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# Induction of yeast apoptosis by an antimicrobial peptide, Papiliocin

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ABSTRACT

Papiliocin is a 37-residue peptide isolated from the swallowtail butterfly *Papilio xuthus*. In this study, we found that Papiliocin induced the accumulation of reactive oxygen species (ROS) and hydroxyl radicals known to be important regulators of apoptosis in *Candida albicans*. To examine the relationship between the accumulation of ROS and the induction of apoptosis, we investigated the apoptotic effects of Papiliocin using apoptotic markers. Cells treated with Papiliocin showed a series of cellular changes normally seen in cells undergoing apoptosis: plasma membrane translocation of phosphatidylserine from the inner to the outer membrane leaflet, measured by Annexin V staining, dissipation of the mitochondrial membrane potential, observed by $DiOC_6(3)$ staining; and the presence of active metacaspases, measured using the CaspACE FITC-VAD-FMK, as early apoptotic events. In addition, DNA condensation and fragmentation, which is important marker of late stage apoptosis, was seen by DAPI and TUNEL assay. Therefore, these results suggest that Papiliocin leads to apoptosis in *C. albicans* via ROS accumulation.



## 1. Introduction

A wide variety of organisms, such as bacteria, fungi, plants, insects, birds, crustacean, amphibians, mammals, and humans produce antimicrobial peptides as a major component of the natural host defense molecules [1]. Antimicrobial peptides are generally defined as having more than 100 amino acids with positively charged residues (net charge of +2 to +9) and amphiphilicity [2,3]. They exhibit an unusually broad spectrum of antimicrobial activity (against both Gram-negative and Gram-positive bacteria as well as fungi, eukaryotic parasites, enveloped viruses, and multidrug-resistant microorganisms) and do not easily induce resistance compared to conventional antibiotics [4]. Although the exact molecular mechanism of antimicrobial peptides remains unclear, it is generally accepted that interaction with the cytoplasmic membrane is a key step for antimicrobial activity resulting in increased permeability and leakage of cytoplasmic components [5,6]. Furthermore, several recent reports have suggested that antimicrobial peptides, such as plant defensin RsAFP2 [7], human lactoferrin [8], and bee venom melittin [9], exert their antimicrobial properties by promoting apoptosis in the human fungal pathogen *Candida albicans* [10].

Apoptosis is a highly regulated cellular suicide program, which is crucial for development and homeostasis in metazoan organisms, resulting in the removal of unwanted, mutated, damaged, or simply dispensable cells without an inflammatory reaction [11,12]. However, it became evident that apoptosis might not only occur in multicellular, but also in unicellular organisms [13]. In yeast, apoptotic cells show typical apoptotic markers as higher eukaryote apoptotic cells, such as externalization of phosphatidylserine, accumulation of ROS, chromatin condensation and fragmentation, and degradation of the DNA [14].

Papiliocin (RWKIFKKIEKVGRNVRDGIIKAGPAVAVVGQAATVVK-$NH_2$), a 37-residue antimicrobial peptide, was derived from the swallowtail butterfly *Papilio xuthus* larvae. This peptide was shown to contain significant antimicrobial activities against several human pathogenic bacterial and fungal strains [15]. Our study investigated whether Papiliocin could exert an antifungal effect by inducing apoptosis as another mechanism in *C. albicans*.

## 2. Materials and methods

### 2.1. Peptide synthesis

Peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry [16,17]. The

*Abbreviations:* DHR, dihydrorhodamine; HPF, hydroxyphenyl fluorescein; FITC, fluorescein isothiocyanate; PI, propidium iodide; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; DAPI, 4′-6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling.

