Release of calcium from intracellular stores and subsequent uptake by mitochondria are essential for the candidacidal activity of an N-terminal peptide of human lactoferrin

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Objectives: Earlier studies showed that mitochondrial damage is a hallmark of the candidacidal activity of an N-terminal peptide of human lactoferrin, further referred to as hLF(1–11). Since uptake of Ca²⁺ by mitochondria may be essential for their activation, the aim of this study was to define the role of Ca²⁺ in killing of Candida albicans by the hLF(1–11) peptide.

Methods: The effect of compounds interfering with Ca²⁺ homeostasis on the hLF(1–11)-induced candidacidal activity, changes in mitochondrial membrane potential, and reactive oxygen species production were evaluated using a killing assay, rhodamine 123 staining, and 2',7'-dichlorofluorescein diacetate, respectively. The increase in cellular Ca²⁺ content was measured using 45Ca²⁺.

Results: Our results revealed that Ruthenium Red, which inhibits the mitochondrial Ca²⁺-uniporter and the voltage-sensitive Ca²⁺ release from internal stores, blocked (P < 0.05) the hLF(1–11)-induced candidacidal activity as well as changes in the membrane potential of mitochondria, and reactive oxygen species production. Oxalate, which precipitates Ca²⁺ in intracellular organelles, decreased (P < 0.05) the peptide-induced changes in the membrane potential of mitochondria, reactive oxygen species production, and candidacidal activity. Furthermore, the Ca²⁺ ionophore ionomycin combined with high CaCl₂ concentrations enhanced the hLF(1–11)-induced candidacidal activity. Moreover, hLF(1–11) caused an influx of Ca²⁺ from the extracellular medium into C. albicans reaching a three-fold increase at 2h, whereas no increase was found in unexposed cells. In agreement, the Ca²⁺-chelator EGTA blocked the peptide-induced candidacidal activity.

Conclusions: Ca²⁺ release from intracellular stores, probably through subsequent mitochondrial Ca²⁺ uptake, is essential for the hLF(1–11)-induced candidacidal activity.

Keywords: lactoferrin peptide, mitochondrial Ca²⁺-uptake, mitochondrial membrane potential, reactive oxygen species production

Introduction

Candida albicans, a yeast often present as a commensal on skin and mucosal surfaces, can cause opportunistic infections mainly in immunocompromised patients. Candida spp. are the fourth most commonly isolated microorganisms from bloodstream infections in the United States and, despite intensive treatment with current drugs, the mortality rate is approximately 40%. These data encourage the development of new classes of antifungal agents. In this connection, antimicrobial proteins and peptides, such as human lactoferrin (hLF)- and histatin-derived peptides, seem promising candidates. Lactoferrin is a 77 kDa protein present on mucosal surfaces and in the specific granules of neutrophils from which it is released upon an inflammatory
stimulus. In addition to the ability of hLF to bind iron, pepsinolysis releases lactoferricin H, a 47 amino acid peptide derived from the N terminus of hLF. We recently demonstrated that a synthetically prepared peptide comprising the first 11 N-terminal residues of lactoferricin H, further referred to as hLF(1–11), exerts potent candidacidal activity. This candidacidal activity as well as that displayed by histatin-based peptides involves mitochondrial damage in yeasts, resulting in the synthesis and secretion of ATP, reactive oxygen species, and reactive nitrogen species. A general killing mechanism employed by various antimicrobial peptides. The mechanism(s) leading to mitochondrial damage involved in C. albicans death upon exposure to these antimicrobial peptides is(are) uncertain; however, influx of Ca\textsuperscript{2+} into the mitochondria from the cytosol may be critical to these processes. Regulation of the free cytosolic Ca\textsuperscript{2+} (Ca\textsuperscript{2+}\textsubscript{cyt}) in yeasts involves: (i) Ca\textsuperscript{2+} release from internal stores, mainly vacuoles, in which the free Ca\textsuperscript{2+} concentration reaches 1.3 mM, compared to 10 \mu M in the endoplasmic reticulum; (ii) Ca\textsuperscript{2+} import into the cytosol from outside involving selective Ca\textsuperscript{2+} channels in the plasma membrane; and (iii) pumping of Ca\textsuperscript{2+} from the cytosol into vacuoles mediated by two transporters with complementary actions, Vcx1p, a low-affinity Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger that rapidly sequesters Ca\textsuperscript{2+} into the vacuole, and Pmc1p, a high-affinity Ca\textsuperscript{2+} ATPase required for maintaining low cytosolic Ca\textsuperscript{2+} concentrations.

