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Roles of the Hsp90-Calcineurin Pathway in the Antifungal Activity of Honokiol S

Kai Liao[1] and Lingmei Sun[2*]

[1]*Department of Pathology and Pathophysiology, Medical School of Southeast University, Nanjing 210009, P.R. China*
[2]*Department of Pharmacology, Medical School of Southeast University, Nanjing 210009, P.R. China*

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*Corresponding author
Phone: +86-25-83272525;
Fax: +86-25-83272525;
E-mail: sunlingmei@seu.edu.cn

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Honokiol, a bioactive compound isolated from the cone and bark of *Magnolia officinalis*, has been shown to have various activities including inhibition of the growth of *Candida albicans*. We investigated the roles of the Hsp90-calcineurin pathway in the antifungal activity of honokiol. The pharmacologic tool was employed to evaluate the effects of Hsp90 and calcineurin in the antifungal activity of honokiol. We also evaluated the protective effects of the calcineurin inhibitor cyclosporin A (CsA) on honokiol-induced mitochondrial dysfunction by the fluorescence staining method. The Hsp90 inhibitor potentiated the antifungal activity of honokiol. A *C. albicans* strain with the calcineurin gene deleted displayed enhanced sensitivity to honokiol. However, co-treatment with calcineurin inhibitor CsA attenuated the cytotoxic activity of honokiol due to the protective effect on mitochondria. Our results provide insight into the action mechanism of honokiol.

Keywords: *Candida albicans*, honokiol, Hsp90, calcineurin, cyclosporin A, mitochondria

Introduction

The heat shock response is considered to be among the most fundamentally important and ubiquitous stress responses in nature [1]. Many heat shock proteins (HSPs) are molecular chaperones that play an important role in promoting the folding, assembly, or cellular localization of client proteins [1]. Hsp90, a specialized chaperone that regulates the form and function of many key signal transducers, enables the emergence and maintenance of drug resistance in diverse fungal species [2]. Inhibition of Hsp90 reverses drug resistance in diverse human fungal pathogens, rendering resistant infections responsive to antifungal treatment. Hsp90 function can be abrogated by natural products, such as geldanamycin (GdA) and radicicol, as well as by diverse chemical scaffolds that have been developed to target Hsp90's key role in enabling malignant transformation [3]. Hsp90 regulates drug resistance by stabilizing the protein phosphatase calcineurin, which is the target of the natural products and immunosuppressants tacrolimus (FK506) and cyclosporin A (CsA) [3].

Calcineurin is a heterodimer composed of a catalytic and a regulatory subunit [4, 5]. It is also known as protein phosphatase 2B and is activated through the binding of Ca^{2+}-calmodulin (CaM) [4]. Among the known serine/threonine protein phosphatases, calcineurin is the only phosphatase that requires Ca^{2+} and CaM for its enzymatic activity and exhibits restricted substrate specificity [4, 5]. Calcineurin is a target of the immunosuppressive agents FK506 and CsA, mediated via their respective immunophilins, FK-binding protein 12 (FKBP12) and cyclophilin A [4, 5]. Calcineurin inhibitors are attractive as new antifungal agents owing to their specific mode of action from other antifungal classes (polyenes, triazoles, and echinocandins) that would target the top of a critical cell signaling pathway, efficacy against emerging azole- and echinocandin-resistant strains, and synergistic nature with existing antifungals, such as the echinocandin antifungal caspofungin [6–8].

Honokiol, a natural product found in the cone and bark of *Magnolia officinalis*, has various activities and has been used in traditional medicine to treat anxiety, thrombosis, and emesis [9]. Honokiol has been found to target the

mitochondrial respiratory chain complex I (CI), inducing reactive oxygen species (ROS) accumulation, disruption of intracellular redox homeostasis, irreversible oxidative modifications of lipid, protein, or DNA, and activation of autophagy or the apoptosis signaling pathway [10, 11]. In the present work, we investigated the roles of the Hsp90-calcineurin pathway in the antifungal activity of honokiol. Findings from this study will be helpful to understand the action mechanism of honokiol against *Candida albicans*.