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assembled peptide was repeatedly washed with diethyl ether and dissolved in 10 ml of 0.1 mM ammonium bicarbonate, 50 ml of $H_2O$, and 50 ml of acetonitrile. The mixture was dried under a vacuum after salts were excluded and was purified using reversed-phase preparative HPLC on a Waters 15-μm Delta Pak $C_{18}$ Column (19 × 30 cm). The purity of the peptide was checked by analytical reversed-phase HPLC on an Ultrasphere $C_{18}$ column, (4.6 × 25 cm) (Beckman, Fullerton, CA, USA). The molecular weight of the synthetic peptide was determined using a matrix-assisted laser desorption ionization (MALDI) mass spectrometer [18,19].

### 2.2. Intracellular ROS measurements

Intracellular ROS production was determined by incubating the cells ($2 \times 10^8$/ml) with 30 μM Papiliocin or 2.5 mM $H_2O_2$ for 2 h at 28 °C, followed by incubation with 5 μg/ml DHR-123 at the end of each experiment. Samples were quantitatively analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Intracellular hydroxyl radical (OH·) accumulation was measured by incubating the cells with Papiliocin or $H_2O_2$ in PBS containing 5 μM HPF for 2 h at 28 °C. Subsequently, the cells were washed twice in PBS and analyzed by flow cytometry.

### 2.3. Annexin V staining

Protoplasts of *C. albicans* were stained with FITC-labeled Annexin V and PI using a FITC-Annexin V apoptosis detection kit. *C. albicans* cells were harvested by centrifugation and digested with a lysis enzyme (20 mg/ml) in potassium phosphate buffer (PPB, 1 M sorbitol, pH 6.0) for 2 h at 28 °C. Protoplasts were incubated with Papiliocin or $H_2O_2$ for 2 h at 28 °C. Subsequently, the cells were washed with PPB and incubated in Annexin binding buffer containing 5 μl/ml of FITC-Annexin V and 5 μl of PI for 20 min. Cells were then analyzed by flow cytometry.

### 2.4. Measurement of mitochondrial membrane potential and metacaspase activation

Fungal mitochondrial plasma membrane depolarization was analyzed by $DiOC_6(3)$ staining. *C. albicans* cells ($2 \times 10^8$/ml) were harvested and incubated with Papiliocin or $H_2O_2$ for 2 h at 28 °C. Subsequently, the cells were washed with PBS, and incubated with 2 ng of $DiOC_6(3)$ for 30 min. Cells were analyzed by flow cytometry.

Metacaspase activity was measured using CaspACE FITC-VAD-FMK *In Situ* Marker (Promega) and analyzed by flow cytometry.

### 2.5. Analysis of late apoptotic markers

Nuclear condensation and fragmentation were analyzed by DAPI staining. For nuclear staining, cells were washed twice with PBS, permeabilized, and incubated in the dark with 1 μg/ml of DAPI for 20 min. Cells were then examined by fluorescence microscopy.

DNA strand breaks in *C. albicans* cells were analyzed by the TUNEL method [38]. Cells were washed in PBS, permeabilized in a permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) on ice, and washed with PBS. DNA ends were labeled with an *in situ* cell death detection kit for 1 h at 37 °C. The stained cells were examined by fluorescence microscopy, (Axio Imager A1 and Axio Cam MR5; Carl Zeiss).

## 3. Results

### 3.1. Papiliocin induces an increase in the total intracellular levels of ROS, specifically hydroxyl radicals

Recent studies suggest that the accumulation of ROS induces and regulates the apoptotic pathway in yeast [20,21]. To confirm the production and accumulation of intracellular ROS induced by Papiliocin, we utilized the ROS sensitive dye DHR-123, which has been used as a general indicator of cellular ROS levels [22]. Multiple ROS directly oxidize DHR-123 to a highly stable, fluorescent derivative rhodamine-123 [23] in such a way that

