Hydrogen Peroxide Induces Hyphal Differentiation in *Candida albicans*¹

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In this study, we demonstrate that hyphal differentiation is induced by the subtoxic concentration of exogenous H₂O₂ in *Candida albicans*. This finding is confirmed by the changing intracellular concentration of H₂O₂. In order to induce the same level of differentiation, low concentrations of exogenous H₂O₂ are required for the null mutants of the thiol-specific antioxidant and catalase, while higher concentrations are needed for cells treated with ascorbic acid, an antioxidant chemical.

Hydrogen peroxide (H₂O₂) directly affects various redox systems to regulate cell differentiation, proliferation, death, signal transduction, and ion transport (3, 12, 13, 19, 20) at subtoxic concentrations (23, 27–29). Therefore, the homeostatic maintenance of H₂O₂ at low levels should be tightly regulated (1, 9, 28). The yeast *Candida albicans* is a pleomorphic human pathogen. An important virulence factor is the morphological transition involving hyphae formation (6, 16, 24), which is regulated by signaling pathways, including the cyclic AMP/protein kinase A and mitogen-activated protein kinase pathways (4, 7,

![FIG. 1. Hyphal induction by exogenous H₂O₂.](A) Microscopic images of H₂O₂-induced hyphae. Wt cells were grown on YPD solid plates supplemented with the indicated concentrations of H₂O₂ at 30°C for 6 days. Representative colonies were photographed with a stereomicroscope (top). Cells in the mid-log phase were cultured in YPD liquid medium containing H₂O₂ for 6 h at 30°C and observed with a light microscope (bottom). (B) Cytotoxicity of H₂O₂. Standardized cell suspensions were challenged with the indicated concentrations of H₂O₂ for 30 min, plated onto YPD solid medium, and incubated at 30°C for 2 days. The survival rate was expressed as a percentage of the number of colonies in the presence of H₂O₂ divided by the number of colonies in the absence of H₂O₂. (C) Efficiency of hyphal differentiation. Cells were grown on YPD solid medium containing the indicated concentrations of H₂O₂ and incubated at 30°C for 6 days. The percentage of hyphal differentiation was expressed as the number of hyphal colonies divided by the total number of colonies.

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due to the cytotoxic effects of H$_2$O$_2$, which was inferred by the mM concentration, however, the cells were severely swollen augmented in a dose-dependent manner (Fig. 1A). At the 10 (30, 31) and the catalase TSA1 C. albicans scavenging genes, the thiol-specific antioxidant (Fig. 1C).

Thus, ascorbic acid lowered the intracellular concentration of H$_2$O$_2$ and inhibited hyphal differentiation. Also, efficient hyphal differentiation in the presence of ascorbic acid required H$_2$O$_2$ sensitive than the wt over the concentration range (data not shown). The decrease was achieved by the addition of ascorbic acid, an antioxidant chemical (14, 15) (see Fig. 4 and 5).

The enhanced sensitivity of the mutants to exogenous H$_2$O$_2$ was presumably caused by an increase in the concentration of intracellular H$_2$O$_2$. The relative amount of intracellular H$_2$O$_2$ was measured by visualizing fluorescent dichlorodihydroflurorescein (DCF) produced by esterase and H$_2$O$_2$ from 5-chloromethyl-2′,7′-DCF diacetate (CM-H$_2$DCFDA) (Invitrogen, Carlsbad, CA). At exogenous H$_2$O$_2$ concentrations of 0.2 mM and 1 mM, fluorescent intensity was enhanced to some degree in the tsa1Δ and cat1Δ mutants and in the tsa1Δ cat1Δ mutant (Fig. 3A). When the intensities were converted to arbitrary units for quantitative comparison, the intracellular H$_2$O$_2$ concentration increased about 1.5-fold in the tsa1Δ and cat1Δ mutants and about twofold in the tsa1Δ cat1Δ mutant compared with that of the wt (Fig. 3B).

The hyphal differentiation efficiencies of the wt and null mutants were compared using 0.2 mM exogenous H$_2$O$_2$. As shown in Fig. 3C, efficiency was considerably enhanced from 5% in the wt to about 25% in the tsa1Δ and cat1Δ mutants and to about 35% in the tsa1Δ cat1Δ mutant. This efficiency was obtained when the wt cells were treated with 1 mM H$_2$O$_2$ (Fig. 1C). The effective promotion of hyphal differentiation at a low concentration of exogenous H$_2$O$_2$ in mutants in which intracellular H$_2$O$_2$ increased indicated that H$_2$O$_2$ is a genuine inducer of C. albicans hyphal differentiation. When the functional CAT1 gene was reintroduced, the percentage of hyphae reduced to the level between the wt and the cat1Δ mutant.