Materials and Methods

Materials

Honokiol (5,5′-diallyl-2,4′-dihydroxybiphenyl) was obtained from Xi'an Yuquan Biological Technology Co., Ltd (China) and its purity was over 98% as analyzed by high-performance liquid chromatography. GdA and CsA were purchased from Shanghai Aladdin Bio-Chem Technology Co., LTD (China) and their purities were over 98%. Glusulase, lyticase, DCFH-DA (2′,7′-dichlorofluorescein diacetate), JC-1 (5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolocarbocyanine iodide), and other molecular-grade chemicals were obtained from Sigma Chemicals (USA).

Microorganisms

DAY364 (Δ*cnb1*/Δ*cnb1*, mutant strain lacking the calcineurin B regulatory subunit) and MCC85 (Δ*cnb1*/Δ*cnb1*+*CNB1*, mutant strain with a constitutive calcineurin B) *C. albicans* strains were cultured in YPD (yeast extract/peptone/dextrose) broth [4]. YPD-uracil (100 ml of YPD plus 400 µl of 0.1 mol/l uracil) was used as an agar (2%) or liquid medium for culturing wild-type (WT) strain CAI4. The strains were stored as frozen stock with 15% (v/v) glycerol at –80°C. Before each experiment, cells were freshly revived on YPD plates from the stock.

Quantification Analysis by Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

C. albicnas WT strain CAI4 was grown overnight in YPD medium and diluted to a cell density of 1.0×10^7 cells/ml. The cells were incubated at 30°C for 4 h followed by centrifugation at 4°C. The total RNAs were isolated using the hot phenol method [10]. Approximately 1 µg of total RNA was used to synthesize cDNA using AMV reverse transcriptase (Promega, USA). Primer sequences for the housekeeping gene 18S rRNA and *HSP90* are shown in Table S1. The qRT-PCR and data analysis were performed as previously described [10].

Sensitivity Determination

The sensitivity of *C. albicans* toward the compounds was tested by the broth microdilution method according to CLSI standard M27-A3 [12].

Measurement of ROS Generation

C. albicans WT strain CAI4 was grown overnight in YPD medium and diluted to a cell density of 1.0×10^7 cells/ml. The cells were incubated at 30°C for 4 h. Cellular ROS generation was evaluated by flow cytometry (Becton-Dickinson Immunocytometry Systems, USA) with DCFH-DA staining as described previously [13, 14].

Analysis of Early Markers of Apoptosis

C. albicans CAI4 cells were treated with honokiol (60 µM) with or without CsA (10 µM) for 4 h. The cells were harvested and washed with sorbitol buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM potassium phosphate, pH 6.8), and then digested with 2% glusulase and 15 U/ml lyticase in sorbitol buffer for 2 h at 28°C. Protoplasts were harvested by centrifugation at 1,000 ×*g* for 10 min. The protoplasts of *C. albicans* were stained with the Annexin V FITC apoptosis detection kit (Beyotime Biotechnology, China) to assess cellular integrity and the externalization of phosphatidylserine (PS) as described earlier [10].

Mitochondrial Membrane Potential (mtΔψ)

The mtΔψ is an important parameter of mitochondrial function and an indicator of cell health. For determinations of mtΔψ, the fluorescent dye JC-1 was used and the method was performed as described previously [10].

