Candida albicans-Conditioned Medium Protects Yeast Cells from Oxidative Stress: a Possible Link between Quorum Sensing and Oxidative Stress Resistance

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Candida albicans, the most frequent fungal pathogen of humans, encounters high levels of oxidants following ingestion by professional phagocytes and through contact with hydrogen peroxide-producing bacteria. In this study, we provide evidence that C. albicans is able to coordinately regulate the oxidative stress response at the global cell population level by releasing protective molecules into the surrounding medium. We demonstrate that conditioned medium, which is defined as a filter-sterilized supernatant from a C. albicans stationary-phase culture, is able to protect yeast cells from both hydrogen peroxide and superoxide anion-generating agents. Exponential-phase yeast cells preexposed to conditioned medium were able to survive levels of oxidative stress that would normally kill actively growing yeast cells. Heat treatment, digestion with proteinase K, pH adjustment, or the addition of the oxidant scavenger alpha-tocopherol did not alter the ability of conditioned medium to induce a protective response. Farnesol, a heat-stable quorum-sensing molecule (QSM) that is insensitive to proteolytic enzymes and is unaffected by pH extremes, is partly responsible for this protective response. In contrast, the QSM tyrosol did not alter the sensitivity of C. albicans cells to oxidants. Relative reverse transcription-PCR analysis indicates that Candida-conditioned growth medium induces the expression of CAT1, SOD1, SOD2, and SOD4, suggesting that protection may be mediated through the transcriptional regulation of antioxidant-encoding genes. Together, these data suggest a link between the quorum-sensing molecule farnesol and the oxidative stress response in C. albicans.

Candida albicans is a normal inhabitant of the oral cavity and the gastrointestinal and genitourinary tracts, where it persists in equilibrium with the host’s microflora; however, alterations in the physiological or immunological status of the host can lead to opportunistic infections ranging from mild mucosal lesions to life-threatening systemic disease (12, 51). The success of C. albicans as an opportunistic pathogen stems in part from its ability to adapt to the many site-specific environmental, and potentially toxic, challenges within the human body. For example, C. albicans frequently encounters high levels of reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide, and hydroxyl radicals, from both endogenous and exogenous sources (44). ROS can damage almost every essential cellular component, resulting in enzyme inactivation, membrane disruption, mutations, and ultimately cell death (9). Recent studies have implicated ROS as a central regulator of programmed cell death in Saccharomyces cerevisiae (42), C. albicans (54), and Aspergillus fumigatus (48). When exposed to toxic levels of hydrogen peroxide, C. albicans displays several apoptosis-like markers, including externalization of phosphatidylserine, nuclease-mediated double-strand DNA breakage, and condensation of chromatin into the nuclear envelope (54).

The major source of exogenous oxidative stress for pathogenic fungi is the phagocytic cells of the host’s immune system. Phagocytic cells play a key role in both innate and acquired resistance to mucosal and systemic candidiasis (2, 32, 64). Optimal microbial killing requires the production of metabolites as well as the action of various enzymes and peptides contained within the secretory granules of phagocytes (33, 64). More specifically, the generation of reactive oxygen and nitrogen intermediates (for example, hydrogen peroxide, superoxide anions, nitric oxide, nitric acid, peroxynitrite, and hypochlorous acid) appears to play an important role in pathogen killing by neutrophils. Although phagocyte-derived oxidants are a principal source of oxidative stress for invading pathogens, other mechanisms of oxidant production exist. A number of microorganisms, for example, Enterococcus faecalis (25), Lactobacillus species (67), and alpha-hemolytic streptococci (3), produce extracellular ROS. Oral streptococci (Streptococcus oralis, Streptococcus mitis, Streptococcus sanguis, and Streptococcus gordonii) have been shown to release hydrogen peroxide into the surrounding medium, where accumulated levels are reported to reach 0.45 to 9.8 mM (3, 15, 58). Because hydrogen peroxide-generating bacteria can be inhibitory or toxic to adjacent fungal cells, hydrogen peroxide released by these sources is likely to limit the proliferation of Candida within the host (14).

