J. Microbiol. Biotechnol. (2009), **19**(8), 803–809
doi: 10.4014/jmb.0812.662
First published online 2 March 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Baicalein Induces Programmed Cell Death in *Candida albicans*

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Received: December 7, 2008 / Accepted: January 13, 2009

Recent evidence has revealed the occurrence of an apoptotic phenotype in *Candida albicans* that is inducible with environmental stresses such as acetic acid, hydrogen peroxide, and amphotericin B. In the present study, we found that the Chinese herbal medicine Baicalein (BE), which was one of the skullcapflavones, can induce apoptosis in *C. albicans*. The apoptotic effects of BE were detected by flow cytometry using Annexin V-FITC and DAPI, and it was confirmed by transmission electron microscopy analysis. After exposure to 4 μg/ml BE for 12 h, about 10% of *C. albicans* cells were apoptotic. Both the increasing intracellular levels of reactive oxygen species (ROS) and upregulation of some redox-related genes (*CAP1*, *SOD2*, *TRR1*) were observed. Furthermore, we compared the survivals of CAP1 deleted, wild-type, and overexpressed strains and found that Cap1p attenuated BE-initiated cell death, which was coherent with a higher mRNA level of the *CAP1* gene. In addition, the mitochondrial membrane potential of *C. albicans* cells changed significantly (p<0.001) upon BE treatment compared with control. Taken together, our results indicated that BE treatment induced apoptosis in *C. albicans* cells, and the apoptosis was associated with the breakdown of mitochondrial membrane potential.

Keywords: *Candida albicans*, Baicalein, programmed cell death, mitochondrial membrane potential

Candida albicans is a pathogenic fungus that causes severe disease in immunocompromised individuals [10, 11, 13, 22]. Recent evidence has revealed the occurrence of an apoptotic phenotype in *C. albicans* that is inducible with environmental stresses such as acetic acid, hydrogen peroxide, and amphotericin B [26]. During programmed cell death, *C. albicans* cells undergo a series of physiological changes, including chromatin condensation, nuclear fragmentation, loss of mitochondrial membrane potential, increased production of reactive oxygen species (ROS), and the exposure of phosphatidylserine in the outer leaf of the plasma membrane [25].

BE is a compound that was originally extracted from the *Scutellaria baicaleinsis* root [16], and it is a promising herbal medicine in Chinese Pharmacopoeia. BE exhibits antioxidant, antibacterial [8, 9, 17], antiviral, and antifungal activities [24, 29, 31, 35, 36]. Additionally, BE is able to inhibit hydrogen-peroxide-induced apoptosis *via* expression of the ROS-dependent heme oxygenase 1 gene [18, 19]. Although we have demonstrated that BE could dramatically decreased biofilm production by *C. albicans* [5], the effect of BE on planktonic *C. albicans* cells remains uncertain.

In this study, we found that BE inhibited planktonic *C. albicans* cells by inducing programmed cell death. Furthermore, we compared the survivals of CAP1 deleted, wild-type, and overexpressed strains and found that Cap1p attenuated BE-initiated cell death, which was coherent with a higher mRNA level of the *CAP1* gene. We also showed an association between the breakdown of mitochondrial membrane potential and apoptosis induced by BE.

Materials and Methods

Strains, Reagents, and Growth Media

The *C. albicans* strains used for this study are listed in Table 1. BE and amphotericin B (AMB) (Sigma-Aldrich, St. Louis, MO, U.S.A.) were dissolved in dimethyl sulfoxide (DMSO) and stored at −80°C. Media used in this study included yeast extract peptone dextrose (YPD, 1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) and phosphate-buffered saline (PBS) [10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride (pH 7.4)]. *C. albicans* strains were grown in YPD medium at 30°C with constant shaking (200 rpm).

Annexin V Analysis

Programmed cell death was detected by flow cytometry using Annexin V-FITC (Annexin-V-FLUOS Staining Kit; Roche, Germany).