Cytochrome *c* (Cyt*c*) Release

Isolation of mitochondria was performed according to the published literature [15]. Cells grown in YPD broth at 30°C to early stationary phase were diluted to 1×10^7 cells/ml with fresh YPD broth and incubated with different concentrations of compounds for 4 h. After centrifugation, the pellet was resuspended in homogenization buffer (50 mM Tris, pH 7.5, 2 mM EDTA, and 1 mM phenylmethysulfonyl fluoride). Glass beads (0.45 ± 0.5 mm diameter) were added to this suspension, and then homogenized using a FastPrep homogenizer (Fastprep FP120; Savant Instruments, USA). After that, the homogenization was supplemented with 2% glucose and centrifuged at 2,000 ×*g* at 4°C for 5 min to remove the unbroken cells and glass beads. The supernatants were collected and centrifuged at 30,000 ×*g* for 45 min, and the supernatant was used for assay of Cyt*c* released from the mitochondria to the cytoplasm. The pellet was used for the determination of Cyt*c* remaining in the mitochondria. The protein quantity was estimated with a BCA protein assay kit (Beyotime Biotechnology, China). Mitochondria were suspended at 2 mg/ml. After being reduced by 500 mg/ml ascorbic acid at room temperature for 5 min, the quantities of Cyt*c* in the supernatants and mitochondria were determined by measuring the absorbance at 550 nm with the BioTek Synergy 4 microplate reader (BioTek Instruments Inc., USA).

Statistical Analysis

All data were presented as the mean ± standard error of the mean (SEM). Graphs were generated using Microsoft Excel (Microsoft Corp., USA). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., USA). Differences between groups were determined

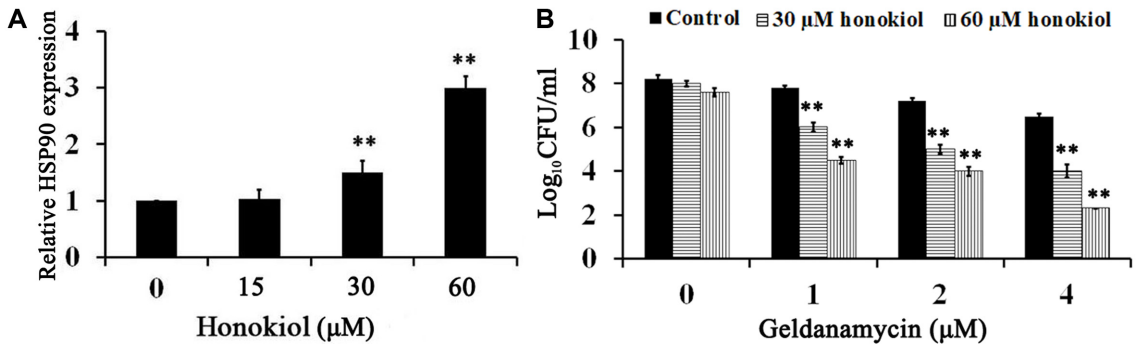

Fig. 1. Hsp90 plays a crucial role in the honokiol tolerance of *C. albicans*.
(**A**) *HSP90* transcript levels increase following honokiol exposure. (**B**) GdA increased the sensitivity of *C. albicans* to honokiol. $^{**}p < 0.01$.

using analysis of variance. A p value <0.05 was considered statistically significant.

Results

Hsp90 Plays a Crucial Role in the Honokiol Tolerance of *C. albicans*

Under normal conditions, molecular chaperones such as Hsp90 have numerous roles in cellular processes, including normal protein folding during translation, refolding of accumulated proteins, and the regulation of protein degradation. In response to various environmental stresses, Hsp90 is upregulated to refold damaged molecules and/or to prevent their irreversible aggregation with other proteins. Given Hsp90's role in azole and echinocandin resistance, we postulated that Hsp90 might also be required for the basal tolerance of *C. albicans* to honokiol. In this study, we observed elevated expression of *HSP90* in response to honokiol exposure in a concentration-dependent manner (Fig. 1A). Inhibitors of Hsp90 have been shown to diminish calcineurin signaling in *Saccharomyces cerevisiae* and to synergistically inhibit growth in combination with azoles. To determine the impact of compromising Hsp90 function on tolerance to honokiol, we used the inhibitor GdA that binds with high affinity to Hsp90's unusual adenosine triphosphate (ATP)-binding pocket and inhibits ATP-dependent chaperone function. We used the concentration of GdA (1 μM) that abrogates resistance to honokiol, but has no impact on growth on its own. When GdA was used at 2 or 4 μM, it slightly suppressed cell growth. As shown in Fig. 1B, pharmacological impairment of Hsp90 by increasing concentrations of GdA increased the antifungal activity of honokiol as expected. The findings suggest that Hsp90 plays a crucial role in the honokiol tolerance of *C. albicans*.