Fungal cells have evolved specific strategies to neutralize ROS (primary defense) and to repair or remove oxidized molecules (secondary defense) (reviewed in references 16, 17, and 45). In response to phagocytic attack, C. albicans initiates highly coordinated changes in its transcriptional program,
which include (i) a switch from glycolysis to gluconeogenesis, (ii) activation of fatty acid degradation, (iii) downregulation of oxidative stress responses and DNA damage repair (39, 40, 57). Induction of the oxidative stress response typically leads to the synthesis and activation of both antioxidant enzymes (superoxide dismutase, catalase, and flavohemoglobin) and nonenzymatic metabolites (trehalose, mannitol, and melanin). Not surprisingly, several studies have found a correlation between inactivation of the antioxidant stress response and decreased survival of C. albicans following oxidant attack (28, 43, 49, 68).

While the oxidative stress response has been characterized in some detail at the transcriptional level in S. cerevisiae, little is known about the molecular mechanisms responsible for resistance to oxidative stress in C. albicans. In this study, we show that C. albicans is able to coordinately regulate the oxidative stress response at the global cell population level by releasing substances into the medium, which impart on adjacent cells an increased resistance to oxidative stress. We show that farnesol, a heat-stable quorum-sensing molecule, is partly responsible for this protective response. Together, the results presented herein suggest that autoregulatory molecules contribute to oxidative stress resistance in the human pathogen C. albicans.

MATERIALS AND METHODS

**Growth conditions.** C. albicans SC5314 and ATCC MYA-2430 (also known as strain A72) were maintained as frozen glycerol stocks at −80°C and cultured monthly on Sabouraud dextrose (SAB; Difco) agar at 30°C. For routine cultures were suspended in prewarmed SD minimal medium at an OD600 of 0.05, were used immediately.

**Conditioned medium preparation.** C. albicans SC5314 was grown aerobically in SD or RPMI medium for 24 h at 30°C or 37°C, respectively, and C. albicans A72 was grown in glucose-phosphate-proline (34) medium at 30°C for 24 h with and without 2 μg/ml micafloxazol (Sigma). Microscopic analysis confirmed the presence of yeast (SD and glucose-phosphate-proline media) or hyphal (RPMI) cells following overnight growth. After centrifugation at 2,500 × g for 15 min, the supernatant was adjusted to pH 6 and passed through a 0.22-μm-pore-size filter. Sterile filtered supernatants (designated as conditioned or spent medium herein) were used immediately.

**Hydrogen peroxide, menadione, and plumbagin sensitivity assay.** Overnight cultures were suspended in prewarmed SD minimal medium at an OD600 of 0.05, and cells were allowed to grow at 30°C until an OD600 of 0.15 was reached. The culture was divided equally, centrifuged at 2,500 × g for 10 min, and resuspended in an equal volume of fresh or Candida-conditioned medium. Following 90 min of incubation at 30°C, cells were harvested, washed with phosphate-buffered saline (PBS), and resuspended in SD minimal medium at an OD600 of 0.3. The culture was then challenged with 1.25 mM hydrogen peroxide, 0.6 mM menadione, or 0.05 mM plumbagin (final concentrations). Samples were taken before and after the addition of each stimulus at various times, diluted, and plated onto SAB plates. Viable counts were determined following incubation at 30°C for up to 48 h, and survival was expressed as a percentage of the viable cells at time zero.