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Table 1. *C. albicans* strains used in this study.

Strain	Parental Strain	Genotype	Reference
SC5314			[12]
CAF2-1	SC5314	*ura3Δ::imm434/URA3*	[12]
CJDADH	CJD21	*cap1D ::hisG/cap1D ::hisG/URA3*	[6]
CJDCAP1	CJD21	*cap1D ::hisG/cap1D ::hisG/CAP1-URA3*	[6]

To compare the activity of BE with apoptotic drugs, we chose AMB as a positive agent. *C. albicans* cells were grown in YPD to exponential phase (OD_{600}=0.5) and treated with different dosages of BE and AMB for 12 h. After treatment, the cells (1×10^6) were resuspended in 100 µl of Annexin-V-FLUOS labeling solution (containing propidium iodide (PI) and Annexin V-FITC), and incubated for 15 min at 25°C in the dark. The cells were rinsed three times with PBS, resuspended in 100 µl of PBS, and analyzed by flow cytometry (FACSCalibur, U.S.A.).

DAPI Staining

C. albicans cells were grown in YPD to exponential phase (OD_{600}=0.5) and treated with 8 µg/ml BE for 12 h, harvested by centrifugation (3,000 rpm, 5 min), and washed with PBS .The cells were then fixed with 70% ethanol. Subsequently, the cells were rinsed three times with PBS and then treated with 1 µg/ml of DAPI at 30°C for 20 min in the dark. The cells were subsequently observed with a Leica TCS sp confocal scanning laser microscope equipped with argon and He–Ne lasers.

Transmission Electron Microscopy Analysis

C. albicans cells were grown in YPD to exponential phase (OD_{600}=0.5) and treated with 8 µg/ml BE for 12 h. The cells were harvested by centrifugation (3,000 rpm, 5 min), washed with phosphate/magnesium buffer (40 mM K_2HPO_4/KH_2PO_4, pH 6.5, 0.5 mM $MgCl_2$), resuspended in 2.5% (v/v) glutaraldehyde in phosphate/magnesium buffer, and fixed overnight at 4°C. After glutaraldehyde fixation, the cells were rinsed twice in 0.1 mM phosphate/citrate buffer (pH 5.8) and resuspended in phosphate/citrate buffer with 10 U/ml lyticase for 90 min at 37°C. After digesting the cell wall, protoplasts were washed and fixed with 2% (w/v) osmium tetroxide for 2 h and then incubated with 1% (w/v) aqueous uranyl acetate. After dehydration with ethanol, the samples were transferred to propylene oxide, infiltrated with 50% (v/v) propylene oxide and 50% (v/v) Epon for 30 min, and then infiltrated with 100% Epon overnight. Cells were transferred to gelatin capsules with 100% Epon and incubated at 60°C for 48 h. Finally, thin sections were cut and stained with uranyl acetate and lead acetate. Micrographs were taken with a Zeiss EM 10C electron microscope.

Measurement of Reactive Oxygen Species

Intracellular levels of ROS were measured with DCFH–DA (Molecular Probes, U.S.A.). Briefly, *C. albicans* cells in exponential phase (OD_{600}=0.5) were collected by centrifugation and washed three times with PBS. Subsequently, the cells were adjusted to 2×10^7 cells/ml. After being incubated with 20 µg/ml of DCFH–DA for 30 min at 30°C, the cells were exposed to different concentrations of BE and incubated at 30°C with constant shaking (200 rpm). At specified interval, cell samples were observed with a Leica TCS sp2 confocal scanning laser microscope with excitation at 485 nm and emission at 520 nm. Alternatively, 1 ml of cell suspension was harvested and 100 µl of the supernatant was transferred to the wells of a flat-bottom microplate (BMG Microplate, 96-well, Blank) to detect fluoresence intensities on the POLARstar Galaxy (BMG, Labtech, Offenburg, Germany) with excitation at 485 nm and emission at 520 nm. Each experiment was performed in triplicate.