Calcineurin Mutant Is Sensitive to Honokiol

Hsp90 regulates drug resistance by stabilizing the protein phosphatase calcineurin. Compromising calcineurin function phenocopied compromising Hsp90 function. To test if calcineurin is involved in the toxic action of honokiol, we tested calcineurin mutant (Δ*cnb1*/Δ*cnb1*) and calcineurin-reconstituted (Δ*cnb1*/Δ*cnb1*+*CNB1*) strains. The calcineurin mutant was more sensitive to honokiol than the WT or calcineurin-reconstituted strains (Fig. 2). The MIC_{50} value of honokiol for *cnb1/cnb1* was 15 μM, whereas for the WT or *cnb1/cnb1+CNB1* strain it was 60 μM. These results suggested that the key mediator of Hsp90-dependent honokiol resistance is calcineurin.

An Inhibitor of Calcineurin Attenuates the Antifungal Activity of Honokiol

CsA is a natural product of bacteria and fungi, respectively, with potent immunosuppressiveand antimicrobial activities. Despite differing chemical structures, their mechanisms of

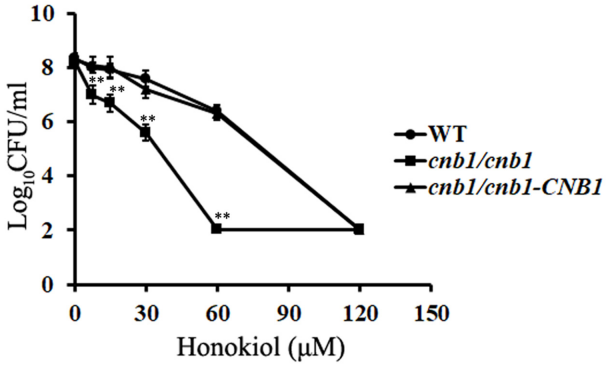

Fig. 2. Mutation of calcineurin potentiated the antifungal activity of honokiol.
Test strains: (■) CAI4, (▲) DAY364 (Δ*cnb1*/Δ*cnb1*), (●) MCC85 (Δ*cnb1*/Δ*cnb1*+*CNB1*). $^{**}p<0.01$.

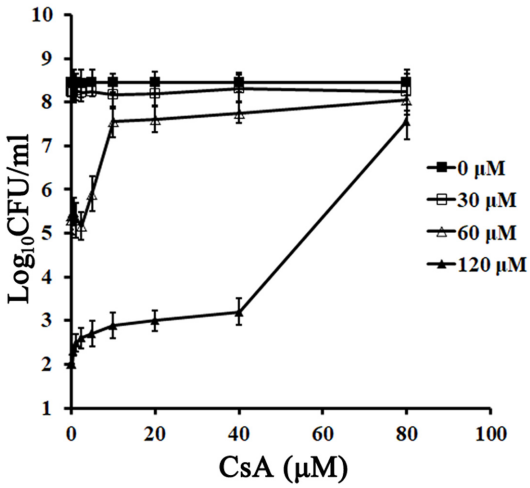

Fig. 3. Attenuation of honokiol-induced cytotoxicity by cyclosporin A (CsA) against *C. albicans* CAI4.
Test concentration: (■) control, (□) 30 μM honokiol, (△) 60 μM honokiol, (▲) 120 μM honokiol.

action and cellular effects are very similar, resulting in the inhibition of the protein phosphatase calcineurin. We next examined whether CsA potentiates the antifungal activity of honokiol by targeting calcineurin. CsA exhibited no activity against *C. albicans* when grown under the tested concentrations, which was consistent with data reported in the literature [16]. Contrary to expectations, increasing the immunosuppressive drug CsA did not increase the death of *C. albicans* cells treated with honokiol and instead prevented the death of the cells (Fig. 3).