This study was undertaken to gain more insight into the role of Ca\textsuperscript{2+} in mitochondrial activation (changes in membrane potential, reactive oxygen species production) preceding mitochondrial dysfunctioning and the subsequent death of C. albicans upon exposure to hLF(1–11).

Materials and methods

Lactoferrin peptide

The synthetic peptide corresponding to residues 1–11 (GRRRR-SVQWCA; 1374 Da) of human lactoferrin, further referred to as hLF(1–11), was prepared and purified as described previously. The purity of the peptide exceeded 88%, as determined by reversed-phase high performance liquid chromatography. Stocks of peptide at a concentration of 1 mg/mL of 0.01% acetic acid (HAc; pH 3.7) were stored at −20°C and dried in a Speed-Vac immediately before use (Savant Instruments Inc., Farmingdale, NY, USA).

Chemicals

CaCl\textsubscript{2}·2H\textsubscript{2}O was purchased from Merck (Darmstadt, Germany), Ruthenium Red and ethylene glycol-bis(β-aminoethyl-ether)-N,N,N\textsuperscript{2},N\textsuperscript{2}-tetracetic acid (EGTA) from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Standard oxalate solution and ionomycin were obtained from Sigma Chemical Co. (St Louis, MO, USA). The oxalate standard solution and stocks of EGTA (1 M) and ionomycin (100 mM), prepared in water were stored at 4°C until use. A stock of Ruthenium Red (10 mM) was prepared in 50% DMSO and stored at −20°C. Calcium chloride (50 mM) was freshly prepared in 10 mM sodium phosphate buffer, pH 7.4.

Source of C. albicans strain

Fluconazole-resistant C. albicans strain Y01-19 was purchased from Pfizer Inc. (Groton, CT, USA). Fluconazole resistance (MIC>256 mg/L) was evaluated with the Etest (Oxoid Unipath Ltd., Basingstoke, UK). Yeasts were cultured overnight in Sabouraud broth (Oxoid) at 37°C and subcultured for 2.5 h on a rotary wheel at 37°C.

Treatment of C. albicans with inhibitors

Calcium chloride (50 mM) or EGTA (10 mM) were added simultaneously with the peptide to C. albicans. In addition, C. albicans cells were pre-incubated with ionomycin (10 \mu M up to 50 mM), Ruthenium Red (30 \mu M) or oxalate (340 \mu M), for 15 min at 37°C before addition of the peptide. These optimal concentrations were established in preliminary experiments to avoid toxic effects of these compounds on C. albicans.

Assay for candidacidal activity of hLF(1–11)

An in vitro assay was used to assess the candidacidal activity of hLF(1–11). Briefly, yeast cells were harvested in mid-log phase by centrifugation (1500 g, 10 min), washed twice in sodium phosphate buffer, and diluted to a concentration of 1 × 10\textsuperscript{4} cfu/mL of buffer. Next, equal volumes of this suspension and various concentrations of hLF(1–11) were mixed in small plastic snap-cap vials. After incubation for 2 h at 37°C, the vials were transferred onto ice and the number of viable blastoconidia was determined by plating serial dilutions of each sample on Sabouraud agar. Results, expressed as cfu C. albicans per mL, are means plus standard deviation (S.D.).

