20 h at 37°C. The retained inhibitory capacity of degraded A1PI, analyzed by using trypsin as the test enzyme, pointed out to SAP1, SAP2, SAP3, SAP4 and SAP9 as proteases leading to the loss of A1PI inhibitory properties (Fig. 1B).

The SDS-PAGE analysis (Fig. 2, left panel) correlated the above enzymatic activity tests with visible cleavage of A1PI molecule by the SAP representatives mentioned above. At a more acidic pH, SAPs produced by both morphological forms of *C. albicans* were able to cleave A1PI. However, at a broader pH range, A1PI was cleaved by SAPs that are mainly associated with the yeast-like form of *C. albicans* (SAP1, SAP2, SAP3 and SAP9). The last one, SAP9, is also expressed by the hyphal form, but is rarely secreted into the medium and has a predominant membrane localization (Naglik et al., 2003). The hyphae-associated SAP4 was comparably effective in A1PI degradation at pH 5, confirming the results presented for A1PI treated with the SAP-containing supernatants.

To localize the points of SAP-dependent cleavage within A1PI molecule, the amino acid sequences of the main products were identified by in-gel digestion of SDS-PAGE-separated bands with trypsin, followed by LC-MS/MS analysis. This analysis revealed that the cleavage of A1PI was mainly limited to the hydrolysis of K343-G344 bond, resulting in a formation of a stable product (dA1PI) with the sequence shortened by 51 amino acid residues in comparison to the native A1PI form (Fig. 2, right panel). The shorter peptide was further degraded into smaller variants, without any effect on the dA1PI properties.

SAP-degraded A1PI changes its inhibitory activity towards neutrophil elastase, present in NET structures, and looses the ability to bind IL-8

The main target for homeostatic function of A1PI in human organism is NE that during microbial infection can be spread as the NET constituent at the sites of neutrophil contact with microbes. First, we tested if NE localized to NET structures during their isolation is still active and possibly available for and inhibited by A1PI (Fig. 3A). The comparable results of inhibition obtained for native and NET-associated NE corroborated such model. Moreover, the treatment of NET-embedded NE with A1PI degraded by the most potent SAPs (dA1PI) led to the partial restoration of NE activity as compared to treatment with native A1PI (Fig. 3B).

The intensification of NET production at sites of bacterial or fungal infection occurred as a results of the action of additional stimuli, such as IL-8 which is produced by incoming or surrounding cells. However, the availability of IL-8 can be modulated by its binding to A1PI. On the other hand, a degradation of IL-8 by any SAP

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**Figure 1.** Changes of A1PI inhibitory properties upon treatment with *C. albicans* aspartic proteases.

A1PI was degraded (dA1PI) at different pH by cell-free supernatants, containing SAPs produced by *C. albicans* cells, cultured in YPD or RPMI medium (A) or treated by purified SAPs, overexpressed in *P. pastoris* (B). The reaction was monitored for residual A1PI inhibitory capacity against trypsin at an enzyme:inhibitor molar ratio of 1:1, using Nα-benzoyl-l-arginine 4-nitroanilide hydrochloride as substrate. The activity of uninhibited trypsin under the same experimental conditions was taken as 100%.

**Figure 2.** Identification of the cleavage products of A1PI processing by *Candida* aspartic proteases.

**Left panel:** SDS-PAGE analysis of A1PI degradation products generated during A1PI contact with individual SAPs at an enzyme:substrate molar ratio of 1:20 in 50 mM citrate buffer, pH 5 (A) and 6 (B) at 37°C for 20 h. A1PI and its degradation product (dA1PI) were visualized by silver staining of SDS-PAGE gels. **Right panel:** Mass-spectrometry identification of A1PI degradation products with indication of major cleavage points by NE (black arrow) and SAP9 (gray arrows). The sequence underlined represents the known cleavage region for bacterial proteases.
Candida albicans proteases inactivate human α1-proteinase inhibitor at different incubation time, pH and IL-8:SAP ratio was not observed (data not presented). To test the influence of A1PI degradation on A1PI ability to form a complex with IL-8, we used an ELISA method in which the cytokine unbound to A1PI was detected. The IL-8 binding by A1PI that prevents IL8 detection at the experimental conditions of ELISA test, was observed (Fig. 4). However, the degraded A1PI (dA1PI) blocked efficiently the complex formation with IL-8, whose molecules were still detected in solution during the experiments.

Human cell destruction by NETs can be influenced by A1PI and modulated by C. albicans SAPs.

