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Membrane Perturbation Induced by Papiliocin Peptide, Derived from *Papilio xuthus*, in *Candida albicans*

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Previously, papiliocin was isolated from the swallowtail butterfly *Papilio xuthus* and its antimicrobial activity was suggested. In this study, the antifungal mechanism of papiliocin against *Candida albicans* was investigated. Confocal laser scanning microscopy (CLSM) and 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence analysis indicated that papiliocin disturbed the fungal plasma membrane. Moreover, the assessment of the release of FITC-dextran (FD) from liposomes further demonstrated that the antifungal mechanism of papiliocin could have originated from the pore-forming action and that the radius of the pores was presumed to be anywhere from 2.3 to 3.3 nm.

Keywords: Papiliocin, antimicrobial peptide, antifungal mechanism, *Candida albicans*

Previously, the cDNA cloning and characterization of a novel gene encoding for a cecropin-like peptide, papiliocin (RWKIFKKIEKVGRNVRDGIIKAGPAVAVVGQAATV VK-NH_2) derived from the larvae of the swallowtail butterfly *Papilio xuthus*, were reported. Furthermore, the antimicrobial activity of chemically synthesized papiliocin was investigated. Papiliocin was shown to contain significant antimicrobial activities against both Gram-positive and Gram-negative bacterial strains and the fungal strain *Candida albicans* [8]. In fact, *C. albicans*, not only an opportunistic but also a pathogenic fungal strain, is known to cause deeply invasive mycoses, including candidiasis [10]. There were also several studies regarding the antifungal agents for treating the diseases caused by *C. albicans* [6, 19]. Therefore, in this study, the antifungal mechanism of papiliocin against *C. albicans* (ATCC 90028) was investigated and suggested.

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Solid-Phase Peptide Synthesis

Peptide synthesis was carried out by Anygen Co. (Gwangju, Korea). The following procedure for peptide synthesis are offered by Anygen Co. The assembly of peptides consisted of a 60-min cycle for each residue at ambient temperature as follows: (i) the 2-chlorotrityl [or 4-methylbenzhydrylamine (MBHA) amide] resin was charged to a reactor and then washed with dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF), respectively; (ii) a coupling step with vigorous shaking was done, using a 0.14 mM solution of Fmoc-L-amino acids and Fmoc-L-amino acids preactivated for approximately 60 min with a 0.1 mM solution of 0.5 M HOBt/DIC in DMF. Finally, the peptide was cleaved from the resin using a trifluoroacetic acid (TFA) cocktail solution at ambient temperature [4, 13, 18].

Analysis and Purification of the Peptide Using HPLC

Analytical and preparative reverse-phase high performance liquid chromatography (HPLC) runs were performed with a Shimadzu 20A or 6A gradient system. Data were collected using an SPD-20A detector at 230 nm. Chromatographic separations were achieved with a 1%/min linear gradient of buffer B in A (A=0.1% TFA in H_2O; B=0.1% TFA in CH_3CN) over 40 min at flow rates of 1 ml/min and 8 ml/min using Shimadzu C_{18} analytical (5 μm, 0.46 cm×25 cm) and preparative C_{18} (10 μm, 2.5 cm×25 cm) columns, respectively.

Confocal Laser Scanning Microscopy (CLSM)

To investigate the intracellular distribution of papiliocin in *C. albicans*, CLSM was first performed. Exponential phased cells were treated with the fluorescein isothiocyanate

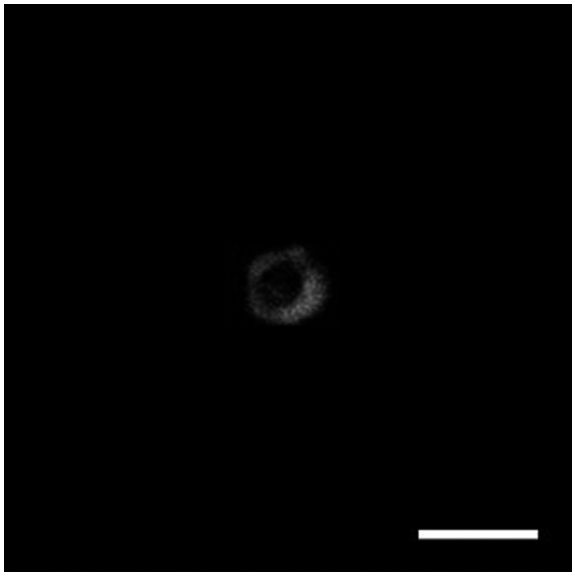

Fig. 1. Confocal microscopy of *C. albicans* cells treated with FITC-labeled papiliocin.
The bar corresponds to 5 μm.