**RNA extraction and relative RT-PCR.** C. albicans was exposed to fresh or conditioned medium as described above, and RNAs were prepared using standard methodology (60). The quantity and quality of RNA were measured spectrophotometrically at 260 nm and 280 nm. Equal amounts of total RNA (2 μg) were reverse transcribed into CDNA using a Retroscript kit (Ambion). PCRs were performed initially using primers designed against the C. albicans elongation factor 1α gene (EF1α forward primer, 5'-GAAGAATTCCTTTGCTTGAC; reverse primer, 5'-GATCAGGCGCCGAAATGCG) to ensure that equal amounts of cDNA were used for each sample (59). If required, the amount of starting cDNA template was then adjusted accordingly. PCR analysis was performed with the following forward and reverse primers designed against the C. albicans superoxide dismutase (SOD) and catalase (CAT) genes: for SOD1, 5'-TTGAAACAGAAATCGGAATCC and 5'-AGGCCATGACCCCAAGACGAG; for SOD2, 5'-ACACCTGGTCTTGGTGAAC and 5'-GGCCTATCCAAGACCTTGAAAT; for SOD4, 5'-CCAGCTTTATTTGTTGACTGG and 5'-AACAGCTTTGTTGATGTT and 5'-TACTTGATTGATGATGTTG for CAT1, 5'-ACACAGGAAATCCAAATGAG and 5'-GGCCTATCCAAGGCTTTGGAG. After initial denaturation at 95°C for 2 min, the samples were subjected to 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C (EF1α and CAT1), 58°C (SOD4), or 60°C (SOD1) for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 2 min. PCRs lacking reverse transcriptase were subjected to PCR amplification to check for the presence of contaminating genomic DNA. In addition, the primers for EFB1 amplification were designed to flank an intron, thereby ensuring that the products were derived from CDNA as opposed to genomic DNA. Reverse transcription-PCR (RT-PCR) samples were resolved by agarose gel electrophoresis. The predicted sizes of RT-PCR products were as follows: EFB1, 242 bp; SOD1, 396 bp; SOD2, 437 bp; SOD4, 254 bp; and CAT1, 579 bp.

**Statistical analysis.** Student’s t test was used to determine statistical significance between the experimental groups. Differences were considered significant if the P value was <0.05.

RESULTS AND DISCUSSION

Hydrogen peroxide sensitivity is growth phase dependent. S. cerevisiae stationary-phase cells are much less sensitive to oxidative stress than exponentially growing cells (29). Older cells not only tolerate higher levels of oxidative stress but also require higher concentrations of hydrogen peroxide to induce apoptosis (19, 42). Given that C. albicans encounters oxidative stress following ingestion by professional phagocytes and through contact with hydrogen peroxide-producing bacteria, we were interested in determining whether the C. albicans response to oxidants was similar to that of S. cerevisiae. To this end, we subjected C. albicans exponential- or stationary-phase
cells to externally added hydrogen peroxide and tested the viability of the cultures after 60 min of exposure (Fig. 1). Early-exponential-phase yeast cells were found to be significantly more susceptible to hydrogen peroxide than stationary-phase cells (15% versus 112% survival, respectively). As C. albicans proceeded through each phase of growth (the lag, exponential, and stationary phases), there appeared to be an accompanying increase in resistance to hydrogen peroxide. Our results therefore indicate that C. albicans cells exhibit a growth phase-dependent resistance to hydrogen peroxide that is similar to that of the budding yeast S. cerevisiae.

Conditioned medium protects exponential-phase cells from the lethal actions of hydrogen peroxide. Several mechanisms have been proposed to explain the reduced sensitivity of S. cerevisiae stationary-phase cells to oxidants. It has been shown, for example, that accumulation of the antioxidant metabolite trehalose during stationary phase can lead to increased resistance to oxidative stress (65, 66). Previous studies have also suggested that oxidant sensitivity diminishes as cells enter stationary phase because cells are exposed to higher levels of endogenous ROS and thus generate an adaptive response (46). An alternative explanation can be derived from bacterial studies describing a link between quorum sensing (cell density-dependent molecules) and the stress response (5, 18, 23). We therefore investigated whether exposure to high levels of autoinducers (conditioned medium) would impart on cells an increased resistance to oxidative stress. Early-exponential-phase C. albicans yeast cells were exposed to fresh or conditioned medium (90 min), washed, and then treated with hydrogen peroxide (Fig. 2A). The cell survival rate for C. albicans early-exponential-phase cells exposed to conditioned medium was significantly higher than that for control cells pretreated with fresh medium (101% and 11% survival, respectively). Similar results were found when yeast cells were exposed to conditioned medium generated from hyphal (RPMI at 37°C) cultures (Fig. 2A). Therefore, the ability to produce the protective factor(s) was not morphology dependent. Additional experiments confirmed that conditioned medium protects cells from oxidative stress in a dose-dependent manner (Fig. 2B). Yeast cells pretreated with conditioned medium which had been diluted with a volume of fresh medium and then challenged with a lethal dose of hydrogen peroxide exhibited lower survival rates than cells treated with undiluted samples (Fig. 2B). It is important to note that since the cells were washed prior to oxidant challenge, the presence of free radical scavengers is unlikely to be the factor(s) responsible for resistance. The results do suggest that conditioned medium has the ability to protect yeast cells from the lethal effects of hydrogen peroxide in a dose-dependent manner.