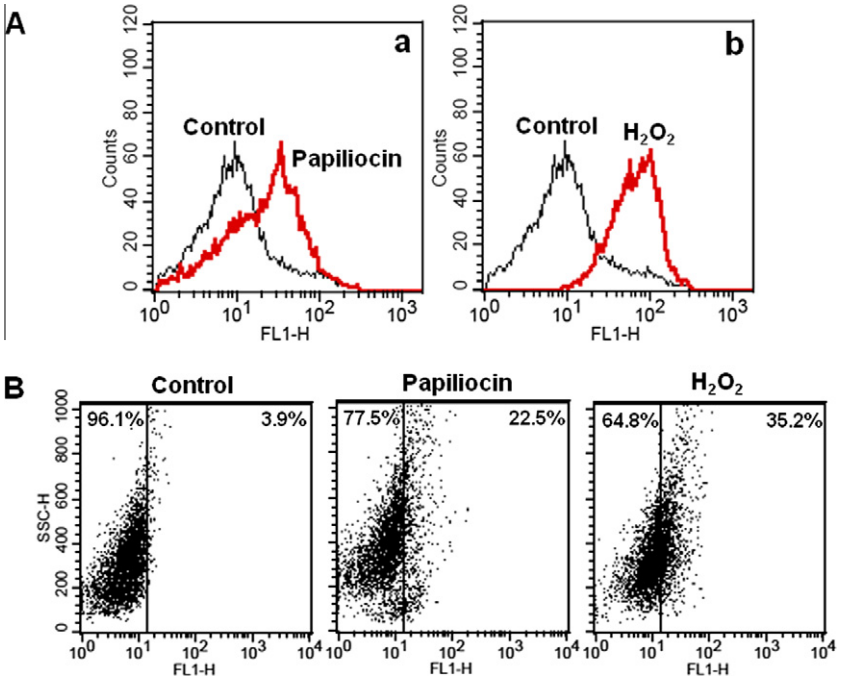


**Fig. 1.** Flow cytometric analysis showing the increase of ROS generation. (A) Histograms show the fluorescence intensity of stained DHR-123: (a) Papiliocin-treated cells and (b) $H_2O_2$-treated cells. (B) The fluorescence intensities of stained HPF after treatment with Papiliocin or $H_2O_2$ for different cell populations.

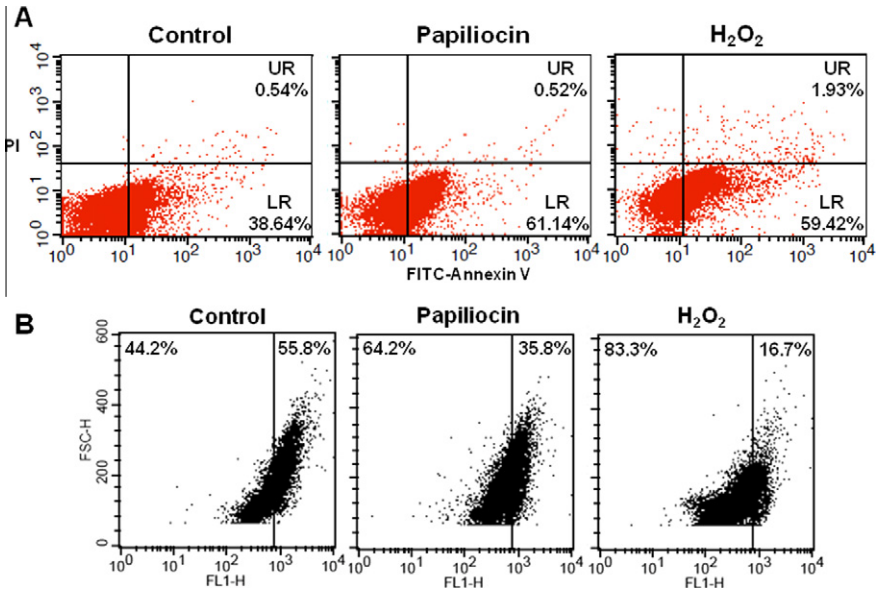


**Fig. 2.** Effect of Papiliocin on externalization of phosphatidylserine at the cytoplasmic membrane and mitochondrial membrane depolarization. (A) Phosphatidylserine externalization shown by FITC-Annexin V staining in Papiliocin- or $H_2O_2$-treated *C. albicans* cells. Cells were measured with a flow cytometer. (B) Flow cytometric analysis of mitochondrial membrane depolarization. Cells were stained with $DiOC_6(3)$.