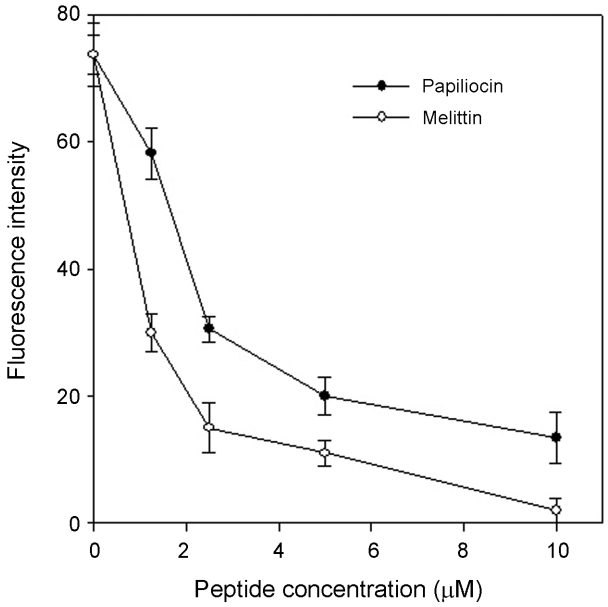

Fig. 2. DPH intensity after the peptides treatment.
Subcultured *C. albicans* cells containing peptides (1.25, 2.5, 5, and 10 μM) were incubated at 28°C for 2 h. The error bars represent the standard deviation values for three independent experiments, performed in triplicate.

(FITC)-labeled papiliocin and incubated for 5 min at 28°C. After incubation, cells were harvested by means of centrifugation at 10,000 rpm for 5 min and washed with ice-cold phosphate-buffered saline [PBS (pH 7.4)]. The visualization and localization of the peptide were detected by a laser scanning spectral confocal microscope (ZEISS LSM 5 EXCITER, Carl Zeiss, Germany) [16]. The result showed that FITC-labeled papiliocin swiftly interacted with the cells and was collected on the fungal cell surface (Fig. 1). This finding suggested that the target site of papiliocin could be the cell membrane or cell wall, the principal components of the cell surface.

1,6-Diphenyl-1,3,5-hexatriene (DPH) Fluorescence Analysis

To elucidate the mechanism of papiliocin, the changes of membrane dynamics were also investigated by employing DPH (Molecular Probes) as a membrane probe. DPH is a hydrophobic molecule and an intercalation of DPH into membranes is accompanied by the strong enhancement of their fluorescence [3, 5, 20]. The exponential phased cells (2×10^4 cells/ml), containing either papiliocin or melittin (1.25, 2.5, 5, and 10 μM), used as a positive control, were incubated at 28°C for 2 h. Melittin is one of the most well-known membrane-active antimicrobial peptides, possessing potent lytic activity towards not only bacterial but also eukaryotic cells [2]. The control cells were incubated without peptides. Samples of the fungal cultures were fixed by 0.37% formaldehyde, collected, and washed with PBS. The cells were frozen by using liquid nitrogen. For labeling purposes, cells were thawed with PBS and resuspended in PBS. The suspended mixture was incubated at 28°C for 45 min in a 120 μM DPH solution. The fluorescence intensity was measured by using an RF-5301PC spectrofluorophotometer (Shimadzu, Japan) at wavelengths λ_{ex}=350 nm, λ_{em}=425 nm [9, 14]. The decrease of the fluorescent intensity by papiliocin indicated that the cell plasma membrane of *C. albicans* was structurally disrupted when exposed to the peptide (Fig. 2). This also demonstrated that the main target site of papiliocin in fungal cells was the cell plasma membrane. In brief, the results suggest that papiliocin exerts its antifungal activity by means of a membrane-disruptive mechanism.