Real-Time RT–PCR

RNA isolation and real-time RT–PCR were performed as described previously [33]. *C. albicans* cells in exponential phase (OD_{600}=0.5) were treated with 8 µg/ml BE for 6 h before RNA isolation. Experiments were performed with the Chromo4 Real-Time PCR System (Bio-Rad, U.S.A.). SYBR Green I (Takara) was used to monitor the amplified products. Gene-specific primers were designed according to the manufacturer's protocol. Primers for *CAP1* were 5'-ACCGTGAAG GTAAAGAACG-3' and 5'-GCTACCACCAGTATATTTAGCC-3'; primers for *SOD2* were 5'-CAGCACTATCGGAAGTAACTC-3' and 5'-GGCATGTTATCATACTGGAAGG-3'; primers for *TRR1* were 5'-ACATTCCAAGGCAGCCATAC-3' and 5'-AGACCGACGAAGCT GGTTAC-3'; and primers for 18S rRNA were 5'-TCTTTCTTGATT TTGTGGGTGG-3' and 5'-TCGATAGTCCCTCTAAGAAGTG-3'. The changes in SYBR Green I fluorescence in every cycle were monitored by the Opticon software (Bio-Rad, U.S.A.), and the threshold cycle (C_T) above background for each reaction was calculated. The C_T value of 18S rRNA was subtracted from that of the gene of interest to obtain a ΔC_T value. The ΔC_T value of an arbitrary calibrator (*e.g.*, an untreated group) was subtracted from the ΔC_T value for each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$. Triplicate experiments were conducted to generate a mean value.

Cell Death Assays

C. albicans cells from YPD (OD_{600}=0.5) at exponential growth phase were treated with 32 µg/ml BE for certain hours and then harvested for death measurement [25]. Overall viability was assessed by using clonogenic assays based on CFUs forming and plated in triplicate on YPD at 30°C for 48 h.

Measurement of Mitochondrial Membrane Potential

C. albicans cells were grown in YPD to exponential phase (OD_{600}=0.5) and treated with BE or AMB, respectively, for a certain time. The cells were then harvested by centrifugation (3,000 rpm, 5 min) and washed three times with PBS; the concentration was then adjusted to 3×10^6 cells/ml. The cells were treated with 10 µg/ml of 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes, Inc., Eugene, OR, U.S.A.) at 30°C for 15 min in the dark and washed twice with PBS. The cells were then analyzed with a POLARstar Galaxy (BMG, Labtech, Offenburg, Germany) at a 485 nm excitation wavelength and an emission wavelength shifting from green (~525 nm) to red (~590 nm). Mitochondrial membrane potential was determined by the ratio of red to green fluorescence. Each experiment was performed in triplicate.

Statistical Analysis

Experiments were performed in triplicate. Data are presented as mean±SD, and the Student's t test was employed to determine the statistical significance between experimental groups. The difference was considered significant if the P-value was less than 0.001.

RESULTS

Apoptosis in *C. albicans* After BE Treatment

The induction of apoptosis was monitored by flow cytometry using Annexin V-FITC and DAPI. After treatment with 4 μg/ml BE for 12 h, 10% of cells were apoptotic (Fig. 1). When the treatment dosage was increased to 8 μg/ml BE, nearly 20% of cells were apoptotic. The cells of 4 μg/ml AMB exposure, as a positive drug group, were almost necrosis after 12 h. Another familiar apoptotic feature is chromatin condensation and fragmentation, a well-established cytological hallmark of apoptosis. After exposure to BE, abnormal chromatin condensation of *C. albicans* cells were shown by DAPI staining. DAPI staining of cells incubated with 8 μg/ml BE (Fig. 2 B) showed alterations in the shape compared with that of the control (Fig. 2 A). Intracellular DNA damage in cells treated with BE was also examined by transmission electron microscopy. Extensive chromatin condensation along the nuclear envelope was observed in cells treated with 8 μg/ml BE (Fig. 3 B), whereas the nuclei of untreated cells (Fig. 3 A) were homogeneous in shape and density.