CsA Reduces Honokiol-Induced Apoptosis in *C. albicans*

It is believed that honokiol treatment results in higher levels of apoptosis in *C. albicans* [10]. We next examined whether CsA attenuates the antifungal activity of honokiol by inhibiting apoptosis, using FITC Annexin V and PI staining. In apoptotic cells, the membrane phospholipid PS translocated from the cytosolic side of the plasma membrane to the cellular surface [17]. Annexin V binds to exposed apoptotic cell surface PS with high affinity, which can be used as a marker of early apoptosis [17]. Cells in the vehicle (DMSO) and CsA treatment groups were almost hardly stained by FITC Annexin V and PI as evaluated by flow cytometric analysis. Honokiol (60 μM) treatment induced externalization of PS after 4 h incubation, whereas co-treatment with CsA significantly decreased early apoptotic cells from 27.9% ± 5.9% to 3.6% ± 0.8%, indicating that CsA

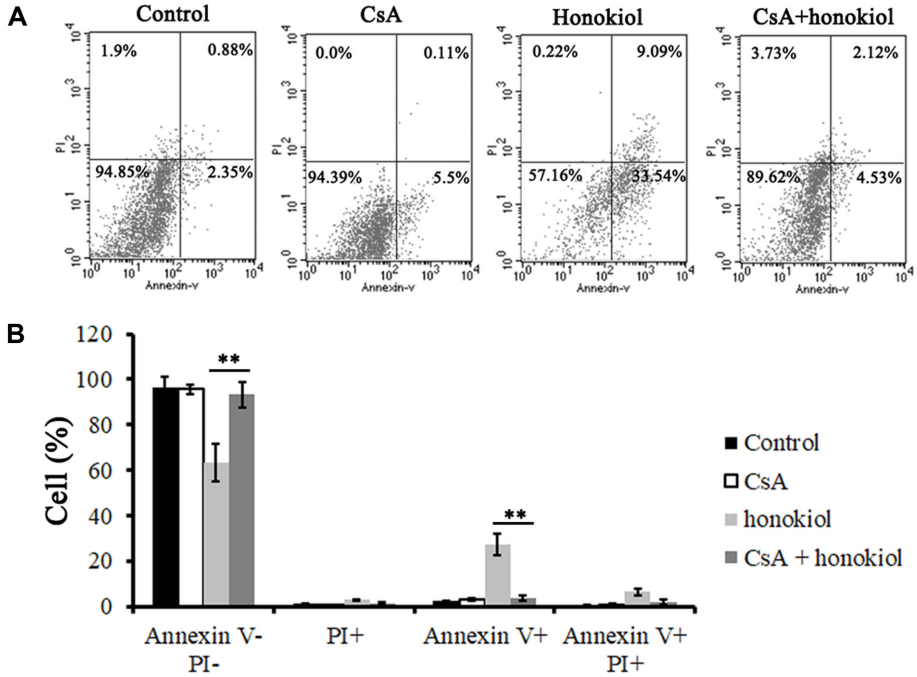

Fig. 4. Attenuation of honokiol-induced apoptosis of *C. albicans* CAI4 by cyclosporin A (CsA).
(**A**) Cell apoptosis was analyzed by flow cytometry. (**B**) Percentages of necrotic, and early and late apoptotic cells. *C. albicans* CAI4 cells were treated with 10 μM CsA, 60 μM honokiol, or their combination for 4 h, and then stained with annexin V-FITC and PI. Annexin positive: early apoptosis; annexin and PI positive: late apoptosis; PI positive: necrosis. $^{**}p < 0.01$.

prevented honokiol-induced apoptosis (Fig. 4).