Conditioned medium protects C. albicans from superoxide anion-generating agents. An oxidative stress response can be

FIG. 2. Survival of early-log-phase C. albicans cells pretreated with fresh medium or 1-day-old culture supernatants and subsequently challenged with hydrogen peroxide or superoxide anion-generating agents. C. albicans cells were grown in SD medium at 30°C until an OD₆₀₀ of 0.15 was reached. Cells were harvested and resuspended in either fresh medium (SD or RPMI) (A to C), filter-sterilized spent medium (A to C), or spent medium that had been diluted with a volume of fresh medium (1:2, 1:5, 1:10, and 1:20) (B). Following 90 min of incubation at 30°C, cells were harvested and washed in PBS, and standardized cell suspensions (1 × 10⁷ cells) were challenged with hydrogen peroxide (1.25 mM for 80 min), menadione (0.6 mM for 60 min), or plumbagin (0.05 mM for 60 min). Yeast cell survival was assessed by dilution and plating on SAB plates. Percentages of survival are expressed as the means ± standard deviations of triplicate samples. A survival rate of >100% reflects the inherent variability associated with the plating process between control and test cultures. **, P < 0.001 for conditioned medium compared to fresh medium-treated samples (Student’s t test). F and S, fresh and spent medium, respectively; N, undiluted sample.
triggered when cells sense an increase in ROS (9). Several redox-cycling agents (menadione and plumbagin) are known to sharply increase intracellular levels of superoxide anions (7, 30). In S. cerevisiae, pretreatment with menadione induces an adaptive response that protects yeast cells from a subsequent challenge with hydrogen peroxide; however, cells treated with hydrogen peroxide are unable to survive menadione exposure (29). Since the response to these redox-cycling agents appears to be distinct, we determined whether C. albicans cells preexposed to conditioned medium were also resistant to the superoxide anion-generating agents menadione and plumbagin. C. albicans cells exposed to fresh medium were significantly more sensitive than conditioned medium-treated cells to both agents (Fig. 2C). This observation indicates that conditioned medium induces resistance to several forms of reduced oxygen.

Addition of the antioxidant alpha-tocopherol to conditioned medium does not neutralize the protective factor. C. albicans has been shown to possess an adaptive stress response to both hydrogen peroxide and superoxide generators (31). Low doses of these compounds can induce a response that protects cells from a subsequent challenge with a higher concentration of the same agent (31). Danley et al. (11) and Schröter et al. (61) have shown that early-log-phase C. albicans cells release ROS into the extracellular environment; however, ROS levels dramatically decline at higher cell concentrations. It is therefore conceivable that conditioned medium may contain sufficient levels of ROS to activate the adaptive stress response, resulting in increased oxidative stress resistance. To address this possibi-
the protective substance is unlikely to be a protein; however, the data do not exclude the possibility that a peptide is the responsible factor. Although *C. albicans* has been reported to secrete a mating type pheromone (4, 38, 53), we do not believe that this diffusible peptide is responsible for increased resistance to oxidative stress. Firstly, most clinical isolates of *C. albicans* (including SC5314) are heterozygous for the *a/a* locus (24, 37) and thus will not secrete mating type-specific pheromones. Secondly, since white-opaque switching is a C. *albicans* is known to produce three QSMs, namely tyrosol, farnesol, and farnesic acid (8, 20, 52). These autoregulatory substances accumulate during cell proliferation, and upon reaching a certain threshold, are known to regulate several cell density-dependent phenomena. In view of the fact that hydrogen peroxide resistance correlates with QSM accumulation, we tested the effect of cell-cell signaling molecules on the ability of cells to withstand oxidative stress.

