Biochemical and Biophysical Research Communications 394 (2010) 170–172

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Melittin induces apoptotic features in *Candida albicans*

Cana Park, Dong Gun Lee *

School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, 1370 Sankyuk-dong, Puk-ku, Daegu 702-701, Republic of Korea

ARTICLE INFO

Article history:
Received 17 February 2010
Available online 24 February 2010

Keywords:
Antimicrobial peptide
Melittin
Antifungal effect
Apoptosis

ABSTRACT

Melittin is a well-known antimicrobial peptide with membrane-active mechanisms. In this study, it was found that Melittin exerted its antifungal effect *via* apoptosis. *Candida albicans* exposed to Melittin showed the increased reactive oxygen species (ROS) production, measured by DHR-123 staining. Fluorescence microscopy staining with FITC-annexin V, TUNEL and DAPI further confirmed diagnostic markers of yeast apoptosis including phosphatidylserine externalization, and DNA and nuclear fragmentation. The current study suggests that Melittin possesses an antifungal effect with another mechanism promoting apoptosis.

1. Introduction

Antimicrobial peptides (AMPs), which are considered as the native line of defense throughout nature, show a high toxicity against both Gram-positive and Gram-negative bacteria as well as fungi, viruses, and mycobacteria [1,2]. Research in this field is of growing interest, as AMPs are typically small (~10–50 residues), cationic, amphipathic peptides, known to permeate microbial membranes by the formation of transmembrane ion permeable pores or by the detergent-like manner, thus inducing leakage of the cellular components across the bilayer [3,4]. On the other hand, recent reports demonstrate that AMPs such as, plant defensin RsAFP2 [5], dermaceptins [6] and human lactoferrin [7], exert their antimicrobial effect *via* inducing apoptosis against microbes.

Melittin is a naturally occurring AMP with remarkable cytolytic potency. It is the principal toxic component of the European honeybee venom, *Apis mellifera*. It is a cationic, amphipathic hexacosa peptide (GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$), with a large hydrophobic region and a stretch of predominantly hydrophilic amino acids at the carboxy terminal. This peptide is widely known to have membrane-active activity by micellization as well as voltage-dependent ion channel formation across the lipid bilayer [8–10]. In this study, it was investigated whether Melittin could exert antifungal effect *via* another mechanism like apoptosis. The results showed that Melittin induced apoptosis against *Candida albicans*. This is the first report that Melittin induces apoptosis in *C. albicans*.

2. Materials and methods

2.1. Peptide synthesis

The peptide was synthesized by the solid-phase method using Fmoc(9-fluorenyl-methozycarbonel)-chemistry [11]. The crude peptide was repeatedly washed with diethylether, dried in a vacuum, and purified using a reversed-phase preparative HPLC on a Waters 15-μm Deltapak C18 column (19 × 30 cm). The purity of the peptide was confirmed by an analytical reversed-phase HPLC on an Ultrasphere C18 column, 4.6 × 25 cm (Beckman, USA). The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization MALDI-mass spectrometer [12].

2.2. C. albicans apoptosis assay

C. albicans cells (2×10^6/ml, YPD) were incubated with 2.5 μM Melittin or 5 mM H_2O_2 for 2 h 30 min at 30 °C. Intracellular ROS accumulation was determined by incubation of cells with 5 μg/ml of DHR-123, which can be oxidized by ROS to become the fluorescent chromophore Rh-123. The cells were analyzed by flow cytometric analysis [13].

Annexin V-FITC labeling was performed following the modified method of F. Madeo [13]. *C. albicans* cells were harvested by centrifugation, washed in 0.1 M potassium phosphate buffer (PPB, pH 6.0), resuspended in PPB containing 1 M sorbitol, and digested with lysing enzyme (20 mg/ml). The cells were washed with 0.1 M PPB containing 1 M sorbitol. The cells were treated with peptide and

Abbreviations: PI, propidium iodide; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling; DAPI, 4′-6-diamidino-2-phenylindole; ROS, reactive oxygen species; DHR, dihydrorhodamine; FITC, fluorescein isothiocyanate.

