Human Salivary Histatin 5 Fungicidal Action Does Not Induce Programmed Cell Death Pathways in *Candida albicans*

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Salivary histatins (Hsts) are potent candidacidal proteins that induce a nonlytic form of cell death in *Candida albicans* accompanied by loss of mean cell volume, cell cycle arrest, and elevation of intracellular levels of reactive oxygen species (ROS). Since these phenotypes are often markers of programmed cell death and apoptosis, we investigated whether other classical markers of apoptosis, including generation of intracellular ROS and protein carbonyl groups, chromosomal fragmentation (laddering), and cytochrome *c* release, are found in Hst 5-mediated cell death. Increased intracellular levels of ROS in *C. albicans* were detected in cells both following exogenous application of Hst 5 and following intracellular expression of Hst 5. However, Western blot analysis failed to detect specifically increased protein carbonylation in Hst 5-treated cells. There was no evidence of chromosomal laddering and no cytochrome *c* release was observed following treatment of *C. albicans* mitochondria with Hst 5. Superoxide dismutase enzymes of *C. albicans* and *Saccharomyces cerevisiae* provide essential protection against oxidative stress; therefore, we tested whether *SOD* mutants have increased susceptibility to Hst 5, as expected if ROS mediate fungicidal effects. Cell survival of *S. cerevisiae* *SOD1/SOD2* mutants and *C. albicans* *SOD1* mutants following Hst 5 treatment (31 μ M) was indistinguishable from the survival of wild-type cells treated with Hst 5. We conclude that ROS may not play a direct role in fungicidal activity and that Hst 5 does not initiate apoptosis or programmed cell death pathways.

Candida albicans and related species are opportunistic pathogens causing oral infections in compromised hosts. Oropharyngeal candidiasis is found in 25 to 30% of head and neck cancer patients receiving radiation (35) and is considered to be an AIDS-defining illness (7, 12). Candidiasis is common in elderly or debilitated patients, denture wearers, and xerostomic individuals. Treatment of candidiasis with triazole antifungal drugs has resulted in the emergence of resistant *C. albicans* strains, particularly in human immunodeficiency virus-infected patients (17, 36). These data point to a pressing need for improved drug therapies.

Salivary histatins (Hsts) are structurally related histidine-rich cationic proteins that are key components of the nonimmune host defense system in the oral cavity (10, 33). Hsts 1, 3, and 5 are the major histatins produced by acinar cells in human major salivary glands (33). In vitro, Hst 5 (24 amino acids) is the most toxic to *C. albicans* at physiological concentrations (15 to 30 μ M) (34, 39, 42). Hst 5 also possesses fungistatic and fungicidal activities against *Candida glabrata*, *Candida krusei*, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans* (40). Importantly, Hst 5 is effective against azole- or amphotericin-resistant strains of these fungi (41), suggesting fundamental differences in their mechanisms of action.

Hst 5 killing is a multistep process characterized by binding with a yeast cell envelope protein, followed by intracellular translocation and efflux of ions, including K^+ , Mg^{2+} , and ATP (19, 43). Extracellular binding of Hst 5 with *C. albicans* is invariably required for toxicity (9), although the primary effec-

tors for Hst 5 appear to be located intracellularly. Thus, intracellular expression of either Hst 5 or Hst 3 in *C. albicans* without exogenous Hst 5 resulted in ATP release in parallel with a substantial loss of cell viability (2). We found that prominent features following Hst 5 treatment of cells were a diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS)-inhibitable reduction in yeast cellular volume and G_1 cell cycle arrest (3). In higher-eukaryote cells, normotonic cell shrinkage due to disordered volume regulation was associated with apoptotic cytochrome *c* release and DNA laddering, which was prevented by DIDS and other volume-regulatory channel inhibitors (32).

Apoptosis has recently been described for unicellular organisms such as *S. cerevisiae* (reviewed by Madeo et al. [31]) and is characterized by a highly coordinated series of intracellular signals that involves the recently discovered yeast caspase-related proteases (30). Oxygen stress appears to be a central regulator of apoptosis (29), associated with changes in cell volume and eventual chromosomal degradation and cell fragmentation. In *S. cerevisiae*, aging and oxidative stress resulted in cells exhibiting classical markers of apoptosis, including the appearance of phosphatidyl serine and annexin in the outer cell membrane, chromatin condensation, and DNA fragmentation (22). Similar apoptotic markers were found in an *S. cerevisiae* strain containing a mutation in the cell division cycle gene *CDC48* (28). Thus, *S. cerevisiae* organisms are capable of exhibiting the typical features of apoptosis that may have a physiological role in eliminating overaged cells.