A number of studies had shown that an excessive production of NETs can lead to cell and tissue destruction (Saffarzadeh et al., 2012), for which the regulation of elastase activity may be crucial. To address this problem, we chose two types of human cell lines, epithelial (A431) and endothelial (HMEC-1), that were treated with isolated NETs and, thereafter, the cell viability was measured using the MTT test. To eliminate possible side effects of different incubation time, pH and IL-8:SAP ratio was not observed (data not presented). To test the influence of A1PI degradation on A1PI ability to form a complex with IL-8, we used an ELISA method in which the cytokine unbound to A1PI was detected. The IL-8 binding by A1PI that prevents IL8 detection at the experimental conditions of ELISA test, was observed (Fig. 4). However, the degraded A1PI (dA1PI) blocked efficiently the complex formation with IL-8, whose molecules were still detected in solution during the experiments.

Figure 3. Influence of SAP-catalyzed A1PI degradation on its inhibitory properties against elastase, embedded in NET structures. Inhibition of native (NE) and NET-associated elastase (NE/NET) by A1PI was compared using various enzyme:inhibitor molar ratios (A). The residual activity of NE was measured with MeOSuc-Ala-Ala-Pro-Val-pNA as substrate. Effect of A1PI degradation by selected SAPs on the inhibitory capacity against NE/NET was carried out under the same conditions using an enzyme:inhibitor molar ratio of 1:1 (B).

Figure 4. IL-8 binding by native (A1PI) and SAP-degraded (dA1PI) elastase inhibitor. Formation of the complexes of A1PI/dA1PI with IL-8 was detected using HUMAN IL-8 ELISA Kit II at a constant amount of IL-8 and varied A1PI/dA1PI ratio.

Figure 5. NET toxicity against human cells and its changes upon cell treatment with A1PI. HMEC-1 and A431 cell monolayers were treated with suspension of NETs at different total protein concentrations (0–50 μg/ml) for 22 h. The viability of the cells was measured by the MTT assay and relatively expressed to non-treated cells (A). The impact of A1PI and dA1PI (degraded by SAP1-S1; SAP3-S3; SAP9-S9) on HMEC-1 cell viability during NET treatment (10 μg/ml) of HMEC-1 cells (B) was determined in the same manner. The molar ratio of A1PI/dA1PI used was calculated according to the amount of NE in NETs, estimated as proposed by Urban et al. (2009). The possible contribution of SAP1, SAP3 and SAP9 to the observed effects was also taken into account (marked S1, S3 and S9, respectively).
effects of destroyed neutrophils on the analyzed cells, the NET structures produced during PMA stimulation of neutrophils were first detached from the neutrophils by MNase treatment that also caused a slight fragmentation of the supporting DNA scaffold. The amount of NETs used in further experiments was determined based on their protein content. The results presented in the Fig. 5A confirmed earlier reports of destructive action of NETs on epithelial and endothelial cells, but in our models the endothelium seemed to be more susceptible to NET presence than epithelial cells.

The observed NET-generated damages to both types of cells can result from actions of many factors, constituting NETs, one of them being elastase. To verify this hypothesis the HMEC-1 monolayer was treated with NETs as described above but at a lower protein concentration, 10 µg/ml. The NET-dependent damages lowered the cell viability to 75%. When the monolayer was treated with NETs previously incubated in the presence of increased concentrations of A1PI, the observed changes showed a gradual increase in cell viability, more or less comparable with the initial conditions, confirming that a large part of the destructive forces of NETs is constituted by elastase (Fig. 5B) whose action can be controlled and even totally blocked in NETs by A1PI. On the other hand, when A1PI was treated with SAPs known to be most potent in A1PI degradation (SAP1, SAP2, SAP9), that produced C-terminally truncated A1PI variant (dA1PI) with the concomitant loss of anti-elastase properties, the protective effect disappeared. With increasing concentrations of dA1PI the cell viability dropped to the initial destructive level. At the same time, the SAPs present at the experimental conditions, can also influence cell viability but the control results performed for pure SAPs showed only a 5–7% contribution of SAP to this effect.

Figure 6. Influences of uncontrolled NET generation on the viability of C. albicans cells and the ability to colonize the NET-damaged epithelial and endothelial cells.

The yeast-like cells were treated with NET proteins (0–30 µg/ml) for 2–16 h and the fungal viability was determined by CFU measurements, in comparison to non-treated cells (A). The colonization of NET-damaged human cells by C. albicans was assayed in two models: sequential (B, C: the human cell treatment with NET, followed by colonization with C. albicans) and simultaneous (D, E: NET impaired both human and pathogen cells at the same time).