* Corresponding author. Fax: +82 53 955 5522.
E-mail address: dglee222@knu.ac.kr (D.G. Lee).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.02.138

compound for 2 h were washed and resuspended in an annexin binding buffer (1 M sorbitol). The cells were incubated with 5 μl of annexin V-FITC/ml and incubated for 20 min prior to the 5 μl of PI.

DNA fragmentation was analyzed by the TUNEL method [14]. The cells treated with peptide and compound for 150 min were washed in a PBS containing 1 M sorbitol. The DNA ends were labeled by using an in situ cell death detection kit.

Nuclear fragmentation was analyzed as DAPI staining. For nuclear staining, cells were washed with PPB, permeabilized, and incubated with 1 μg of DAPI/ml for 10 min. Cells were then harvested and examined by fluorescence microscopy.

For quantitative assessment of stained cells (annexin V, TUNEL), at least 200 cells per sample were examined by fluorescence microscopy, Axio Imager A1 and Axio Cam MR5 (Carl Zeiss).

3. Results and discussion

Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$) is one of the representative antimicrobial peptides isolated from the European honeybee venom, *Apis mellifera* [8]. It has been reported that antibacterial and antifungal property against human pathogenic strains of this peptide *via* membrane-disruption is remarkable. Due to its pronounced cytolytic activity, Melittin is widely used as a positive control to compare the antimicrobial activity of the molecules and as a template for designing hybrid peptides [15,16]. Also, extensive research with regards to its membrane-active mechanisms has been undertaken to reveal the architecture of the membrane perturbation and molecular details of the mechanism [17,18].

3.1. Melittin induces intracellular production of ROS in C. albicans

Recently, studies in the pathway analysis and system biology indicated that regardless of action classification based on drug-target interaction, major classes of bactericidal antibiotics stimulated the production of hydroxyl radicals, which ultimately contributed to induce programmed cell death [19,20]. To determine whether this finding also applied to Melittin in exerting a fungicidal effect, the intracellular production of ROS in the cells treated with Melittin was analyzed. The relative fluorescent intensity of DHR-123, which is oxidized to a fluorescent derivative by intracellular ROS in the cultures treated with Melittin or not, determined by flow cytometry. *C. albicans*, a denoted yeast model system for studying programmed cell death in higher eukaryotes [21], as well as the most prevalent systemic fungal pathogen [22], was used as test organism. A total of 7.8% of cells treated with Melittin was DHR-123 positive, corresponding to the intracellular accumulation of ROS (Fig. 1). The detection of ROS is one of the major stimuli for the induction of apoptosis and is considered one of the crucial elements for cell death found in yeast and higher eukaryotes [13]. It was investigated whether the apoptotic features could be observed in *C. albicans* cultures treated with Melittin. The investigation for the mechanisms of action of Melittin in retaining antifungal activity were followed by examination for programmed cell death reminiscent of apoptotic markers, such as phosphatidylserine externalization.

3.2. Phosphatidylserine externalization in Melittin-treated C. albicans

To determine whether Melittin could induce the apoptotic features in *C. albicans*, based on the MIC value as a criterion, the cells were treated with the peptide under the two concentrations at 1.25 μM as a subinhibitory concentration or at 2.5 μM. Phosphatidylserine is only distributed in the inner leaflet of the lipid bilayer of the plasma membrane, which is maintained by the ABC transporters in *C. albicans*. The phosphatidylserine externalization at the outer leaflet of the cytoplasmic membrane is interpreted as an early marker of apoptosis in *C. albicans* cells as well as mammalian cells [13,22]. To detect the early event of apoptosis and discrete necrosis, *C. albicans* cells exposed to Melittin were co-stained with annexin V-FITC and PI. As shown in Fig. 2A-d, 12% of the cells treated with at the subinhibitory concentration of Melittin were stained as annexin V-positive and PI-negative, displaying similar response to 5 mM H_2O_2, an inducer of apoptosis in yeast cells [23]. When cells were treated with Melittin at the MIC, however, 40.2% of the PI-positive cells were observed (Data not shown). In the cultures treated with peptide at the two times the MIC, the dominant population of the cells was 76% of PI-positive, indicating immediate cell death caused by the membrane defects [15]. It is likely that at the subinhibitory concentration of Melittin, *C. albicans* cells show apoptotic features, while the membrane-active necrotic effect mainly contributes to the antifungal activity of Melittin at the MIC.