Oxidative damage as a result of the generation of reactive oxygen species (ROS) has been correlated with aging cells and with a reduced activity or a lowered abundance of oxidative defenses. The respiratory chain generates ROS (superoxide

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radicals) that oxidize membrane lipids and cellular proteins, causing protein carbonylation. In *S. cerevisiae*, the rate of protein carbonylation is closely linked to levels of ROS and is a function of the degree of coupling of the mitochondrial respiratory chain (1). Thus, a transition from phosphorylating to nonphosphorylating respiration, which may accompany growth arrest or nutrient deprivation, causes an increase in levels of ROS and protein oxidation.

The metalloenzyme superoxide dismutase (SOD) is the primary enzymatic system involved in the removal of ROS and acts by catalyzing the conversion of superoxide anions into O_2 and hydrogen peroxide. *C. albicans* possesses three SOD enzymes that function in the protection of the organism from oxidative stresses. *C. albicans* expresses gene products for cytosolic Cu/Zn-SOD (a *SOD1* gene product) (15) and mitochondrial Mn-SOD (a *SOD2* gene product) (37), which are orthologues of *S. cerevisiae* *SOD1* and *SOD2* genes, respectively. In addition, *C. albicans* also possesses an atypical cytosolic manganese-containing SOD (*SOD3* gene product) that is expressed following prolonged stationary-phase growth conditions (21).

Recently, it has been reported that ROS are generated in *C. albicans* following treatment with Hst 5 (13). However, it is not clear whether this increase in ROS leads to apoptotic processes, including protein oxidation, chromosomal degradation, and cytochrome *c* release, or whether ROS are produced as a secondary effect in growth-arrested cells. Therefore, we looked for the presence of further markers of apoptosis, including protein carbonyl formation, cytochrome *c* release, and chromosomal fragmentation, following Hst 5 exposure and measured the susceptibility of *C. albicans* and *S. cerevisiae* SOD mutants to Hst 5 toxicity.

MATERIALS AND METHODS

Strains and culture conditions. *C. albicans* strain DS1 is a clinical isolate. The following *S. cerevisiae* SOD isogenic mutant strains (with genotypes in parentheses) (5) were generously provided by Daniel Kosman (University at Buffalo, Buffalo, N.Y.): AMR4D (*SOD* wild type), AMR4C ($\Delta sod1\ sod1::URA3$), *DS11* ($\Delta sod2\ sod2::LEU2$), and AMR4B ($\Delta sod1\Delta sod2\ sod1::URA3\ sod2::LEU2$). *C. albicans* *sod1* mutant strain CH104r (wild-type CAI4; $\Delta sod1/\Delta sod1\ sod1::hisG$) was a kind gift from Sa-Ouk Kang (Seoul National University, Seoul, Korea) (14). The DB9 strain, containing integrated genes for the expression of codon-optimized human salivary Hst 5, and the DB10 strain, for the expression of non-codon-optimized Hst 5 (2), were used. The two strains showed identical growth kinetics and exhibited no reduction in cell viability on glucose-containing medium. The induction of Hst 5 expression in DB9 in sucrose-containing medium resulted in a significant loss of cell viability, while the non-codon-optimized strain DB10 exhibited no loss of viability and served as a control strain. All strains were maintained on yeast extract-peptone-dextrose agar plates and recultured monthly from -70°C stock. Yeast extract-peptone-dextrose agar and media were obtained from Difco.

Assay of intracellular oxidation levels in Hst 5-treated yeast cells. Measurement of total intracellular oxidation levels either in *C. albicans* cells treated with exogenous Hst 5 or in cultures of *C. albicans* cells (DB9) expressing endogenous Hst 5 (2) was accomplished by using the oxidant-sensitive probe H_2 -dichlorodihydrofluorescein diacetate (H_2 -DCFDA; Molecular Probes, Eugene, Oreg.). *C. albicans* cells treated with exogenous Hst 5 were cultured in yeast nitrogen base (YNB) medium for 24 h at 30°C , washed, and treated with Hst 5 according to the candidacidal assay described below. To examine ROS levels in cells in which endogenous production of Hst 5 occurs, *C. albicans* strains DB9 (which exhibits intracellular inducible expression of human salivary Hst 5) and DB10 (a nontoxic control) (2) were used. DB9 cells in the late log growth phase ($100\text{-}\mu\text{l}$ volumes containing 10^4 cells) were transferred into 100 ml of YNB medium containing 2% glucose (noninducing medium) or 2% sucrose (inducing medium) and grown for 12 h under optimal conditions for the expression of Hst 5. Cells

grown in 2% glucose served as controls for cells induced to express Hst 5 by growth in 2% sucrose.