Appreciable Variation in Reactive Oxygen Species

One of the typical hallmarks of apoptosis is the production of intracellular ROS. In metazoan programmed cell death, ROS have been shown to participate in both early and late regulatory steps. To examine the production of ROS in apoptotic *C. albicans* cells, the fluorescent reagent DCFH-DA was used. Here, a striking increase in the levels of intracellular ROS was observed in the BE-treated cells compared with control, with a Leica TCS sp2 confocal scanning laser microscope (Fig. 4A). Moreover, coherent results were shown by the POLARstar Galaxy (Fig. 4B). After BE treatment, the intracellular ROS of all the treated cells were increased. Remarkably, the levels of ROS in the cells treated with 32 μg/ml of BE were nearly four times higher than that of the untreated cells after 24 h.

Upregulation of Some Redox-Related Genes

To investigate the mechanism by which BE induces apoptosis in *C. albicans* cells, the expressions of several redox-related genes, including *CAP1* (a key regulatory gene

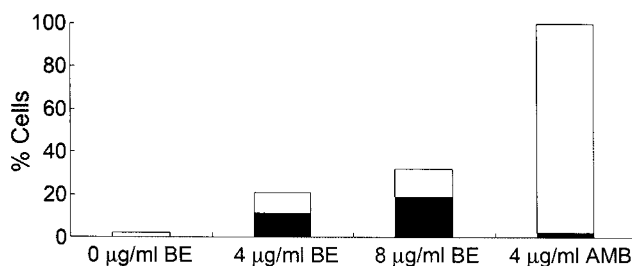

Fig. 1. Induction of apoptosis by BE was detected by flow cytometry using Annexin V-FITC and PI.
To compare the difference with apoptotic agents, AMB was used as a positive control. The percentages of cells that are apoptotic (black bars) and necrotic (white bars) after exposure to 0 μg/ml, 4 μg/ml, or 8 μg/ml BE or 4 μg/ml AMB for 12 h are shown.

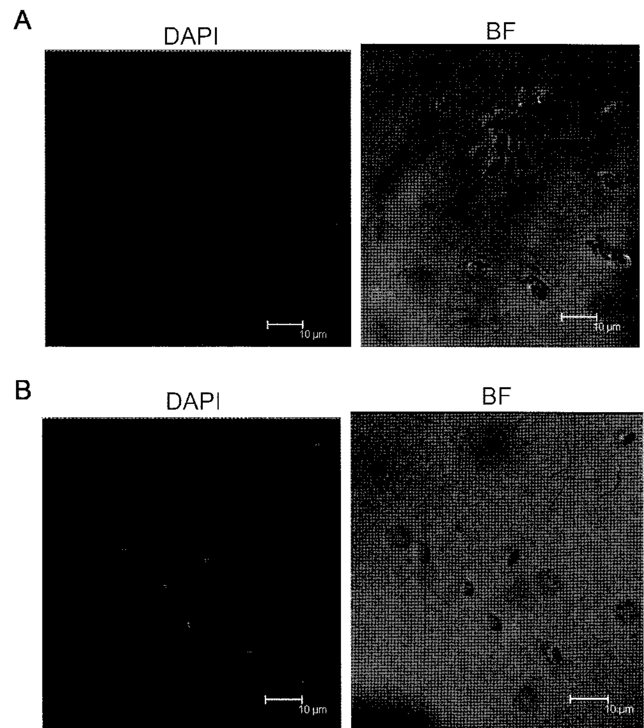

Fig. 2. After BE treatment, abnormal chromatin condensation of *C. albicans* cells were shown by DAPI staining.
DAPI, fluorescence field; BF, bright field. **A.** Untreated control. **B.** Cells treated with 8 μg/ml BE for 12 h. Bar, 10 μm.