3.3. DNA and nuclear fragmentation in Melittin-treated C. albicans

To further confirm the apoptotic features induced in *C. albicans* by the antimicrobial peptide Melittin, the TUNEL assay was conducted in order to detect apoptotic DNA fragmentation by labeling 3′-OH termini with modified nucleotides catalyzed by terminal deoxynucleotidyltransferase. The labeling of DNA breaks by TUNEL, one of the reliable methods for the identification of apoptotic cells, is utilized to visualize the apoptotic phenotype cells [15,24]. 9% of TUNEL-positive cells were observed in the population treated with Melittin (Fig. 2B-a). DAPI staining of Melittin-treated cells showed the distributed nuclear fragments (Fig. 2B-d). In untreated cultures, the nucleus appears as single

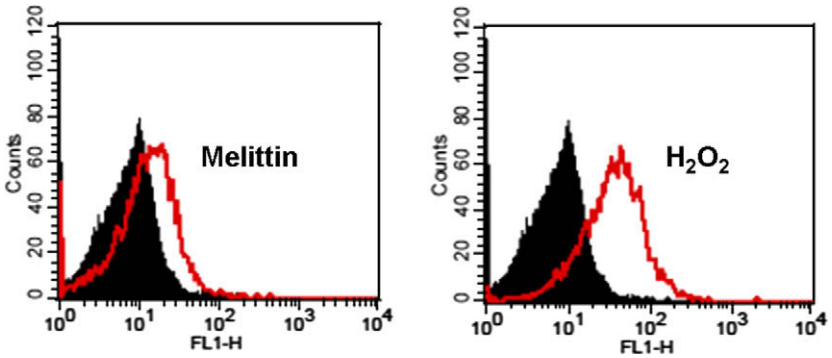

Fig. 1. ROS accumulation in Melittin-treated *C. albicans* cells. Flow cytometric analysis of cells treated with Melittin incubated with DHR-123.