Following incubation of cells with exogenous Hst 5 (*C. albicans* DS1) or endogenous Hst 5 (*C. albicans* DB9), cells were washed and suspended in ice-cold lysis buffer (10 mM sodium phosphate buffer [pH 7.4] supplemented with protease inhibitors). Whole cells (2×10^7) were broken by glass bead disruption at 2°C by use of the FASTRNA system (Bio 101) and centrifuged at $3,500 \times g$, and the supernatants from total cell lysates were collected. Cell extracts were normalized by protein concentration (MicroBCA assay; Pierce). Cell extracts ($100\text{-}\mu\text{l}$) were incubated with $5\text{-}\mu\text{M}$ H_2 -DCFDA activated by deacetylation according to the manufacturer's instructions (0.4 M KOH-40% MeOH for 20 min immediately prior to use) for 1 h at 30°C in the dark. The total fluorescence of each cell lysate was measured (excitation λ , 490 nm; emission λ , 526 nm) with a Hitachi F-2000 fluorescence spectrometer. As additional controls of oxidant levels in our assays, we utilized oxidized- and reduced-bovine serum albumin (BSA) controls to assess positive and negative detection levels, respectively, with H_2 -DCFDA for each assay. Oxidized BSA was obtained by incubating BSA fraction V (5 mg/ml) in the presence of $FeCl_3$ in 50 mM potassium phosphate buffer (K_2HPO_4 - KH_2PO_4) (pH 7.4) for 30 min at room temperature. Reduced protein was prepared by incubating BSA in the presence of 10 mM $NaBH_4$ and 25 mM ascorbate for 30 min at room temperature. Oxidized- and reduced-protein samples were dialyzed extensively against 10 mM potassium phosphate buffer (pH 7.4).

Measurement of protein carbonyl content. Oxidative damage to cellular proteins can be evaluated by the detection of carbonyl groups generated in some amino acid side chains, as has been described for *S. cerevisiae* following its subjection to oxidative-stress conditions (4). Protein carbonyls were detected by the dinitrophenylhydrazine (DNPH) derivatization method (4, 25, 26). Cells for analytical Western blot experiments were grown and treated with Hst 5 or with 1 mM H_2O_2 as described above, and cell lysates were prepared by glass bead disruption (9). The protein contents of cell extracts were adjusted so that they were equal (MicroBCA assay; Pierce), and extracts ($500\text{-}\mu\text{g}$ each) were incubated in 10% trifluoroacetic acid-10 mM DNPH for 25 min at room temperature. Samples were then neutralized with 2 M Tris-30% glycerol-0.01% bromophenol blue-1 mM β -mercaptoethanol and loaded onto 10% polyacrylamide gels. Gel-separated proteins were transferred to polyvinylidene difluoride and probed with anti-2,4-dinitrophenol (anti-DNP) polyclonal antibody (D9656; Sigma Chemical Co., St. Louis, Mo.). Colorimetric detection of DNP-derivatized proteins was done by use of goat anti-rabbit horseradish peroxidase and peroxidase developing reagents (Sigma Chemical Co.). Oxidized- and reduced-BSA samples were used as internal controls as described above.