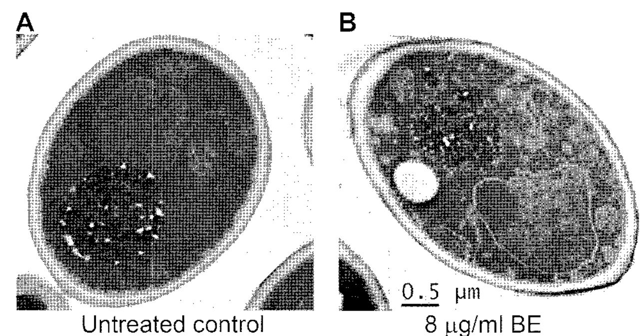

Fig. 3. Transmission electron micrographs show morphological changes characteristic of apoptosis in cells treated with BE.
A. Untreated control. **B.** Cells treated with 8 μg/ml BE for 12 h. Bar, 0.5 μm.

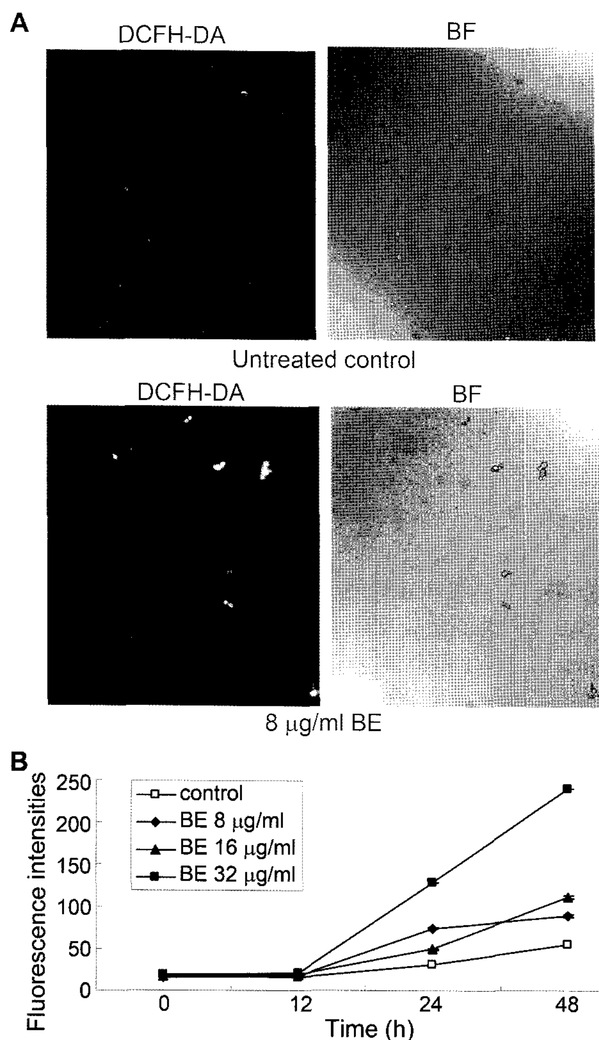

Fig. 4. ROS were detected with DCFH-DA (DCFH-DA, fluorescence field; BF, bright field).
A. The intracellular ROS of cells were observed with a Leica TCS sp2 confocal scanning laser microscope with excitation at 485 nm and emission at 520 nm after exposure to BE. **B**. Changes in ROS levels were observed at different times, after exposure to various doses of BE, on the POLARstar Galaxy with excitation at 485 nm and emission at 520 nm. The ROS production was presented by fluorescence intensities.

of oxidative stress response), *SOD2* (manganese superoxide dismutase gene), and *TRR1* (thioredoxin reductase gene), were monitored by real-time RT–PCR. After exposing to 8 μg/ml BE for 3 h, all three redox-related genes were found to be upregulated. In particular, the upregulation of the *CAP1* gene was about 24-fold. The expressions of *SOD2* and *TRR1* were upregulated 3-fold and 9-fold, respectively (Fig. 5).