The effects of a decreased intracellular H$_2$O$_2$ concentration on hyphal differentiation were examined in the presence of ascorbic acid, which reduces the number of intracellular reactive oxygen species in some organisms. When wt cells were cultured under the full differentiation conditions (YPD plus 10% fetal bovine serum [FBS], 37°C), the level of intracellular H$_2$O$_2$ increased about sevenfold, from 8 to 65 arbitrary units (Fig. 4B and C). However, the addition of 50 mM or 100 mM ascorbic acid to the medium reduced the amount of intracellular H$_2$O$_2$ to the same or a lower level of serum depletion (Fig. 4B and C). Microscopic examination revealed that hyphal differentiation was markedly inhibited by ascorbic acid (Fig. 4A). Although the mechanisms of H$_2$O$_2$-induced hyphal transition are unclear, it is highly possible that increased intracellular H$_2$O$_2$ might be partly or completely involved. We further confirmed the above effects in the tsa1Δ, cat1Δ, and tsa1Δ cat1Δ mutants. When 50 mM ascorbic acid, an antioxidant chemical, was added to the medium 30 min after the treatment of mutant cells with different concentrations of exogenous H$_2$O$_2$, hyphal differentiation was induced even at otherwise toxic concentrations: 10 mM for the wt, 4 mM for the tsa1Δ and cat1Δ mutants, and 1 mM for the tsa1Δ cat1Δ mutant (Fig. 5). Thus, ascorbic acid lowered the intracellular concentration of H$_2$O$_2$ and inhibited hyphal differentiation. Also, efficient hyphal differentiation in the presence of ascorbic acid required exogenous H$_2$O$_2$.

The above results suggest that the mere increase of intracellular H$_2$O$_2$ is insufficient for complete hyphal differentiation. The intracellular H$_2$O$_2$ concentration of cells cultured in

![FIG. 2. Construction of CAT1 null mutants and a revertant. The CAT1 genes of the wt and the tsa1Δ mutant (30, 31) were disrupted using URAS-dpl200 (32), yielding the cat1Δ and tsa1Δ cat1Δ mutants, respectively. The sense and antisense primers were nucleotide positions 754 to 823 and 2312 to 2381, respectively, of the CAT1 open reading frame (ORF). To construct a revertant, the DNA fragment containing its own promoter, ORF, and terminator was cloned into pLUX, linearized with Nhel, and transformed into the cat1Δ mutant. Southern (A) and Northern (B) analyses were performed to confirm the authenticity of the constructed strains, using the 32P-labeled probe prepared from the MfeI fragment of the CAT1 ORF. For the Southern analyses, genomic DNA was digested with NsiI and NcoI. Lanes 1, parental strains (CAI4 and the tsa1Δ mutant in panels A and B, respectively); lanes 2, strains with one allele disrupted; lanes 3, strains with URAS popped out from the lane 2 strains; lanes 4, null mutants (the cat1Δ and tsa1Δ cat1Δ mutants in panels A and B, respectively); lanes 5, strains with URAS popped out from the cat1Δ mutant; lanes 6, CAT1-reintroduced strains of the cat1Δ mutant.

18, 21, 22). Pathway triggers are variant (8) and include specific carbohydrates or amino acids (5, 26), serum (11), temperature (17), pH (10), N-acetylglucosamine (2), and starvation (7).

Following infection, C. albicans encounters macrophages but survives ingestion by rapidly adopting a hyphal morphology (25). Since the intracellular concentration of H$_2$O$_2$ in a macrophage is intrinsically high, it was presently germane to examine whether H$_2$O$_2$ can induce hyphal differentiation.

**Hyphal differentiation by H$_2$O$_2$.** When wild-type (wt) SC5314 cells were grown on YPD solid or liquid medium containing 0, 0.4, 1, 4, or 10 mM H$_2$O$_2$, the extent of differentiation was augmented in a dose-dependent manner (Fig. 1A). At the 10 mM concentration, however, the cells were severely swollen due to the cytotoxic effects of H$_2$O$_2$, which was inferred by the survival rate (35%) in contrast to the survival rate at 0.4 mM and 1 mM (90%) (Fig. 1B). Interestingly, undifferentiated colonies also appeared at all concentrations, enabling the evaluation of induction efficiency expressed as a percentage of the number of differentiated colonies in the total number of colonies. The induction efficiency was dose dependent as expected, but 100% differentiation did not occur even at 10 mM (Fig. 1C).

Next, we increased or decreased the endogenous intracellular H$_2$O$_2$. The increase was achieved by nullifying two H$_2$O$_2$-scavenging genes, the thiol-specific antioxidant C. albicans TSA1 (30, 31) and the catalase C. albicans CAT1, individually (tsa1Δ or cat1Δ) or simultaneously (tsa1Δ cat1Δ) (Fig. 2). The growth of null mutants was impeded, and mutants were more H$_2$O$_2$ sensitive than the wt over the concentration range (data not shown).
FBS-supplemented YPD was identical to cells grown in YPD in the presence of 4 mM H$_2$O$_2$ (Fig. 4C and 5B), although differentiation was 100% and 60%, respectively (Fig. 1C). This indicates that some factors present in the serum are required for full hyphal differentiation in addition to increased intracellular H$_2$O$_2$. Based on these observations, we propose that hyphal differentiation in *C. albicans* occurs through two separate, but not mutually exclusive, steps: (i) initiation by...
intracellular H$_2$O$_2$ above a certain concentration and (ii) promotion by currently unknown additional factors in serum.

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