CsA Impeded Honokiol-Induced Mitochondrial Dysfunction

ROS generation is an important factor responsible for the fungicidal activity of honokiol [10]. The mitochondrion is the major source of ROS, especially superoxide anions, which are highly involved in mitochondrial dysfunction. We further evaluated whether treatment with CsA could decrease ROS generation induced by honokiol in *C. albicans*. As expected, treatment with honokiol induced ROS generation in a concentration-dependent manner, whereas co-treatment with CsA (10 μM) largely suppressed honokiol-induced ROS generation (Figs. 5A and 5B).

It is reported that CsA is a potent inhibitor of mitochondria permeability transition pore (mPTP), a protein that is formed in the inner membrane of the mitochondria [18, 19]. Induction of mPTP can lead to mitochondrial swelling and cell death through apoptosis or necrosis [19]. Previous reports have shown that honokiol-induced mitochondrial dysfunction was related to apoptosis [10, 11]. Next, we examined whether the protective effect of CsA on *C. albicans* was dependent on mitochondrial functions. JC-1 staining showed that CsA treatment inhibited the honokiol-induced decrease in mtΔψ (Fig. 6A). Cyt*c*, a component of the electron transport chain, is a water-soluble mitochondrial intermembrane-space protein loosely attached to the inner mitochondrial membrane. The release of Cyt*c* from mitochondria to the cytosol, where it activates the caspase family of proteases, is believed to be a primary trigger leading to the onset of apoptosis [20]. Measuring the amount of Cyt*c* leaking from the mitochondria to the cytosol, or out of the cell to the culture medium, is a sensitive method for monitoring the degree of apoptosis [21]. Further studies showed that there was a significant accumulation of Cyt*c* in the cytosol after incubation of the cells with honokiol (60 μM), whereas mitochondrial Cyt*c* was reduced by about 56% ($p < 0.01$) (Fig. 6B). Next, we evaluated whether CsA (10 μM) prevents the mitochondrial Cyt*c* release induced by honokiol (60 μM). As expected, treatment with CsA inhibited the increase of Cyt*c* (cytosolic fraction) induced by honokiol (Fig. 6B).

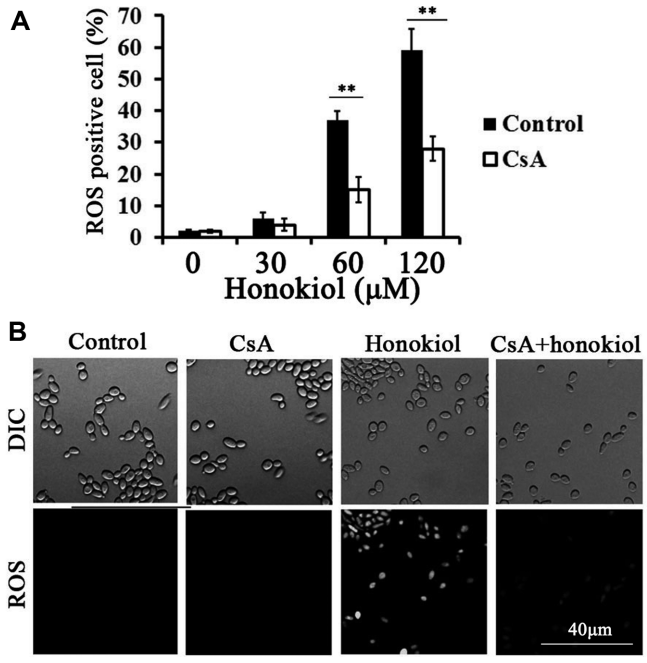

Fig. 5. Treatment with cyclosporin A (CsA) (10 μM) decreased ROS generation induced by honokiol in *C. albicans* CAI4.
(**A**) The percentages of cells that produced ROS in honokiol-treated *C. albicans* as measured by flow cytometry. (**B**) ROS induction in *C. albicans* cells treated with honokiol (60 μM) or CsA (10 μM) as observed by fluorescence microscopy. $^{**}p < 0.01$.