an increase in the fluorescent signal reflects the ROS production. As shown in Fig. 1A, cells treated with Papiliocin exhibited high ROS levels compared to the untreated cells. There was also a significant increase in fluorescence when the cells were treated with 2.5 mM $H_2O_2$. To confirm that these ROS are involved in the intracellular accumulation of reactive hydroxyl radicals known to be common mediators of apoptosis [24], we used the fluorescent probe HPF, which is oxidized by hydroxyl radicals with high specificity [25]. In proportion to the intracellular ROS accumulation after Papiliocin treatment, the level of intracellular hydroxyl radicals also increased in Papiliocin-treated cells (Fig. 1B). These findings indicate that ROS induced by Papiliocin accumulated in the interior of *C. albicans* cells, and most were converted into strong oxidant hydroxyl radicals, which are considered crucial factors in aging and in apoptosis of yeast cells [26]. Thus, further investigations of the antifungal mechanisms of Papiliocin, such as the exposure of phosphatidylserine at the outer surface, were necessary following the confirmation of the apoptotic features in *C. albicans* cells.

### 3.2. Papiliocin induces exposure of phosphatidylserine at the cytoplasmic membrane and mitochondrial membrane depolarization

Early stage apoptosis can be determined with Annexin V-FITC stain, which binds to phosphatidylserine with high affinity in the presence of $Ca^{2+}$ [13], combined with the membrane-impermeable dye PI. As shown in Fig. 2A, the cell population in the lower-right (LR) quadrant, which corresponds to the percentage of early apoptotic cells (Annexin V-positive and PI-negative), increased to 61.14 ± 2% and 59.42 ± 1.7%, respectively, and the percentages for late apoptosis (UR) were 0.52 ± 0.2% and 1.94 ± 0.4% after treating the cells with Papiliocin and $H_2O_2$ for 1 h, respectively. The results indicate that Papiliocin induced apoptotic cell death in *C. albicans* cells. Hence, it was confirmed that the generation and accumulation of intracellular ROS induced by Papiliocin, specifically hydroxyl radicals, was linked to a ROS-dependent apoptotic mechanism in *C. albicans* cells.

To investigate whether Papiliocin can induce a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$), $\Delta\Psi_m$ was measured using a mitochondria-specific voltage-dependent dye, $DiOC_6(3)$, which aggregates into healthy mitochondria and fluoresces green. When the mitochondrial membrane depolarizes, the dye no longer accumulates and instead is distributed throughout the cell resulting in a decrease in green fluorescence [27]. As shown in Fig. 2B, treatment with Papiliocin resulted in a significant decrease in the $\Delta\Psi_m$, in accordance with the pattern induced by experimentally applied $H_2O_2$ used as a positive control. The decrease in the $DiOC_6(3)$ fluorescent signal could reflect the depolarization of the mitochondrial membrane. Therefore, the results suggest that Papiliocin could induce the breakdown of $\Delta\Psi_m$ and the loss of mitochondrial permeability.

### 3.3. Papiliocin induces the activation of metacaspases

To monitor the activation of endogenous metacaspases in *C. albicans* cells treated with Papiliocin, cells were incubated *in vivo* with FITC-labeled VAD-FMK (FITC-VAD-FMK), which binds specifically to the active center of metazoan caspases and yeast metacaspases [28]. As shown in Fig. 3, cells treated with Papiliocin showed a significant increase in fluorescent FITC-VAD-FMK-loaded cells consistent with the positive control cells that were treated with $H_2O_2$. These results suggest that Papiliocin treatment did initially lead to significant generation of strong oxidant hydroxyl radicals, which are well known to be important regulators of yeast apoptosis, proportional to the accumulation of intracellular ROS. Therefore, Papiliocin induces the feature of mitochondria-mediated apoptosis in *C. albicans* cells, including membrane depolarization and metacaspase activation.

