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Protective Effects of Antioxidant Enzymes of *Candida albicans* against Oxidative Killing by Macrophages

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Protective roles of antioxidant enzymes, copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), and catalase of *Candida albicans* against exogenous reactive oxygens and oxidative killing by macrophages were investigated. The initial growth of *C. albicans* was inhibited by reactive, oxygen-producing chemicals such as hydrogen peroxide, pyrogallol, and paraquat, but it was restored as the production of antioxidant enzymes were increased. The growth inhibition of *C. albicans* by reactive, oxygen-producing chemicals was reduced by treating the purified candidal SOD and catalase. Also, in the presence of SOD and catalase, the oxidative killing of *C. albicans* by macrophages was significantly inhibited. These results suggest that antioxidant enzymes, CuZnSOD, MnSOD, and catalase of *C. albicans* may play important roles in the protection of *C. albicans* not only from exogenous oxidative stress but also from oxidative killing by macrophages.

Key words: Antioxidant enzymes, *Candida albicans*, macrophages, oxidative killing

Candida albicans is a medically important yeast-like, dimorphic fungus that is responsible for opportunistic infections in immunocompromised and debilitated patients. In addition to minor localized cutaneous, oropharyngeal and vaginal infection, the organism produces transient or persistent fungemia leading to systemic infections in many organs (13, 19, 28).

As with other fungal pathogens, the primary host defense mechanism against *C. albicans* is centered in a phagocytic response consisting of two types of general microbicidal mechanisms: an oxygen-dependent system and an oxygen-independent system (28). The oxygen-dependent killing mechanism is a respiratory burst. It is a metabolic pathway which functions to produce a group of highly reactive microbicidal agents by the reduction of oxygen. These reactive oxygen intermediates including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical (OH) acted as microbiocidal agents by damaging the microbial cell wall, cell membrane, and nucleic acid (3, 8, 18).

To survive against phagocytic killing, microorganisms should detoxify reactive oxygen intermediates generated from respiratory burst of stimulated phagocytic cells. Therefore, antioxidant enzymes including superoxide dismutase (SOD), catalase, peroxidase, and glutathione peroxidase may be necessary for protecting microorganisms from reactive oxidants (11). Among these antioxidant enzymes, SOD and catalase are known to be important in the defense of microorganisms against oxidative stress. Antioxidant activities of SOD and catalase protect microorganisms such as *Legionella pneumophila, Brucella abortus, Caulobacter crecentus,* and *Neisseria* spp. from phagocytic killing and increase intracellular survival of the microorganisms (25, 27, 30).

In this study, we investigated the defensive function of CuZnSOD, MnSOD, and catalase of *C. albicans* against environmental reactive oxygens and oxidative killing by macrophages.

Materials and Methods

Chemicals and enzymes

Yeast nitrogen base (YNB) and Sabouraud dextrose agar (SDA) were purchased from Difco Laboratories (Detroit, MICH.). Paraquat dichloride,

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nitro blue tetrazolium (NBT), neotetrazolium chloride (NTC), hypoxanthine, pyrogallol, hydrogen peroxide and xanthine oxidase (from milk) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Other chemicals were first grade reagents.

Yeast strain and culture condition

Candida albicans ATCC 36802 was cultivated at 30°C with shaking in YNB broth containing 2% glucose from stock cultures maintained on SDA at 4°C. Cells were harvested at the early stationary growth phase. These conditions yielded only yeast forms, without hyphae or pseudohyphae, when observed by microscopy.

Purification of SOD and catalase

The harvested cells were disrupted twice with a French pressure cell press (SLM Aminco, France) at 12,000 psi. The cell lysate was centrifugated at 10,000 rpm for 15 min at 4°C and the supernatant was collected, filtered through 0.45 μm membrane filter, and used for further studies. The supernatant was concentrated with ammonium sulfate between 40% and 90% saturation at 4°C. The precipitate was collected by centrifugation at 12,000 rpm for 15 min and resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1 M ammonium sulfate followed by dialysis against the same buffer. The dialysate was applied to a Phenyl Sepharose CL-4B column (1.6 by 25 cm) equilibrated with the same buffer and eluted with a linear concentration gradient of 1.0~0 M ammonium sulfate in 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 20 ml/h. The fractions exhibiting SOD and catalase activity were pooled, desalted through dialysis against distilled water at 4°C for 12 h, and lyophilized. For further purifications of MnSOD and catalase, c oncentrated samples obtained from Phenyl Sepharose CL-4B chromatography were dialyzed with 10 mM sodium phosphate buffer (pH 6.5) and applied to a hydroxyapatite column (1.6 by 20 cm) equilibrated with the same buffer. The enzymes were eluted with a linear concentration gradient of 10~500 mM sodium phosphate buffer (pH 6.5) with a flow rate of 30 ml/h. The active fractions were collected, desalted through dialysis against distilled water at 4°C for 12 h, and lyophilized. Purities of isolated enzymes were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out following the method of Laemmli (15).