Measurement of \textsuperscript{45}Ca\textsuperscript{2+} accumulation

Accumulation of Ca\textsuperscript{2+} in C. albicans was measured as previously described. In brief, yeast cells grown in yeast peptone dextrose (YPD) medium (pH 5.5) at 30°C were harvested in mid-log phase by centrifugation (1500 g, 10 min) and 5 × 10\textsuperscript{5} cfu were resuspended in 1.5 mL of fresh YPD medium supplemented with 20 \mu Ci of \textsuperscript{45}CaCl\textsubscript{2} (Amersham Biosciences Europe GmbH, Freiburg, Germany) per mL. Next, these C. albicans cells were exposed at 30°C to 17\mu M hLF(1–11) for various intervals. The reaction was stopped by transferring the cells onto melting ice and C. albicans were harvested onto 2.4 cm GF/F filters (Whatman International Ltd, Kent, UK), washed with ice-cold buffer [10 mM CaCl\textsubscript{2}, 5 mM HEPES–NaOH (Sigma); pH 6.5], dried at 90°C for 1 h, placed in scintillation vials, and processed for liquid scintillation counting in a Packard 1600 TR liquid scintillation counter using OptiFluor (Packard) scintillation cocktail after addition of 10 mL of Ultima Flo-M (Packard BioScience B.V., Groningen, The Netherlands).

Results of the total cell-associated radioactivity are expressed as disintegrations per min (dpm).

Assay for mitochondrial membrane potential

The positively charged fluorescent probe rhodamine 123 (Molecular Probes, Eugene, OR, USA) was used to measure the mitochondrial membrane potential in C. albicans. Briefly, C. albicans in mid-log phase was resuspended in 1 mM potassium phosphate buffer, pH 7.0 and pre-incubated for 10 min at 37°C with 10 \mu M rhodamine 123 in the same buffer. After two washes with buffer, C. albicans cells were treated for 10 min at 37°C with 17 \mu M hLF(1–11) and then analysed on a fluorescence-activated cell sorter (FACS) FACSCalibur (Becton & Dickinson, San José, CA, USA) equipped with an argon laser at 488 nm. Rhodamine 123 median fluorescence intensities (MFI) were measured in the second channel. Data acquisition and analysis were controlled with the CellQuest Pro software and hardware interface.
Results, expressed as the percentage of the MFI for hLF(1–11)-exposed C. albicans, are means ± s.d.

Measurement of reactive oxygen species production

2′,7′-Dichlorofluorescein diacetate (Eastman Kodak Company, Rochester, NY, USA) was used to measure reactive oxygen species production by C. albicans as previously described. In brief, C. albicans cells were harvested in mid-log phase, washed twice as described above, and then diluted to a concentration of 2 × 10⁵ cfu/mL of sodium phosphate buffer. Next, C. albicans cells were pre-incubated for 15 min at 37°C with 100 mM of 2′,7′-dichlorofluorescein diacetate then treated for 15 min at 37°C with various concentrations of hLF(1–11). Immediately before use, 2′,7′-dichlorofluorescein diacetate (100 mM) was dissolved in DMSO and further diluted in buffer. The fluorescence of DCF was measured on the FACSCalibur. Results are expressed as MFI ± s.d.

Statistical analysis

Differences between the values for hLF(1–11)-treated and untreated C. albicans were analysed by the Mann–Whitney U-test. The level of significance was set at P < 0.05. Correlation between the accumulation of Ca²⁺ in C. albicans and the exposure to 17 μM hLF(1–11) in time was calculated using the Pearson correlation test.

Results

Involvement of Ca²⁺ in the hLF(1–11)-induced candidacidal activity

To investigate whether Ca²⁺ is essential for the candidacidal activity of hLF(1–11), the effect of 10 mM EGTA on the candidacidal activity of this peptide was determined in sodium phosphate buffer, pH 7.4. The results revealed that this calcium chelator completely blocked (P < 0.05) the candidacidal activity of the peptide (Figure 1). In addition, the combination of the ionophore ionomycin (10 μM) and CaCl₂ (50 mM) increased (P < 0.05) the candidacidal activity induced by the highest concentration of hLF(1–11) tested. The number of C. albicans cells surviving upon treatment with 67 μM hLF(1–11) amounted to 5.1 ± 2.6/10³ and after exposure to the combination of 17 μM hLF(1–11), whereas no increase was found during this period of analysis in C. albicans not exposed to the peptide.