We initially focused our studies on the isoprenoid alcohol farnesol, which is produced enzymatically from the sterol biosynthetic intermediate farnesyl pyrophosphate (21). Farnesol is reported to accumulate to a maximum level of 10 to 50 μM during stationary phase (20), and only the *E. E* isomer possesses QSM activity (62). Exponential-phase cells pretreated with physiological levels of (*E,E*-farnesol (17.5 and 35 μM) were significantly more resistant than control cells to oxidative stress (Fig. 5A). The addition of farnesol to fresh medium, however, did not restore hydrogen peroxide resistance to the levels seen with *Candida*-conditioned medium. At the concentrations tested (up to 35 μM), farnesol did not alter the growth rate of the cells (data not shown). Furthermore, although farnesol is known to influence the yeast-to-hypha conversion at the concentrations used in this study (20), control and farnesol-treated cells appeared as budding yeast cells before and after exposure to either fresh or conditioned medium. This was not surprising since the conditions used (SD medium, pH 6, at 30°C and RPMI medium, pH 6, at 30°C) do not normally stimulate yeast-to-hypha morphogenesis (13). The inability of farnesol to completely mimic the properties of conditioned medium raises the possibility that other molecules are partly responsible for conferring oxidative stress resistance or that the conditioned medium used contains higher levels of farnesol than those tested.

**TABLE 1. Conditioned medium treated with heat and proteolytic enzymes produces similar levels of protection**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment (pH 2)</td>
<td>98.6 ± 4.96</td>
</tr>
<tr>
<td>Adjusted to pH 6</td>
<td>108.2 ± 9.04</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>85.3 ± 3.63</td>
</tr>
<tr>
<td>Heat</td>
<td>92.4 ± 7.43</td>
</tr>
<tr>
<td>Proteinase K and heat</td>
<td>96.9 ± 1.49</td>
</tr>
</tbody>
</table>

* C. *albicans* cells were prepared as described in the legend to Fig. 2. Conditioned medium was either left untreated (pH 2) or brought to a pH of 6 and subsequently treated with protease K (100 μg/ml for 2 h at 56°C) and/or heat (56°C for 2 h followed by 85°C for 30 min). Hydrogen peroxide susceptibility was determined according to the legend to Fig. 2. Percentages of survival are expressed as the means ± standard deviations of triplicate samples.

In bacteria, cell-to-cell communication, also referred to as quorum sensing, has been shown to be involved in regulating a range of cellular functions, including bioluminescence, virulence factor production, biofilm development, and oxidative stress resistance. Several studies have shown that the *Pseudomonas aeruginosa* quorum-sensing molecules (QSMs) 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) and butyrylhomoserine lactone (C4-HSL) are necessary for optimal resistance to hydrogen peroxide and the superoxide anion-generating agent phenazine methosulfate (5, 18, 23). *C. albi-
subsequently exposed the cells to a lethal dose of hydrogen peroxide (1.25 mM for 60 min). Since the conditioned media were generated from cultures with different cell densities, we normalized the cell survival rate (78% ± 10% and 86% ± 2% for azole-treated and control-treated cells, respectively) to the number of cells originally found in the conditioned medium culture. Cells exposed to conditioned medium from azole-treated cells were 20-fold more resistant to hydrogen peroxide than control-treated cells (data not shown). These results strengthen the hypothesis that the presence of farnesol in the conditioned medium is at least partly responsible for oxidative stress resistance.