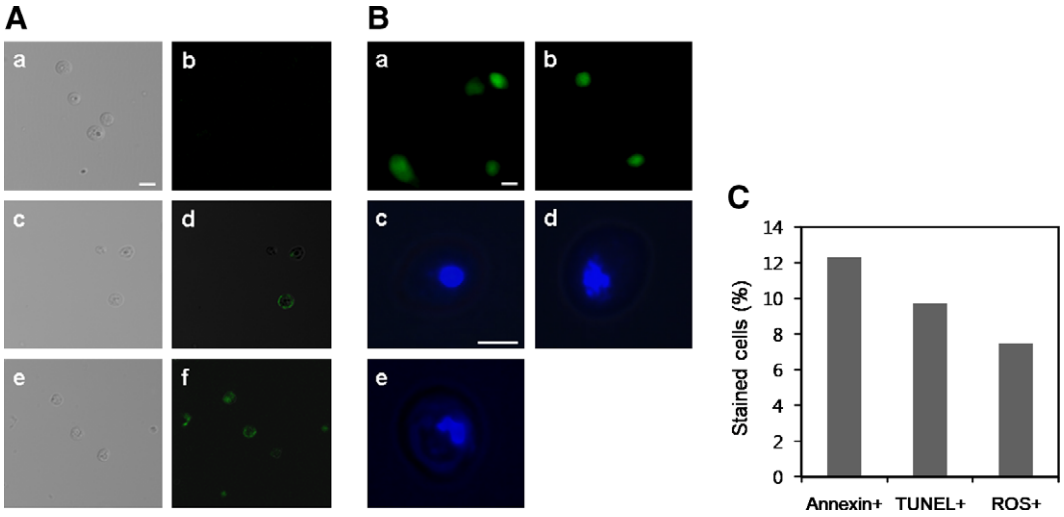

Fig. 2. *C. albicans* apoptosis assay. (A) Phosphatidylserine externalization shown by FITC-annexin V staining in Melittin-treated *C. albicans* cells. *C. albicans* cells were treated with Melittin (c and d), H_2O_2 (e and f) or not treated (a and b). Subpanels (a, c, and e) are phase-contrast micrographs. (B) DNA and nuclear fragmentation shown by TUNEL and DAPI staining in Melittin-treated *C. albicans* cells. *C. albicans* cells were treated with Melittin (a and d), H_2O_2 (b and e) or not treated. (C) Melittin-treated *C. albicans* cells showing apoptotic markers. Annexin+, TUNEL+, and ROS+ refer to the percentage of stained cells.

round spot in the cell (Fig. 2B-c). *C. albicans* is known to activate a programmed cell death with features reminiscent of apoptosis in response to a variety of environmental stimuli such as, NaCl stress [25], acetic acid [26], hydrogen peroxide [21] as well as amphotericin B [15]. In the case of Melittin, even though further investigations are needed, it is currently speculated that at low concentration, the formation of peptide dimer or tetramer, which is linked to the membrane-active activity, was inhibited, Melittin could more easily translocate the plasma membrane, and then bind to intracellular molecules, which might trigger apoptosis in *C. albicans*. Taken together, these results showed that a series of apoptotic features were observed in cultures of *C. albicans* treated with Melittin mainly at the subinhibitory concentration.

In conclusion, this study presents the first investigation for antimicrobial peptide Melittin inducing apoptotic features in *C. albicans*. Although the molecular background of the actions of Melittin has not as yet been fully elucidated, this report suggests another direction, which deserves further studies to define antifungal action mechanisms of Melittin, as well as membrane-disruptive mechanisms.

Acknowledgment

This work was supported by a Grant (CODE 20080401-034-017) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