Effect of hLF(1–11) on Ca²⁺ accumulation in C. albicans

⁴⁵Ca²⁺ accumulation was measured in C. albicans upon exposure to hLF(1–11). The results revealed a linear increase (r = 0.791; P < 0.001) in Ca²⁺ accumulation in C. albicans upon exposure to 17 μM hLF(1–11) reaching a three-fold increase at 2 h (Figure 2), whereas no increase was found during this period of analysis in C. albicans not exposed to the peptide.

Effect of Ruthenium Red on the hLF(1–11)-induced candidacidal activity

Since effects on mitochondria are essential for the candidacidal activity of hLF(1–11), the action of Ruthenium Red, which inhibits the Ca²⁺-uptake by mitochondria and the voltage-sensitive Ca²⁺ release from internal stores, was evaluated. The results revealed that this inhibitor blocked (P < 0.05) the candidacidal activity of hLF(1–11) (Figure 3).

Effect of inhibitors of Ca²⁺ released by intracellular stores on the hLF(1–11)-induced candidacidal activity

To investigate whether Ca²⁺ released by intracellular stores is involved in the candidacidal activity of hLF(1–11), the effects of oxalate, which precipitates Ca²⁺ in intracellular organelles and high extracellular Ca²⁺ (in the mM range), which inhibits Ca²⁺ release from intracellular stores in various cell types, were evaluated. The results revealed that oxalate partially

Figure 1: Effect of EGTA on the dose-dependent killing of fluconazole-resistant C. albicans by the human lactoferrin-derived peptide hLF(1–11). Cells were incubated with various concentrations of the peptide in the presence (filled bars) or absence (open bars) of 10 mM EGTA. After incubation for 2 h at 37°C, the number of viable C. albicans was determined microbiologically. ‘Zero’ means no peptide. Results are means plus s.d. of at least three independent experiments. Significant differences (P < 0.05) by the Mann-Whitney U-test compared with cells exposed to hLF(1–11).

Figure 2: Accumulation of ⁴⁵Ca²⁺ in C. albicans at various time points after addition of the hLF(1–11) peptide (n ≥ 3 independent experiments).

Figure 3: Effect of Ruthenium Red on the hLF(1–11)-induced candidacidal activity.
inhibited (P<0.05), whereas high Ca²⁺ completely blocked the hLF(1–11)-induced killing of C. albicans (Figure 4), indicating that Ca²⁺ release from intracellular stores is crucial in the candidacidal activity of hLF(1–11).

Effect of Ruthenium Red, oxalate, high extracellular CaCl₂ and EGTA on the hLF(1–11)-induced reactive oxygen species production

Next, the effect of previously mentioned pharmacological agents on the hLF(1–11)-induced reactive oxygen species production was studied. The results revealed that Ruthenium Red, oxalate, high extracellular CaCl₂ and EGTA completely blocked reactive oxygen species production (P<0.05) by C. albicans upon exposure to hLF(1–11) (Figure 5).

Discussion

The main conclusion from the present results is that the release of Ca²⁺ from intracellular stores, probably through the subsequent uptake of Ca²⁺ by mitochondria, plays an essential role in the peptide-induced killing of C. albicans. This conclusion is based on the following findings. First, Ruthenium Red which, by interfering with the mitochondrial Ca²⁺-uniporter, inhibits the mitochondrial Ca²⁺-uptake and Ca²⁺ release from intracellular stores are essential for the hLF(1–11)-induced changes in the mitochondrial membrane potential.

Effect of Ruthenium Red, oxalate, high extracellular CaCl₂ and EGTA on the hLF(1–11)-induced reactive oxygen species production

Table 1. Effect of various inhibitors on the hLF(1–11)-induced changes in the membrane potential of mitochondria of fluconazole-resistant C. albicans by rhodamine 123 staining

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Rhodamine 123 staining (%)</th>
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<tbody>
<tr>
<td>hLF(1–11) peptide</td>
<td>no peptide</td>
</tr>
<tr>
<td>None</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ruthenium Red</td>
<td>2 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxalate</td>
<td>13 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>7 ± 3.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGTA</td>
<td>7 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results, expressed as the percentage of the median fluorescence intensity for hLF(1–11)-exposed C. albicans, are means ± S.D. of at least three independent experiments. *Significantly (P<0.05) different from C. albicans not exposed to the hLF(1–11) peptide.