Neutrophils use several strategies to fight against bacterial and fungal infection, including NET formation, but many microorganisms developed different mechanisms to survive. The destruction of the surrounding tissue may add to the fungal survival strategy. To verify this hypothesis, we constructed the models of mutual interactions between C.
Candida albicans cells and epithelial (A431) or endothelial (HMEC-1) cells being in contact with NETs. First, we checked the effects of isolated NETs on the viability of fungal cells (Fig. 6A). Measurements performed for a short period of contact time (2 h) showed about 60% decrease in fungal cell viability, but after a longer time of contact (16 h) the fungal cells recovered their growth, in comparison to the control conditions. Even more, some NET constituents may stimulate the fungal growth as the full neutrophil extract does (data not shown). Next, we analyzed the possible spreading of C. albicans cells over epithelial and endothelial cell monolayer, both influenced by NET treatments during different periods of time (2–4 h) at different NET-protein concentration (0–30 µg/ml). For this approach, two models were used. In the first model, the human cells were first treated with NETs for various time periods, and then NETs were removed and human cell monolayers were covered for 2 h by C. albicans cells, whose attachment and growth were further analyzed by CFU methods. In another model, human and pathogen cells were allowed to be influenced by NETs at the same time in a mutual contact. The results obtained from both models (Fig. 6B–E) clearly indicate that the damaged human cells become more susceptible to further C. albicans colonization. The longer time of NET treatment favored C. albicans colonization. In the light of these results, the usage of a variety of SAPs by the fungus for the deregulation of elastase control due to the SAP-dependent A1PI degradation and for possible modulation of chemotaxis serves to the superior goal, i.e. further propagation of infection. However, excessive production of NETs at the fungal infection site leads to the destruction of both fighters. Once again, the human endothelium seemed to be more sensitive for destruction and susceptible to further colonization by the fungus.

**DISCUSSION**

C. albicans is well adapted to changes occurring in its environment, i.e. the host tissues and cells. In the adaptation processes, C. albicans engages aspartic proteases to degrade tissue barriers, to inactivate host defense molecules, and to digest host proteins for nutrient supply (Naglik et al., 2003). However, SAPs also contribute to the induction of the inflammatory response of the host by stimulation of cytokine expression (Naglik et al., 2003; Schaller et al., 2005), acting directly (Pietrella et al., 2010) or in an indirect way, through a disruption of the complexes involved in maintaining the host’s homeostasis. One of these interactions, important for the biochemical homeostasis in the host organism, is the protease-inhibitor complex formation between neutrophil elastase and its major regulator, A1PI.

Neutrophils, being the first immune cells arriving at the site of infection, are able to extracellularly release their granular proteins or to form NET structures, both resulting in a local eruption of neutrophil serine proteases, mainly NE, that on one hand can inactivate bacterial or fungal virulence factors (Belaaouaj et al., 1998; Tkalec et al., 2000), but on the other hand they are also involved in a variety of inflammatory conditions, including chronic lung diseases (chronic obstructive pulmonary disease, cystic fibrosis, acute lung injury, and acute respiratory distress syndrome) (Bergin et al., 2012). Moreover, NE induces the production of pro-inflammatory cytokines in the lungs, thus augmenting the inflammation (Meyer-Hoffert & Wiedow, 2011), but also inactivates extracellular immune mediators such as immunoglobulins, complement components, and cathelicidin (Tralau et al., 2004; Stromstedt et al., 2009).

Once released, proteolytically active NE is under control of A1PI that is responsible for maintaining the balance, being of a critical importance for the protection of lung tissues (Greene et al., 2008; Reeves et al., 2010; Tuder et al., 2010). Alveolar epithelial cells are known to produce A1PI locally which may contribute to both the anti-protease defense and anti-inflammatory effects within the lung (Venembre et al., 1994). Our work shows here for the first time that such a complex between NE and A1PI can be also formed when NE is present within the NET structures.

For many bacterial infections, the NE-A1PI balance can be disturbed due to the efficient inactivation of A1PI by proteases secreted by microorganisms, resulting in serious tissue damage (Sires et al., 1994; Sponer et al., 1991). As the serpin core possesses a highly ordered structure (Elliott et al., 1998), the exposed reactive-site loop of A1PI is generally the primary target for the proteolytic attack of bacterial enzymes (Rapala-Kozik et al., 1999). Several bacterial proteases were found to operate in a close vicinity to the NE-cleavage site (M358-S359) within this loop, i.e., to hydrolyze peptide bonds between residues F352 and I360 (Rapala-Kozik et al., 1999). In the current study we present for the first time that similarly, aspartic proteases of a fungal pathogen — C. albicans — can also cleave the A1PI reactive loop at the K343-G344 bond, impairing the A1PI inhibitory properties. In the selection of SAPs that are most efficient in A1PI degradation, acidic conditions were preferred. They refer to severe bacterial infections, observed in cystic fibrosis where characteristic feature of the inflammatory sites is a local acidosis, which is attributed to the increase of lactic acid production by the anaerobic, glycolytic activity of infiltrating neutrophils (Lardner, 2001; Coakley et al., 2000). Our results showed that the most efficient inactivation of A1PI was performed by SAP1, SAP2 and SAP3, proteases predominantly produced by the yeast-like forms, contributing to the pathogen dissemination. Hyphae-specific protease SAP4 also functions in the acidic environment and can be important in A1PI degradation during prolonged infections. Additionally, the surface-bound SAP9 which is expressed by both morphological forms contributes to A1PI cleavage. Thus, independently from the reversible switch between the unicellular yeast-like form and the elongated filamentous form, C. albicans is well equipped with the A1PI-degrading power. The action of SAP9 can be of a special importance if the fungus is locally arrested in NET structures. Our data are consistent with previous observations that C. albicans mutants lacking either SAP1 or SAP2 genes caused a lesser tissue damage in infection models and had a significantly reduced potential to stimulate cytokine expression. In contrast, mutants lacking SAP4 to SAP6 induced similar levels of cytokines to those in the wild-type strain (Schaller et al., 2003).