Farnesol (100 μM) has been shown recently to activate the C. albicans HOG1 (hyperosmotic glycerol) mitogen-activated kinase signal transduction pathway (63). Phosphorylation and translocation of Hog1 to the nucleus results in activation of the general stress response and the phenomenon of stress cross-protection (63). Induction of the core stress response allows cells challenged with a mild stress to acquire resistance to a stronger, seemingly unrelated stress. This is in contrast to the adaptive response, in which pretreatment of cells with a non-lethal stress stimulates adaptation that protects cells from a potentially lethal dose of the same stress (31). Low doses of hydrogen peroxide (0.4 mM), however, do not activate C. albicans Hog1, indicating that adaptation to an oxidative stress is not mediated through the Hog1 stress-activated kinase pathway (63). Since farnesol can increase endogenous levels of ROS (41) and can activate Hog1, it is possible that farnesol may stimulate cell survival through a Hog1-independent adaptive response to oxidative stress and/or through activation of the Hog1-dependent general stress response.

Tyrosol [2,4-(hydroxyphenyl)-ethanol], another C. albicans QSM, has been reported to interfere with the phagocytic respiratory burst (10) and can act as an antioxidant scavenger (47). Since tyrosol can act as an antioxidative agent, it was of interest to test whether the protective factor or signaling molecule in conditioned medium was tyrosol. The addition of tyrosol to fresh medium (0 to 25 μM) did not change the cells’ susceptibility to hydrogen peroxide (Fig. 5B). Chen et al. (8) reported that C. albicans grown in synthetic minimal medium at 30°C accumulates tyrosol to a maximum level of ~5 μM. Therefore, the levels tested were greater than those typically found in conditioned medium. In summary, these experiments indicate that in contrast to tyrosol, the C. albicans QSM farnesol may confer a capacity to resist an oxidative insult.

**Conditioned medium induces the expression of antioxidant-encoding genes.** To protect against the damaging effects of ROS, cells have evolved specific defense mechanisms which involve the synthesis and/or activation of protective enzymes or molecules (45). In P. aeruginosa, quorum-sensing circuits are essential for the optimal transcription of two superoxide dismutase genes (sodA and sodB) and the major catalase gene katB (18). C. albicans has enlisted several classes of antioxidant enzymes to defend against a variety of ROS; however, superoxide dismutases (Sod1-6p) and catalase (Cat1p) are the primary enzymes involved. We therefore analyzed, by relative RT-PCR, the expression of genes encoding the enzymatic mechanisms responsible for eliminating hydrogen peroxide and superoxide in cells exposed to conditioned medium. Relative RT-PCR analysis of RNA samples extracted from C. albicans cells exposed to fresh or conditioned medium revealed
differences in the expression patterns of key antioxidant enzymes (Fig. 6). The expression of catalase (CAT1; also known as CTT1), and to a lesser extent, superoxide dismutase (SOD1, -2, and -4) was increased during exposure to conditioned medium (Fig. 6). Commercial farnesol (up to 35 μM), however, did not induce any noticeable change in the levels of antioxidant gene expression (data not shown). These results suggest that conditioned medium contains a factor that is capable of regulating at the transcriptional level antioxidant-encoding genes with activities responsible for detoxifying both superoxide and hydrogen peroxide. Antioxidant genes are expressed divergently under different growth conditions, and exposure to certain stresses can induce their expression (35, 43, 50). Alterations in the expression profiles of these genes may therefore be a consequence of nutrient depletion, exposure to metabolic waste products, or unknown stressors present in the conditioned medium.

C. albicans catalase, which promotes the conversion of hydrogen peroxide to water and molecular oxygen, has been shown to be essential for peroxide resistance and protection against macrophage killing (68). Interestingly, the induced superoxide dismutases (encoded by SOD1-6) are located in both the cytoplasm (Sod1p and Sod4p) and the mitochondrial intermembrane space (Sod2p) (27, 43, 56). Previous studies have shown that the sod1 and sod2 null mutants both display heightened sensitivity to menadione (26, 28); however, additional phenotypes suggest that Sod2p is primarily responsible for scavenging intracellularly produced superoxides and that Sod1p plays an important role in removing extracellular, macrophage-generated peroxide (26, 28). Although SOD4 (also known as orf19.2062 and orf6.7493) is regulated during phenotypic switching (36), a role for this isozyme remains to be established. Together, these results clearly emphasize the importance of enzymatic defense mechanisms and provide a possible explanation for conditioned medium protection against oxidative stress.

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