Cytochrome *c* release from *C. albicans* mitochondria. Purified *C. albicans* yeast mitochondria were prepared essentially as described elsewhere (11). *C. albicans* cells were cultured in 1 liter of YNB medium for 18 h at 30°C , collected by centrifugation at $500 \times g$, and washed twice with distilled water and once with 1 M sorbitol. Cells were resuspended in 10 ml of spheroplasting buffer (1 M sorbitol, 25 mM EDTA, 100 mM Na-citrate [pH 5.8]) to which Zymolyase 20T (2.5 mg per g [wet weight] of yeast cells) was added, and cells were incubated for 2 h at 30°C with gentle shaking. Spheroplast formation was monitored microscopically and by lysis of osmotically sensitive cells in 10% sodium dodecyl sulfate (SDS); under these conditions, more than 90% of the cells were converted to spheroplasts. Spheroplasts were centrifuged at $500 \times g$ for 5 min and washed twice with 1 M sorbitol. Cells were resuspended in 10 ml of ice-cold breaking buffer (0.6 M sorbitol, 20 mM HEPES, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [pH 7.4]) and broken in a glass homogenizer on ice by use of at least 200 strokes. The cell homogenate was centrifuged at $500 \times g$ at 4°C for 5 min, and the supernatant was saved. The supernatant was centrifuged at $12,000 \times g$ at 4°C for 15 min, and the pellet was washed twice and resuspended in assay buffer (10 mM Tris-HCl, 0.6 M mannitol, 2 mM EGTA [pH 6.8]).

Isolated *C. albicans* mitochondria (0.9 mg of protein/ml) were incubated in 20 μl of assay buffer (10 mM Tris-HCl, 0.6 M mannitol, 2 mM EGTA [pH 6.8]) for 10 or 30 min at 25°C in the presence or absence of Hst 5 (31 μM) or H_2O_2 (1 mM; positive control). After incubation, mitochondria were pelleted by centrifugation at $12,000 \times g$ at 4°C for 15 min. The supernatant and pellet fractions were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting with rabbit anti-yeast cytochrome *c* (8). Horseradish peroxidase-linked goat anti-rabbit immunoglobulin G was used as the secondary antibody, and enhanced-chemiluminescence substrate was used for the detection of yeast cytochrome *c*.

Chromosomal fragmentation studies. Gross morphological changes in *C. albicans* genomic DNA following endogenous Hst 5 production were assessed by use of the DB9 strain. Only DB9 cells expressing endogenous Hst 5 were assayed for DNA fragmentation, since the amount of exogenous Hst 5 needed to treat a

sufficient number of cells for genomic DNA isolation was limited. DB9 cells were grown in 200 ml of YNB medium containing 2% glucose (noninducing medium) or 2% sucrose (inducing medium) and grown for 24 h. Induced and noninduced cells were harvested, cell numbers were equalized, and genomic DNA was isolated by the modified, gentle spheroplast lysis procedure (16). The isolated DNA was loaded and run on 1.5% agarose gels (Life Technologies, Bethesda, Md.) and stained with SYBR green nucleic acid dye (Cambrex BioScience Rockland, Inc., Rockland, Maine), and chromosomal-DNA patterns were visualized at a wavelength of 300 nm by UV transillumination.

Candidacidal assay. The antifungal activity of Hst 5 with *C. albicans* and *S. cerevisiae* SOD mutant strains was assayed by our standard microdilution plate method (19). Briefly, *C. albicans* cells were grown in YNB medium, washed twice with 10 mM sodium phosphate buffer ($\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$) (pH 7.4), resuspended (2.5×10^5 cells/ml), and incubated for 1 h with Hst 5 (31 μM). Cells were diluted, plated onto Sabouraud dextrose agar, and incubated for 24 h at 37°C. Cell survival was calculated as the number of colonies recovered from Hst 5-treated cells divided by the number of colonies recovered from control cells times 100. All candidacidal assays were performed in triplicate. Student's *t* test was used to determine statistical significance between groups.

RESULTS

Hst 5 increases total intracellular levels of reactive oxygen radicals. The production of ROS is one of many early changes implicated in apoptosis. To determine whether the exogenous addition of Hst 5 to cells or the intracellular expression of Hst 5 induces increases in intracellular levels of ROS, we measured total intracellular ROS both in cells immediately after extracellular Hst 5 treatment and in DB9 cells following the induction of the expression of Hst 5. ROS production for Hst 5-induced cells was measured after 12 h, since this induction time corresponds to maximal cell death. DB10 cells were used as a control for levels of nonspecific ROS production that could be caused by protein expression. Artificially oxidized- and reduced-BSA samples were used to establish background levels of fluorescence related to protein oxidation. The addition of Hst 5 to *C. albicans* cells was found to triple the total intracellular levels of ROS from 234 ± 12 to 657 ± 56 relative fluorescence units. A similar increase in total cellular ROS in DB9 cells grown under inducing conditions (an increase from 193 ± 18 to 592 ± 44 relative fluorescence units, approximately three times the amount detected in DB9 cells grown under noninducing conditions) was noted. DB10 cell extracts showed no significant increase in ROS levels over background following the induction of nontoxic protein, suggesting that elevated ROS levels are specific to the intracellular expression of Hst 5. These results are in agreement with findings initially reported by another group (13) and extend those data to show that intracellular Hst 5 alone can increase ROS levels in cells. We next questioned whether elevated ROS following Hst 5 treatment causes oxygen stress and directly leads to apoptotic cell death.