Preparation of FITC-Dextran Loaded Liposomes and Leakage Assay

To investigate the extent of membrane damage induced by papiliocin, the release of FITC-labeled dextran (FD) of various sizes (FD4, FD10, and FD20) from liposomes was measured, composed of phosphatidylcholine (PC)/ phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ ergosterol [5:4:1:2 (w/w/w/w)], mimicking the membranes of *C. albicans* [1]. This leakage assay by employing fluorescent dyes having different molecular masses is a method to determine the pore formation mechanism in the membrane perturbation process [11, 15]. All FDs were purchased from Sigma Chemical Co. (U.S.A.). The average molecular mass of FD4, FD10, and FD20 is 3.38, 10.1, and 14.8 kDa, respectively. The hydrodynamic radius (Stokes radius) of FD4, FD10, and FD20 is known to 1.4, 2.3, and 3.3 nm, respectively [7]. To prepare FD-entrapped liposomes,

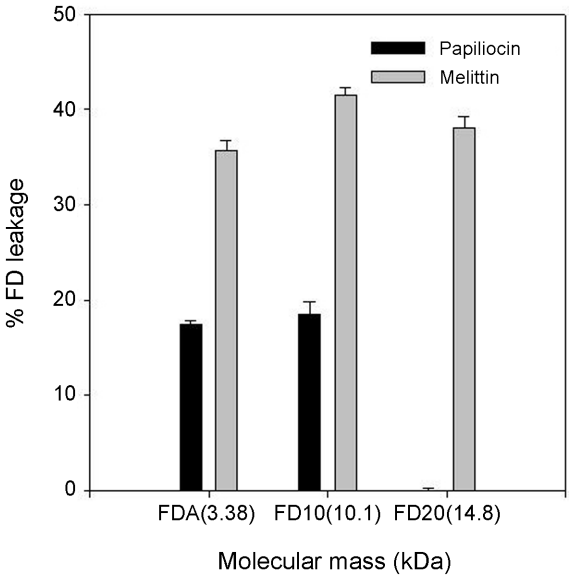

Fig. 3. Percentage of FITC-dextrans (FD) leakage induced by the peptides from PC/PE/PI/ergosterol [5:4:1:2 (w/w/w/w)] liposome. The error bars represent the standard deviation values for three independent experiments, performed in triplicate.

buffer I (1 ml of 50 mM potassium phosphate, pH 7.4, with 0.1 mM EDTA) containing 2 mg/ml of FD was sonicated for 30 min with 20 mg/ml of lipid [PC/PE/PI/ergosterol at 5:4:1:2 (w/w/w/w)] solution in chloroform, on ice. The chloroform was removed by using a rotary vacuum evaporator for 2 h at 25°C, resulting first in the formation of a viscous gel and then a liposome suspension. Buffer I (2 ml) was added and the suspension was evaporated further for the removal of eventual traces of chloroform. The liposome suspension was then centrifuged and washed for several cycles at 13,000 rpm for 30 min to remove unentrapped FD. For the assay, 10 μM of papiliocin or 5 μM of melittin were treated in a suspension of FD-loaded liposomes. The mixture (3 ml, final volume) was stirred for 10 min in the dark and then centrifuged at 13,000 rpm for 20 min. The supernatant was recovered and its fluorescence intensity was recorded by measuring at wavelengths λ_{ex}=494 nm, λ_{em}=520 nm, with an RF-5301PC spectrofluorophotometer (Shimadzu, Japan). Twenty μl of 10% Triton X-100 was added to the vesicles to determine 100% dye leakage [12, 17]. The percentage of dye leakage was calculated as follows: % dye leakage=$100\times(F-F_0)/(F_t-F_0)$, where F represents the fluorescent intensity achieved by the peptides treatment, and F_0 and F_t represent the fluorescent intensities without the peptides treatment and with Triton X-100 treatment, respectively. As displayed in Fig. 3, melittin released, on average, 35.64% of FD4, 41.45% of FD10, and 38.11% of FD20. Papiliocin released 17.46% of FD4 and 18.53% of FD10; however, it did not induce any release of FD20. This result indicated that papiliocin could make pores in the model membranes and that the radius of the pores was anywhere between 2.3 nm and 3.3 nm. Furthermore, this result also confirmed that papiliocin contained a membrane-disruptive mechanism.

In summary, the antifungal mechanism of action of papiliocin is suggested. Several studies indicate that papiliocin exerts its antifungal activity against *C. albicans* by the membrane-active mechanism. Although investigation of the exact mechanism is further required, this study suggests that papiliocin may be applied for the development of novel potent antifungal agents.

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