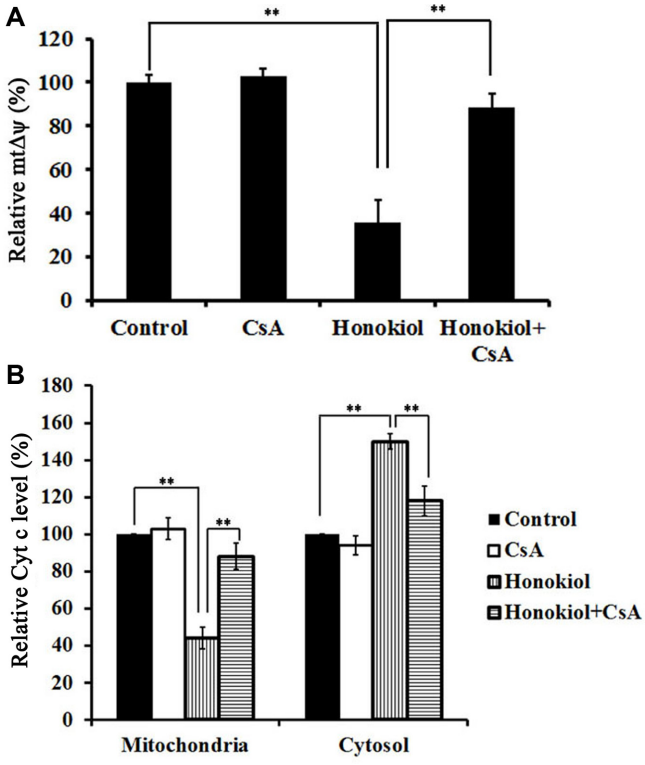

Fig. 6. Treatment with cyclosporin A (CsA) prevented mitochondrial injury induced by honokiol in *C. albicans* CAI4.
(**A**) The effect of CsA (10 μM) on the honokiol (60 μM)-induced collapse of mtΔψ in *C. albicans*. (**B**) The effect of CsA (10 μM) on honokiol (60 μM)-induced Cyt*c* release from mitochondria in *C. albicans*. $^{**}p < 0.01$.

Discussion

C. albicans is the most frequently encountered *Candida* species in the clinic and is the fourth most common cause of hospital-acquired infectious diseases, with mortality rates approaching 50% [22]. Treatment of invasive fungal infections remains notoriously challenging, due in large part to the limited availability of clinically useful antifungal drugs [1].

Natural products have provided an unparalleled source of therapeutic agents that have revolutionized modern medicine [23]. Honokiol, a natural product found in the cone and bark of *M. officinalis*, has various activities and has been used in traditional medicine to treat anxiety, thrombosis, and emesis [24, 25]. Honokiol has been found to have a remarkable inhibitory effect on *C. albicans* via targeting of the mitochondrial respiratory chain CI, resulting in ROS accumulation and mitochondrial dysfunction [10, 11]. Hsp90 is a molecular chaperone that is induced by stress in eukaryotes and regulates the folding and transport of client proteins [2]. In fungi, Hsp90 has been demonstrated to mediate drug (such as azole) resistance and biofilm formation in diverse fungal species [22]. In the present study, honokiol treatment induced a substantial increase in the expression of *HSP90*, and an Hsp90 inhibitor enhanced the antifungal efficacy of honokiol against *C. albicans*, indicating that Hsp90 mediated honokiol tolerance (Fig. 1). Hsp90 regulates drug resistance by stabilizing the protein phosphatase calcineurin, which is the target of the immunosuppressant CsA [1]. Calcineurin B is encoded by a single gene (*CNB1*) in *C. albicans* and is known to be essential for calcineurin activity [26]. As expected, the *cnb1/cnb1* mutant lacking calcineurin was hypersensitive to honokiol as compared with the WT and calcineurin-reconstituted strains (Fig. 2).