Hst 5 does not alter intracellular levels of protein carbonylation. A major effect of intracellular reactive oxygen radicals is the secondary production of oxidized proteins. Amino acids are targets for such oxidation and are detected on the basis of intracellular protein carbonylation, an irreversible and highly deleterious modification of these proteins. Thus, concomitant with ROS measurement, we examined the protein carbonylation contents of yeast cell lysates from strains DB9 and DB10 grown under inducing and noninducing conditions by DNPH derivatization followed by DNP Western blotting. The results shown in Fig. 1 are representative of multiple experiments with

FIG. 1. Protein carbonylation in *C. albicans* following intracellular expression of Hst 5. Protein carbonylation in cells before (lane 1) and after (lane 2) the expression of Hst 5 (strain DB9) and before (lane 3) and after (lane 4) the expression of nontoxic Hst 5 (strain DB10) was detected by DNPH derivatization. *C. albicans* cell lysates were prepared by glass bead disruption, and protein extracts from cells were treated with DNPH to derivatize oxidized amino acids. Gel (10% SDS-PAGE)-separated proteins were probed with anti-DNP polyclonal antibody. Approximately 6 μg of protein was loaded per lane. A nonspecific increase in protein carbonylation accompanies the expression of either Hst 5 (lane 2) or its nontoxic analogue (lane 4).

strains DB9 and DB10. Although growth conditions for the expression of either Hst 5 (lane 2) or the nontoxic Hst 5 derivative (lane 4) increase the levels of total cellular protein carbonylation compared with those in noninduced cells (lanes 1 and 3), no differences between levels of oxidized protein in induced DB9 and DB10 cell extracts expressing protein were evident. Thus, intracellular Hst 5 does not cause a specific increase in protein carbonylation. Instead, placing cells in growth conditions for the induction of nonnative proteins resulted in some degree of oxidative stress that was unrelated to the toxicity of the expressed protein. Protein carbonylation was also assessed for *C. albicans* cells treated with exogenous Hst 5 as described above for the ROS assays or with 1 mM H_2O_2 . No difference in carbonyl content in extracted total cellular proteins was found in cells either treated or not treated with Hst 5, while a generalized increase in protein carbonyl content was observed following H_2O_2 treatment (data not shown).

Assessment of chromosomal-DNA integrity. Cells undergoing apoptosis or programmed cell death show signs of changes in DNA integrity, usually evidenced by discrete laddering on agarose gels. To determine whether intracellular production of Hst 5 resulted in apoptotic chromosomal changes, chromosomal DNA was prepared from the DB9 and DB10 strains grown under inducing and noninducing conditions. DNA samples were run on 1.2% agarose gels and stained with the DNA-specific dye SYBR green I. As shown in Fig. 2, no changes in chromosomal DNA were noted for either strain DB9 (lanes 1 and 2) or strain DB10 (lanes 3 and 4), regardless of whether inducing (lanes 2 and 4) or noninducing (lanes 1 and 3) growth conditions were used. Indeed, the DNA showed marked stability, as evidenced by a lack of any fragmentation, even when

FIG. 2. Expression of intracellular Hst 5 in *C. albicans* does not cause chromosomal fragmentation. Chromosomal DNA was isolated from cells expressing Hst 5 (lane 2) or a nontoxic Hst 5 analogue (lane 4) and compared with DNA from uninduced strains (lanes 1 and 3). DNA samples were run on 1.2% agarose gels and stained with the DNA-specific dye SYBR green I. No fragmentation in chromosomal DNA was evident in strains expressing either toxic (lane 2) or nontoxic (lane 4) Hst 5.

samples were isolated from cells grown for up to 48 h (data not shown).