`<sup>b</sup>`Significantly different (P<0.05) from cells exposed to the peptide but not incubated with the pharmacological agent.

The role of mitochondrial Ca²⁺-uptake and Ca²⁺ release from intracellular stores in the hLF(1–11)-induced changes in the mitochondrial membrane potential was further investigated using rhodamine 123 and FACS analysis.<sup>5</sup> The results revealed that (i) the hLF(1–11) peptide induced a significant (P<0.05) increase in rhodamine 123 fluorescence in C. albicans, compared with unexposed cells, and that (ii) Ruthenium Red, oxalate, high extracellular CaCl₂, and EGTA completely blocked (P<0.05) the hLF(1–11)-induced change in the mitochondrial rhodamine 123 staining (Table 1), suggesting that mitochondrial Ca²⁺-uptake and Ca²⁺ release from intracellular stores are essential for the hLF(1–11)-induced changes in the mitochondrial membrane potential.

Discussion

The main conclusion from the present results is that the release of Ca²⁺ from intracellular stores, probably through the sub-sequent uptake of Ca²⁺ by mitochondria, plays an essential role in the peptide-induced killing of C. albicans. This conclusion is based on the following findings. First, Ruthenium Red which, by interfering with the mitochondrial Ca²⁺-uniporter, inhibits the mitochondrial Ca²⁺-uptake and blocks the peptide-induced killing of C. albicans. However, it should be realized that Ruthenium Red also affects the voltage-sensitive Ca²⁺ release pathway in intracellular stores<sup>25</sup> and therefore it cannot be excluded that such an effect explains (in part) the inhibition of the hLF(1–11)-induced killing of C. albicans by this pharmacological agent. In this connection, our preliminary data demonstrating that carbonyl cyanide m-chlorophenylhydrazone (100 µM), which also inhibits Ca²⁺ uptake by mitochondria,<sup>25</sup> blocked the hLF(1–11)-induced killing (data not shown), suggest that Ca²⁺ uptake by mitochondria is involved in
the candidacidal activity induced by this peptide. Furthermore, Ruthenium Red decreased the hLF(1–11)-induced changes in the mitochondrial membrane potential and reactive oxygen species production, both important in the peptide-induced candidacidal activity. Second, we have shown that inhibition of Ca2+ release from intracellular stores, such as vacuoles, by oxalate partially decreased the candidacidal activity of hLF(1–11). High Ca2+ concentrations, which (in the mM range) inactivate Ca2+-chelator EGTA (dotted bars) or no inhibitor (open bars). Results are means plus S.D. of median fluorescence intensities (MFI) of at least three independent experiments. *Significant differences (P<0.05) compared to cells exposed to hLF(1–11).

![Figure 5](http://jac.oxfordjournals.org/)

Figure 5. Effect of Ruthenium Red, oxalate, high extracellular CaCl2 and EGTA on hLF(1–11)-triggered reactive oxygen species production by fluconazole-resistant C. albicans. Cells were pre-incubated with 100 μM 2',7'-dichlorofluorescein diacetate and then treated for 15 min at 37°C with various concentrations of hLF(1–11) in the presence of 30 μM Ruthenium Red (filled bars), 340 μM oxalate (diagonally hatched bars), 50 mM CaCl2 (vertically hatched bars), 10 mM EGTA (dotted bars) or no inhibitor (open bars). Results are means plus S.D. of median fluorescence intensities (MFI) of at least three independent experiments. *Significant differences (P<0.05) compared to cells exposed to hLF(1–11).

Cellular Ca2+ content at 2 h, whereas no increase in Ca2+ accumulation was found in cells not exposed to the peptide or exposed to a control peptide (data not shown).

Together, the present data indicate that the hLF(1–11) peptide causes intracellular stores to release Ca2+ into the cytosol as well as an influx of Ca2+ from the extracellular medium; this may result in an influx of Ca2+ into mitochondria, a change in the mitochondrial membrane potential and the formation of the reactive oxygen species. Further studies are needed to prove that the hLF(1–11) peptide-induced Ca2+ influx into mitochondria may lead to the destruction of the energized mitochondria.

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