### 3.4. Papiliocin induces DNA damage, a feature of a late apoptosis

To investigate whether Papiliocin displays features of the late-stages of apoptosis in *C. albicans*, we evaluated DNA damage, such as nuclear DNA condensation and fragmentation, visualized by DAPI and TUNEL assay, respectively. Fig. 4A shows that *C. albicans* cells exposed to Papiliocin exhibited a significant amount of changes in the nuclear DNA, as assessed by the DNA-binding fluorescent dye DAPI. A strong blue fluorescence (Fig. 4A, b) indicated a

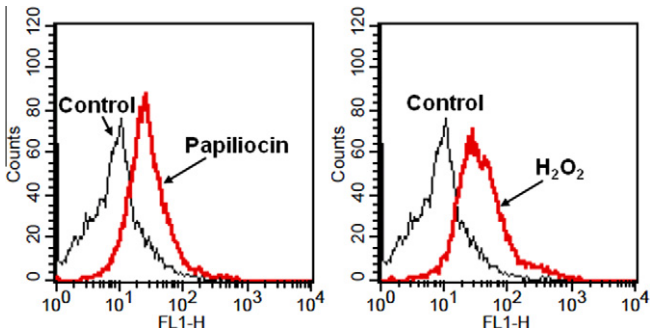


**Fig. 3.** Effect of Papiliocin on the activity of metacaspase in *C. albicans* cells. Cells were stained with FITC-VAD-FMK, and analyzed by flow cytometry.

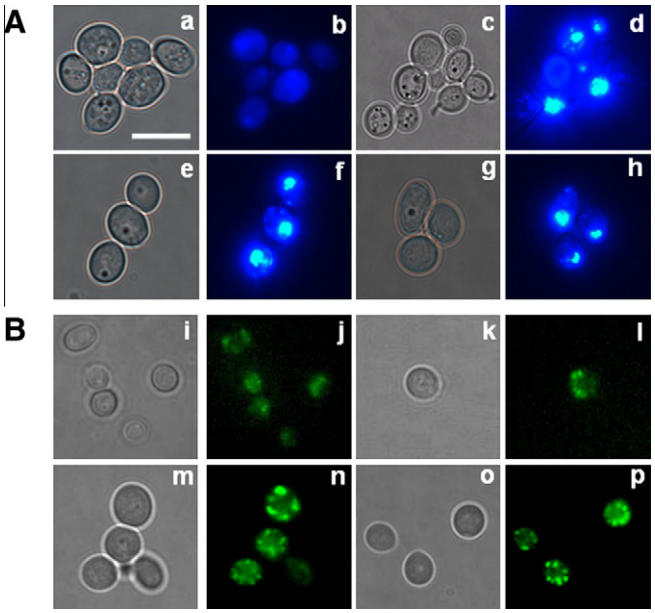


**Fig. 4.** DNA and nuclear fragmentation shown by DAPI and TUNEL staining. Representative micrographs showing cells stained with DAPI (A) and TUNEL (B). Subpanels a and b are the controls. The cells were treated with Papiliocin (c, d, and i–l) or $H_2O_2$ (e–h and m–p). Bar, 10 μm.

greater degree of typical apoptotic DNA condensation and fragmentation in the nuclei of *C. albicans* cells exposed to Papiliocin than in the intact nuclei of normal control cells. Similar results were obtained by TUNEL assay, which stains nuclear DNA strand breaks at the late-stages of apoptosis. In cells exposed to the peptide Papiliocin (Fig. 4B, i–l), the proportion of TUNEL-positive nuclei was significantly increased compared to that of the untreated control population (data not shown). At the same time, cells exposed to 2.5 mM $H_2O_2$ revealed both an increase in TUNEL-positive nuclei and intensive green fluorescence spots (Fig. 4B, m–p). These observations mean that exposure of *C. albicans* cells to Papiliocin results in apoptotic DNA damage, which is considered an important phenomenon of late stage apoptosis in yeast.