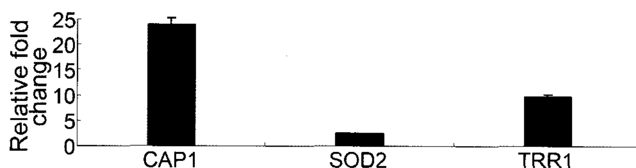

Fig. 5. Changes in redox-related gene expression after treatment with 8 μg/ml BE for 6 h were detected by real-time RT–PCR.
Gene expression was calculated as the fold increase relative to the control group. Data are shown as the mean±SD of three independent experiments.

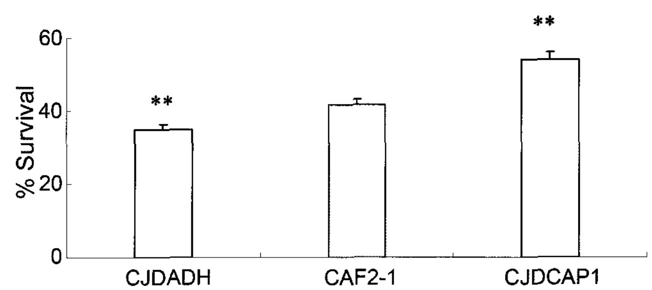

Fig. 6. Genetic activation of CAP1 attenuates BE-initiated cell death, whereas inactivation of CAP1 accelerates killing.
Survival of CAP1-deleted strain, parental strain, and CAP1 overexpressed strain after exposure to 32 μg/ml BE for 3 h. Overall viability was assessed by using clonogenic assays based on CFUs formed from plating on YPD at 30°C for 48 h. Data are the mean±SD for three independent experiments. **, $p<0.001$ compared with the parental strain CAF2-1.

Caplp Attenuates BE-induced Apoptosis

Because of the high-level expression of the three genes at mRNA level in real-time RT–PCR, we investigated one of these redox-related proteins in BE-induced programmed cell death; for example, Caplp. Here, we used the CAP1-deleted strain (CJDADH), wild-type strain (CAF2-1), and CAP1 overexpressed strain (CJDCAP1).As shown in Fig. 6, the survivals were 35%, 41.7%, and 54% in CJDADH, CAF2-1, and CJDCAP1, respectively, after exposing to 32 μg/ml BE for 3 h. From these results, we observed a significant ($p<0.01$) higher survival in CJDCAP1 than that of CAF2-1 and a markedly lower survival in CJDADH ($p<0.001$). The higher survival of CJDCAP1 strain was a result of higher expression of Caplp, which was closely related to upregulation of the *CAP1* gene in the mRNA level.

Loss of Mitochondrial Membrane Potential

To further determine the mechanism of apoptosis induced by BE treatment in *C. albicans*, mitochondrial function was assessed by measuring mitochondrial membrane potential using the fluorescent reagent JC-1. After exposing cells to 16 μg/ml BE for 12 h, the intracellular mitochondrial membrane potential decreased to half of that of the control significantly ($p<0.001$) (Table 2). Additionally, we monitored the mitochondrial function changes of AMB and observed the same tendency. In contrast to AMB, BE owned a weaker ability of inducing apoptosis, as shown in Fig. 1.

Discussion

BE is a potent skullcapflavone medicine that exhibits a range of biological activities. In this study, we found that

Table 2. Mitochondrial membrane potential in the BE and AMB treated groups.

Time (h)	Ratio		
	Control	16 μg/ml BE	4 μg/ml AMB
0	37.19±1.04	31.41±2.85	30.54±0.09
3	26.88±1.62	27.82±1.39	15.38±0.12**
12	28.06±0.80	14.89±0.18**	1.41±0.01**

Mitochondrial membrane potential was determined by the ratio of red to green fluorescence intensity. Fluorescence intensity was measured with the POLARstar Galaxy. Results are the mean±SD for three independent experiments.
**, $p<0.001$ compared with the control at the same time point.