The same data had shown that plasma A1PI binds to circulating neutrophils and is localized to the outer surface of the cell membrane which may facilitate the ability of A1PI to regulate key neutrophil inflammatory responses including cell chemotaxis (Bergin et al., 2010). By forming the complex with IL-8, A1PI inhibits CXCR1 engagement, with a negative impact on the downstream CXCR1 signaling events. Our present results show that the degradation of A1PI by fungal proteases affects not only the interaction with elastase but also the complex formation between A1PI and IL-8. This chemokine interacts with glycosylated part of A1PI molecule via charge complementation (Bergin et al., 2010; Reeves et al., 2010). Although the SAP-truncated A1PI retains all
the binding sites for IL-8, the interaction is weaker by ca. 50%, as compared to native A1PI molecule. This may be related to possible changes in A1PI conformation that can occur during proteolysis of reactive site loop, limiting access to IL-8 binding sites (Knäuper et al., 1990).

Our results correspond to the findings of Pietrella et al. (2010) who demonstrated a strong ability of SAP1, SAP2 and SAP6 to induce inflammatory cytokines, and are consistent with a low capacity to induce inflammation found in null mutants lacking either S.API or SAP2 (Schaller et al., 2005). Such uncontrolled local accumulation of IL-8 can be involved in the recruitment of neutrophils to the site of infection and is observed under conditions of persistent neutrophilic airway inflammation (Harril et al., 2007). For example, during the initial stages of acute lung injury and acute respiratory distress syndrome, a massive accumulation of neutrophils in the vasculature of the lung was also identified (Cheng et al., 2013).

The migration of neutrophils to sites of infection is beneficial for the host because of their ability to kill pathogens. However, the presence of neutrophils at the infection foci may also be deleterious when an excessive, uncontrolled amount of NE is released into the extracellular medium, leading to lung matrix degradation. Pathologic effects of NE are also associated with microvascular injury, causing endothelial damage and increased capillary permeability that may enhance the translocation of microorganisms across the endothelial cell barrier and initiate bacteremia (Moraes et al., 2003). In epithelial and endothelial cell models, used in the present study, we also found that NET-associated NE partially influences the viability of both cell types. The effect is most pronounced when NE activity is no longer controlled by SAP-truncated A1PI. But at the same moment, a destruction of pathogen also occurred. Such effects raise a question if the local imbalance in the host protein homeostasis is really cost-effective for C. albicans cells, where parts of the population could be killed during deregulation of the host homeostasis. However, our models of NET-dependent impairment of epithelial and endothelial cells clearly indicated that the cells presented a surface more accessible for C. albicans colonization. Even the partial destruction of C. albicans cells in this situation cannot influence the total population of pathogen cells. Although the observation on a short time scale demonstrated drastic fungal killing effects, very often shown in published reports, during longer time of colonization, the remaining and still alive fungal cells can restore the whole population or even stimulate its growth by a yet unidentified stimulator, liberated during neutrophil breakdown, as was also noted by Urban et al. (2006).

Moreover, C. albicans is often associated with the polymicrobial community that is more resistant to any treatment, as it was shown in pneumonia and cystic fibrosis, where C. albicans interacts with typical pneumonia pathogens, such as Pseudomonas aeruginosa (Mear et al., 2013; Adar et al., 2011). It is probable that in such situations, especially in pulmonary fungal infections which have been systematically studied in recent years (Chotirmall & McElvaney, 2014; Roux et al., 2013; Wahab et al., 2014), the deregulation of well-balanced homeostatic protein–protein complexes of the host is more profitable for maintaining the pathogen community.

Taken together, the results obtained in the present study reveal that C. albicans SAPs are able to disturb A1PI-NE complex formation, thus influencing epithelial and endothelial cell colonization and adding another process of cytokine enhancement to the virulence attributes of this fungal pathogen.

Competing interests

The authors declare that they have no competing interests.

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