References

[1] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[2] R.E. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (2006) 1551–1557.
[3] H.A. Pereira, Novel therapies based on cationic antimicrobial peptides, Curr. Pharm. Biotechnol. 7 (2006) 229–234.
[4] H. Jenssen, P. Hamill, R.E. Hancock, Peptide antimicrobial agents, Clin. Microbiol. Rev. 19 (2006) 491–511.
[5] A.M. Aerts, D. Carmona-Gutierrez, S. Lefevre, G. Govaert, I.E. François, F. Madeo, R. Santos, B.P. Cammue, K. Thevissen, The antifungal plant defensin RsAFP2 from radish induces apoptosis in a metacaspase independent way in *Candida albicans*, FEBS Lett. 583 (2009) 2513–2516.
[6] C.O. Morton, S.C. Dos Santos, P. Coote, An amphibian-derived, cationic, alpha-helical antimicrobial peptide kills yeast by caspase-independent but AIF-dependent programmed cell death, Mol. Microbiol. 65 (2007) 494–507.
[7] M.T. Andrés, M. Viejo-Díaz, J.F. Fierro, Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of K^+-channel-mediated K^+ efflux, Antimicrob. Agents Chemother. 52 (2008) 4081–4088.
[8] E. Habermann, Bee and wasp venoms, Science 177 (1972) 314–322.
[9] K. Matsuzaki, S. Yoneyama, K. Miyajima, Pore formation and translocation of melittin, Biophys. J. 73 (1997) 831–838.
[10] S. Toraya, T. Nagao, K. Norisada, S. Tuzi, H. Saitô, S. Izumi, A. Naito, Morphological behavior of lipid bilayers induced by melittin near the phase transition temperature, Biophys. J. 89 (2005) 3214–3222.
[11] R.B. Merrifield, Solid phase synthesis, Science 232 (1986) 341–347.
[12] P. Jungblut, B. Thiede, Protein identification from 2-DE gels by MALDI mass spectrometry, Mass Spectrom. Rev. 16 (1997) 145–162.
[13] F. Madeo, E. Fröhlich, K.U. Fröhlich, A yeast mutant showing diagnostic markers of early and late apoptosis, J. Cell Biol. 139 (1997) 729–734.
[14] A.J. Phillips, I. Sudbery, M. Ramsdale, Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*, Proc. Natl. Acad. Sci. USA 100 (2003) 14327–14332.
[15] C. Park, D.G. Lee, Fungicidal effect of antimicrobial peptide arenicin-1, Biochim. Biophys. Acta 1788 (2009) 1790–1796.
[16] J.M. Saugar, M.J. Rodríguez-Hernández, B.G. de la Torre, M.E. Pachón-Ibañez, M. Fernández-Reyes, D. Andreu, J. Pachón, L. Rivas, Activity of cecropin A-melittin hybrid peptides against colistin-resistant clinical strains of Acinetobacter baumannii: molecular basis for the differential mechanisms of action, Antimicrob. Agents Chemother. 50 (2006) 1251–1256.
[17] S.C. Park, M.H. Kim, M.A. Hossain, S.Y. Shin, Y. Kim, L. Stella, J.D. Wade, Y. Park, K.S. Hahm, Amphipathic alpha-helical peptide, HP (2–20), and its analogues derived from *Helicobacter pylori*: pore formation mechanism in various lipid compositions, Biochim. Biophys. Acta 1778 (2008) 229–241.
[18] M.T. Lee, W.C. Huang, F.Y. Chen, H.W. Huang, Mechanism and kinetics of pore formation in membranes by water-soluble amphipathic peptides, Proc. Natl. Acad. Sci. USA 105 (2008) 5087–5092.
[19] M.A. Kohanski, D.J. Dwyer, B. Hayete, C.A. Lawrence, J.J. Collins, A common mechanism of cellular death induced by bactericidal antibiotics, Cell 130 (2007) 797–810.
[20] M.A. Kohanski, D.J. Dwyer, J. Wierzbowski, G. Cottarel, J.J. Collins, Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death, Cell 135 (2008) 679–690.
[21] K.U. Fröhlich, F. Madeo, Apoptosis in yeast-a monocellular organism exhibits altruistic behaviour, FEBS Lett. 473 (2000) 6–9.
[22] P.G. Pappas, J.H. Rex, J. Lee, R.J. Hamill, R.A. Larsen, W. Powderly, C.A. Kauffman, N. Hyslop, J.E. Mangino, S. Chapman, H.W. Horowitz, J.E. Edwards, W.E. Dismukes, NIAID mycoses study group, a prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients, Clin. Infect. Dis. 37 (2003) 634–643.
[23] E. Cabiscol, E. Piulats, P. Echave, E. Herrero, J. Ros, Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*, J. Biol. Chem. 275 (2000) 27393–27398.
[24] I. Wadskog, C. Maldener, A. Proksch, F. Madeo, L. Adler, Yeast lacking the SRO7/SOP1-encoded tumor suppressor homologue show increased susceptibility to apoptosis-like cell death on exposure to NaCl stress, Mol. Biol. Cell 15 (2004) 1436–1444.
[25] P. Ludovico, M.J. Sousa, M.T. Silva, C. Leão, M. Côrte-Real, *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid, Microbiology 147 (2001) 2409–2415.
[26] G.F. Ribeiro, M. Côrte-Real, B. Johansson, Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock, Mol. Biol. Cell 17 (2006) 4584–4591.