BE could induce programmed cell death in *C. albicans* cells. The activities of BE in apoptosis were detected by flow cytometry using Annexin V and DAPI. Additionally, extensive chromatin condensation along the nuclear envelope was confirmed by transmission electron microscopy. In previous literatures, it was reported that exposure of 4–8 μg/ml AMB induced apoptosis in *C. albicans* [26]. Thus, we chose AMB as a positive agent to investigate the activitives of BE. After comparing, we found that BE induced apoptosis with less extent than AMB. In addition, the level of intracellular ROS, the expressions of several important redox-associated genes, and the mitochondrial membrane potential were changed after exposure to BE. Briefly, the intracellular ROS level was elevated, and the expressions of *CAP1*, *SOD2*, and *TRR1* were upregulated after BE treatment. Coherent with a higher mRNA level of the *CAP1* gene, overexpression of Cap1p protein attenuated BE-initiated programmed cell death in CJDCAP1 strain more than that of wild-type CAF2-1 strain. These data indicated that BE treatment induced apoptosis, and the apoptotic effects of BE was associated with the breakdown of mitochondrial function in *C. albicans*.

Since intracellular oxygen radicals are mainly generated in mitochondria and are involved in programmed cell death, we examined endogenous ROS generation. In apoptosis-like progress, we found that intracellular ROS were increased in all treated groups. During programmed cell death, hyperpolarization of the mitochondrial membrane is triggered by apoptotic stimuli. Subsequently, the production of intracellular ROS increases, which initiates mitochondrial fragmentation [27]. Given the massive ROS production that we observed, we postulated that certain reductive molecules might be upregulated to counter the increased intracellular ROS. *CAP1,* which encodes the basic region-leucine zipper (b-Zip) transcription factor Cap1p, is considered to be a key regulatory gene of the oxidative stress response in *C. albicans* [1, 32, 33, 37]. Superoxide dismutases (SODs), which catalyze the direct removal of ROS, play a critical role in antioxidant defense [21, 30]. Thioredoxin systems are also very important in redox homeostasis, with thioredoxin reductase (TRR1) as its key enzyme [7, 14, 28]. Real-time RT–PCR showed that all three genes, *CAP1*, *SOD2*, and *TRR1*, were upregulated. The upregulation of these important genes was consistent with our prediction that the massive production of intracellular ROS subsequently activated redox-related genes. In the present study, we show that Cap1p attenuated BE-initiated apoptosis on the protein level, which was coherent with the upregulation of these redox-related genes on the mRNA level. On the other hand, the massive ROS production initiated the mitochondrial thread-grain transition (mitochondrial fragmentation) and de-energization. Finally, the mitochondrial de-energization results in loss of mitochondrial membrane potential accompanied by the release of cytochrome *c*.

Programmed cell death is a complex progress involving many factors. In yeast, these factors include cell division cycle gene (*CDC48*) [4], apoptosis-inducing factor (AIF) [34], yeast metacaspase (MCA1) [15], messenger RNA decapping associated gene (*LSM*) [23], γ-glutamylcysteine synthetase (GCS1) [3], inositolphosphosphingolipid phospholipase (Isc1p) [2], and Ras pathway signaling [25]. Ras pathway signaling, which plays a key role in the determination of cell fate in mammalian models, accelerates programmed cell death in *C. albicans*. Mitochondria not only participate in yeast apoptosis, but also function as a key determinant of cellular life and death [20]. Here, we monitored the variation of mitochondrial membrane potential, which was the direct indicator of mitochondrial function, and found that it decreased significantly ($P<0.001$) after exposure to BE although the decreasing was not as marked as with AMB. Collectively, these data indicated that BE treatment induced apoptosis in *C. albicans* cells, and the apoptosis was associated with the breakdown of mitochondrial membrane potential.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 30630071, 30870105, 30825041) and National Basic Reasearch Project 2005CB523105 and Shanghai Key Scientific and Technological Program Project Grant (06XD14027) and *Rising-Star* Program (08QA14001). We thank William A. Fonzi for kindly providing *C. albicans* strains SC5314 and CAF2-1.

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