However, pharmacological blockade of calcineurin activity by CsA attenuated the antifungal activity of honokiol (Fig. 3). Annexin V and PI staining showed that the declined cytotoxicity of honokiol induced by CsA was due to inhibition of apoptosis in *C. albicans* (Fig. 4). CsA binds to the cytosolic protein cyclophilin A (CyPA) to form a CsA-CyPA complex, which inhibits the calcium/calmodulin-dependent phosphatase, calcineurin (Fig. 7). CsA also binds to cyclophilin D (CyPD) located in the matrix of mitochondria and is thought to regulate the opening of mPTP, and then inhibits the mPTP opening [18]. After co-treatment with CsA, the antifungal sensitivity of honokiol should be increased by the formation of the CsA-CyPA complex and inhibiting calcineurin (Fig. 7). However, the

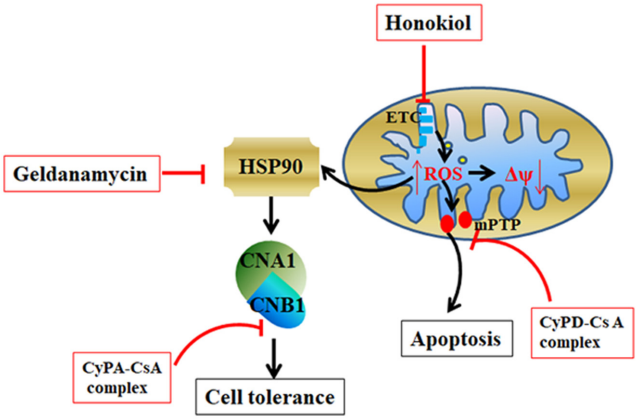

Fig. 7. A model of the Hsp90-calcineurin pathway in the antifungal activity of honokiol.

Honokiol treatment induced a substantial increase in the expression of *HSP90*, and an Hsp90 inhibitor enhanced the antifungal efficacy of honokiol. Hsp90 regulates drug resistance by stabilizing the protein phosphatase calcineurin. cyclosporin A (CsA) binds to the cytosolic protein cyclophilin A (CyPA) to form a CsA-CyPA complex, which inhibits the calcium/calmodulin-dependent phosphatase, calcineurin. CsA also binds to cyclophilin D (CyPD) located in the matrix of mitochondria. Because formation of the CsA-CyPD complex prevents the mPTP from opening, CsA attenuates the antifungal activity of honokiol. ETC, electron transfer chain; mPTP, mitocondria permeability transition pore.

antifungal mechanism of honokiol was related with mitochondrial dysfunction, accompanied by increased cellular superoxide anion and collapse of the mtΔψ [11]. It is reported that the affinity of the mitochondrial site of action for CsA is probably higher than the affinities of other binding sites [18]. After co-treatment with CsA, the formation of the CsA-CyPD complex prevents the mPTP from opening, which may result in the decrease of ROS generation, collapse of the mtΔψ, and Cyt*c* release from mitochondria induced by honokiol treatment (Figs. 5 and 6). We also tested the other immunosuppressive drug FK506, which binds to a small soluble protein, FKBP12, and inhibits calcineurin [27]. Treatment with FK506 also attenuated the cytotoxic activity by blocking the apoptosis induced by honokiol (data not shown). FK506 is reported to show a protective effect against mitochondrial dysfunction and could reduce the mitochondrial-dependent apoptotic cell death induced by 3-nitropropionic acid in neuronal cultures [28].

In conclusion, although Hsp90 has been proposed to promote drug resistance by simulating calcineurin function, inhibitors of calcineurin did not mimic the inhibitor of *HSP90* in the ability to increase the antifungal sensitivity of

honokiol against *C. albicans*. Instead, inhibitors of calcineurin, such as CsA, hindered the efficacy of honokiol treatment by protective effects on the mitochondria.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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