Hst 5 does not cause cytochrome *c* release from isolated mitochondria. Cytochrome *c* release from the mitochondria to the cytosol is a typical feature of apoptotic cell death that may be initiated by ROS. In this regard, acetic acid-induced apoptosis in wild-type *S. cerevisiae* was characterized by cytochrome *c* release that was prevented in mutant cells with a disruption of the cytochrome *c* gene (27). We previously observed that the addition of cytochrome *c* to *C. albicans* cells induces cell death (9), showing the toxicity of cellular cytochrome *c* in our system. Therefore, we investigated whether Hst 5-mediated increases in ROS levels could induce cytochrome *c* release from mitochondria. For these experiments, Hst 5 (31 μ M) was incubated with freshly isolated mitochondria from *C. albicans* cells for 10 and 30 min, and then the supernatant was probed for the release of cytochrome *c* by immunoblotting. No cytochrome *c* was detected in the mitochondrial buffer medium following 10 or 30 min of application of Hst 5 (Fig. 3), while the amount of cytochrome *c* in mitochondrial lysates appeared unchanged. In contrast, cytochrome *c* could be detected in supernatants of mitochondria treated with 1 mM H_2O_2 as a positive control (Fig. 3, lane C). These results show that Hst 5 does not induce the release of cytochrome *c* from mitochondria in vitro, suggesting that apoptosis through cytochrome *c* release from *C. albicans* cells is not consistent with an Hst 5 fungicidal mechanism.

***C. albicans* and *S. cerevisiae* SOD mutants do not have altered susceptibility to Hst 5 killing.** SODs are essential enzymes in cellular defenses against superoxide anions and oxidative stress mediated by ROS. *S. cerevisiae* SOD-deficient mutants are highly susceptible to dioxygen stress (23) and to conditions that generate ROS (24). Likewise, SOD1 provides *C. albicans* with essential protection against oxidative stress, as

FIG. 3. Detection of cytochrome *c* released from *C. albicans* mitochondria following incubation with Hst 5. Mitochondria from *C. albicans* cells were freshly isolated for each experiment, suspended in osmotic buffer, and incubated either with (+) or without (–) Hst 5 (31 μ M) for 10 or 30 min at 25°C. Mitochondria were centrifuged, and both the pellet and the supernatant were subjected to SDS–15% PAGE and Western blotting using anti-yeast cytochrome *c*. Cytochrome *c* (arrow) was detected in the mitochondrial pellet (lanes marked “pellet”) and in whole mitochondria (lane M) both with and without Hst 5 treatment. Cytochrome *c* was detected in supernatants of mitochondria treated with 1 mM H_2O_2 as a positive control (lane C). However, no cytochrome *c* was detected in supernatants from mitochondria that were either treated or not treated with Hst 5 (lanes marked “supernatant”).

shown by the increased sensitivity of *C. albicans* *sod1/sod1* mutants to menadione (14). Therefore, if the observed increase in intracellular ROS following Hst 5 treatment indeed mediates Hst 5's fungicidal activity, then yeast SOD mutants with defects in their ability to eliminate ROS should be more susceptible to the fungicidal activity of Hst 5. We tested this hypothesis by using both *S. cerevisiae* SOD-deficient mutants and *C. albicans* *sod1/sod1* mutants.

The cell survival rate for wild-type *SOD1/SOD2* *S. cerevisiae* following Hst 5 (31 μ M) treatment was $42\% \pm 10\%$ (Fig. 4A), which was very similar to the survival rates for *S. cerevisiae* Δ *sod1* ($45\% \pm 4\%$), Δ *sod2* ($37\% \pm 8\%$), and Δ *sod1* Δ *sod2* ($44\% \pm 5\%$) cells treated with Hst 5 (Fig. 4A). Statistical analysis of the data was performed, since differences in levels of killing by Hst 5 among SOD mutants were small. By Student's *t* test, the *P* values for differences between the wild-type and any of the mutant strains were >0.05 , showing that a loss of SOD function had no effect on the fungicidal activity of Hst 5. Similar results were found when the cell survival rate for the *C. albicans* wild type CAI4 ($14\% \pm 6\%$) following Hst 5 treatment (31 μ M) was compared with that for the *C. albicans* *sod1* mutant strain ($19\% \pm 7\%$) (Fig. 4B). Again, a loss of *SOD1* gene function did not alter the killing profile of the *C. albicans* Δ *sod1*/ Δ *sod1* mutant compared with that of the wild-type strain following 31 μ M Hst 5 treatment (*P* values were >0.05). Thus, no alteration in the sensitivity of SOD mutant cells to Hst 5 was found for either strain, suggesting that ROS do not play a