## 4. Discussion

In this study, we found that the total cellular levels of ROS in *C. albicans* were increased by Papiliocin following the generation of hydroxyl radicals. This phenomenon specifically accompanies apoptotic indicators in *C. albicans* cells. ROS, such as $O_2^{-\cdot}$, $H_2O_2$ and $OH^{\cdot}$, are considered to be crucial regulators of aging, and their accumulation has been proven to play a key role in apoptosis in metazoans and yeast [29]. Apoptosis, a physiological mode of cellular death for the development and homeostasis of metazoan organisms, can be induced by various intra- or extracellular stimuli and via several signaling pathways depending on cell type, cellular environment, and differentiation state [30]. In mammals, apoptotic cells are characterized by a specific series of morphological and biochemical properties, such as the exposition of phosphatidylserine on the cell surface, the characteristic condensation of chromatin to the nuclear envelope, DNA fragmentation, and the formation of membrane-enclosed cell fragmentation called apoptotic bodies [20]. These obviously altruistic functions have recently been described for unicellular organisms. In yeasts, as in mammalian cells, phosphatidylserine is distributed asymmetrically on the inner leaflet of the plasma membrane, and is translocated from the inner to the outer leaflet at the early stages of apoptosis [31]. Annexin V-FITC labeling visualization in *C. albicans* suggests that there is a more fundamental connection between the accumulation of ROS induced by Papiliocin and phosphatidylserine exposure as a typical event of apoptosis (Fig. 2A). Another well-known, characteristic feature of an early cellular event in many cases of apoptosis is the reduction of the mitochondrial membrane potential ($\Delta\Psi_m$), which is induced by a variety of proapoptotic stimuli including withdrawal of growth factors and chemotherapeutic drug treatment [32,33]. Disruption of the $\Delta\Psi_m$ usually leads to the opening of the transition pore of the mitochondrial membrane and releases apoptogenic factors into the cytosol [34]. Metacaspases, which are well-known, apoptogenic factors of yeast, are the functional homologs of metazoan caspases (cysteine-dependent aspartate-specific proteases), which induce apoptosis by cleaving key cellular substrates and plays a role in programmed cell death in yeast [35,36]. Interestingly, the results of the mitochondria-mediated experiments seem to suggest that the Papiliocin-induced apoptotic pathway leads to the disruption of the mitochondria integrity (Fig. 2B) and induction of metacaspase activity (Fig. 3). Furthermore, apoptotic chromosomal DNA condensation and fragmentation are often an integral part of apoptosis in higher organisms [30]. This condensation and fragmentation were detected by a DAPI staining method, but also by the *in situ* TUNEL assay, which detects the exposed free 3′-OH groups in DNA (Fig. 4A and B). The labeling and visualization of DNA breaks by TUNEL is a common method for the identification of apoptotic cells [37]. Using diagnostic assays for detecting apoptotic features, we confirmed that the exposure of *C. albicans* cells to Papiliocin resulted in apoptotic physiological changes via the accumulation of intracellular ROS, specifically hydroxyl radicals at the initial step of the Papiliocin-induced apoptotic pathway.

In this study, the antifungal mode of action of Papiliocin, a 37-residue antimicrobial peptide derived from *P. xuthus* larvae, was investigated. The collective data presented in this study indicates that Papiliocin caused apoptosis in *C. albicans* cells representing several key markers of yeast apoptosis, including an increase of ROS generation, phosphatidylserine externalization, a reduction in the mitochondrial membrane potential, and the presence of active metacaspase and DNA condensation and fragmentation. The apoptotic pathway induced by Papiliocin has not been completely established, but these results support the antifungal property of Papiliocin by promoting apoptosis in *C. albicans*.

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