FIG. 4. SOD mutants of *S. cerevisiae* and *C. albicans* have no differences in susceptibility to Hst 5. The cell survival rates for *S. cerevisiae* SOD mutant strains (A) and *C. albicans* SOD mutant strains (B) following 1 h of incubation with Hst 5 (31 μM) were assayed by the microdilution plate method. Cell survival was calculated with the equation (number of colonies recovered from Hst 5-treated cells/number of colonies recovered from control cells) $\times$ 100. All assays were performed at least three times, and Student's *t* test was used to determine statistical significance between groups. A loss of *SOD1* gene function did not alter the fungicidal activity of Hst 5 in any of the *C. albicans* or *S. cerevisiae* mutants compared to that of the wild-type strain ($P > 0.05$).

direct role in fungicidal activity. Collectively, these data suggest that ROS alone may not mediate the toxic effects of Hst 5 in *S. cerevisiae* or *C. albicans*.

DISCUSSION

We found that the total cellular levels of ROS in *C. albicans* increased both following the exogenous application of Hst 5, in agreement with the results of a previous study (13), and following the intracellular expression of Hst 5. This effect specifically accompanies the fungicidal activity of Hst 5, since the expression of a nontoxic Hst 5 analogue in cells does not increase ROS levels. ROS play a central role in promoting oxidative stress and may trigger apoptotic or programmed cell death processes. Apoptosis in unicellular organisms has recently gained recognition with the discovery of yeast caspases (30). Like in higher-eukaryote multicellular organisms, ROS induction of yeast apoptosis involves a regulated cascade of events that includes cytochrome *c* release and the translation of caspase and other regulatory proteins. The application of

oxidants such as hydrogen peroxide or acetate to *S. cerevisiae* elicits ROS-mediated apoptosis that can be inhibited by cycloheximide (29). Recently, it was shown that the addition of alpha factor to *S. cerevisiae* cells of the opposite mating type induces ROS-mediated programmed cell death that is prevented by cycloheximide (38). Neutrophil oxidants were shown to induce hyphal DNA fragmentation that mediates fungicidal effects (6). These data show that, as in multicellular eukaryotic organisms, classical yeast apoptosis induced by toxic substances or pheromones requires protein synthesis and is mediated by ROS.

Although Hst 5-induced toxicity shares some features of apoptosis, including G₁ cell cycle arrest (3) and ROS formation, the present data show that none of the other apoptotic markers, including protein carbonylation, DNA fragmentation, and cytochrome *c* release from mitochondria, accompany Hst 5-induced cell death. Furthermore, the pretreatment of *C. albicans* cells with cycloheximide results in no protection from Hst 5 cell death (9), showing that the expression of proapoptotic proteins required for the cell death cascade does not occur.

Instead, our data suggest that ROS formation may be secondary to Hst 5 processes that impair cellular metabolism or ion homeostasis. Conditions that cause yeast cell growth arrest are known to elevate ROS levels. Thus, cell stasis as a consequence of starvation-induced growth arrest in *S. cerevisiae* increased cellular levels of ROS (1). Recent evidence suggests that ROS are generated by metabolic conditions and that they have subsequent stimulatory or inhibitory functions in other systems, such as ion transport (reviewed by Kourie [20]). In this regard, low intracellular potassium increased ROS levels, which directly stimulated gene expression for NaK-ATPase activity (44). Thus, ROS themselves may contribute to cellular signaling. The prominent release of cellular ions, including ATP and potassium ions from *C. albicans*, following Hst 5 treatment may initiate cellular production of ROS in an attempt to reestablish homeostasis rather than triggering cell death.

Our data showing that SOD mutants of *S. cerevisiae* or *C. albicans* are no more susceptible to Hst 5 killing than wild-type cells also argue against a primary role of ROS in mediating cell death. Recently, it was shown that both miconazole and fluconazole induce elevated cellular ROS levels (18). Azole antimycotics inhibit the biosynthesis of yeast cell membrane ergosterol and disrupt cell membrane function. In contrast, Hst 5 shares none of the mechanistic pathways of azole antifungal drugs. Yet both Hst 5 protein and azole drugs lead to elevated ROS levels in treated *C. albicans* cells, further suggesting that ROS formation is a consequence of previous cellular damage initiated by these antifungal agents. The collective data presented here suggest that the appearance of ROS in Hst 5-treated cells is secondary to the major toxic effect of Hst 5 and that Hst 5-induced cell death does not occur through apoptosis.

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