Enzyme assay of SOD and catalase

SOD activity was assayed by the inhibition of NTC reduction using a xanthine-xanthine oxidase system as described by Noritaka *et al.* (22). One milliliter of the reaction mixture was composed of 50 mM sodium phosphate buffer (pH 7.5), 0.2 mM hypoxanthine, 0.2 mM NTC, 0.8% Triton X-100, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mU of xanthine oxidase. The enzyme activity was measured at 540 nm using a spectrophotometer (Beckman DU-600, USA). One unit of SOD activity was defined as the amount of SOD needed to cause 50% inhibition in the rate of NTC oxidation at 37°C. Discrimination of CuZnSOD and MnSOD was used by 3 mM KCN, an inhibitor of CuZnSOD. Assay of catalase activity was performed by the method of Roggenkamp *et al.* (24). In brief, catalase activity was measured spectrophotometrically at 240 nm using a reaction system consisting of 50 mM potassium phosphate buffer (pH 7.2) and 40 mM H_2O_2 in a final volume of 1 ml. One unit of enzyme activity was defined as the amount of the enzyme which catalyzes the degradation of 1 μM of H_2O_2 per min at 37°C. The time required for 240 nm to decrease from 0.450 to 0.400 corresponds to the decomposition of 3.45 moles of H_2O_2 in the 3 ml assay.

Effects of oxidants on *C. albicans* growth and production of antioxidant enzymes

To investigate the effects of oxidants on the growth of *C. albicans* and production of antioxidant enzymes, *C. albicans* was cultivated in YNB medium containing different concentrations of hydrogen peroxide, pyrogallol, or paraquat. The concentrations of hydrogen peroxide were 0.5 and 2.5 mM, and those of pyrogallol and paraquat were 2 and 10 mM, respectively. For the normal control, cells were cultivated in YNB medium without any reagents. After cultivation at 30°C with shaking, cell densities of the aliquot removed at the indicated time were monitored at 600 nm. Cells were obtained by centrifugation at 10,000 rpm for 15 min at 4°C and disrupted using glass beads (0.5 mm Dia. Biospec Products, Inc., Bartlesville, OK) with vigorous vortexing. The cell pellet was removed by centrifugation at 10,000 rpm for 15 min at 4°C and enzyme activities in the supernatant were measured. All the experiments were performed in triplicate and the standard deviation were calculated.

Disc assay for *C. albicans* growth under exogenous oxidants

Approximately 10^8 yeast cells were added to 3 ml of 0.8% agar solution and the mixture was overlaid on SDA plates. Because *C. albicans* has a strong resistance to paraquat, SDA plates containing 0.05% amphotericine B were used to analyze any effects

by paraquat. This helped *C. albicans* damaged easily by paraquat. After the agar solidified, sterile disc papers (6 mm-diameter) were placed on the plates. Sixty units or 120 U of the purified candidal catalase were added to the disc prior to adding 5 μl of 30% H_2O_2. One hundred fifty units or 300 U of the purified candidal SOD was added to the disc before adding 5 μl of 30% pyrogallol, 10 μl of 30% paraquat or xanthine-xanthine oxidase, respectively. After incubation at 30°C for 48 h, the growth was observed. The control group was not treated with either SOD or catalase. All the experiments were performed in triplicate and the standard deviation was calculated.

In vitro assay for survival of *C. albicans* within macrophages

To investigate the effects of SOD and catalase on survival of *C. albicans* within macrophages, an *in vitro* assay was carried out with activated monocytic line (J774A.1, (ATCC TIB67). Macrophage cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2. For monolayer inoculation, 24-well tissue culture plates were seeded with 1 ml of culture medium containing 2×10^5 cells. After incubation for 24 h, subconfluent monolayers were washed out with PBS and further incubated in 1 ml of culture medium without antibiotics for 24 h. Infection was carried out by adding 2×10^4 of *C. albicans* cells in the same medium per well. For the experimental group, 1,000 U of the purified catalase or the same units of the purified SOD were added prior to infection, respectively. The number of intracellularly viable *C. albicans* was determined at 1 and 4 h after infection. After aspiration of the medium above adherent J774A.1 cells, inoculated monolayers were washed with PBS twice and incubated for 30 min in 1 ml of sterile, distilled water for osmotic lysis of the macrophages. This procedure disrupted the macrophage cells without affecting the viability of *C. albicans*. Lysis of cells was confirmed by microscopic observation. The lysate was diluted 1:50 in PBS (pH 7.4) and plated on SDA plates to estimate the effects of SOD and catalase on the survival of *C. albicans* within macrophages. To determine the phagocytic rate of *C. albicans* by macrophages, the removed medium was collected and plated on SDA plates by the same method as described above. After 24 h incubation at 30°C, colonies on the plate were counted and the number was defined as colony forming unit (CFU). Percent phagocytosis of *C. albicans* cells was calculated by the equation, 100 – (coculture CFU/non-coculture CFU × 100). All the experiments were performed in triplicate and the mean number and standard deviation of CFU in three independent experiments were calculated.

Results

Effects of exogenous oxygen radicals on *C. albicans* growth and the production of antioxidant enzymes

To determine the effects of exogenous oxygen radicals on the growth of *C. albicans* and the production of antioxidant enzymes, *C. albicans* was cultivated in the presence of either redox cycle agents such as pyrogallol and paraquat or hydrogen peroxide. Under such oxygen radical stressed conditions, growth of *C. albicans* was significantly retarded during the early growth phases, but growth was restored at exponential and stationary phases, which were similar to those of yeast culture without exogenous oxygen stress (Fig. 1). To determine the relativity of growth restoration and production of antioxidant enzymes, activities of SOD and catalase in each culture condition were assayed and compared. *C. albicans* grown under oxygen radical-stressed conditions showed higher activities of SOD and catalase than those under normal control (Table 1). These indicate that these enzymes are overexpressed to combat exogenous radicals and are important for the survival of *C. albicans*

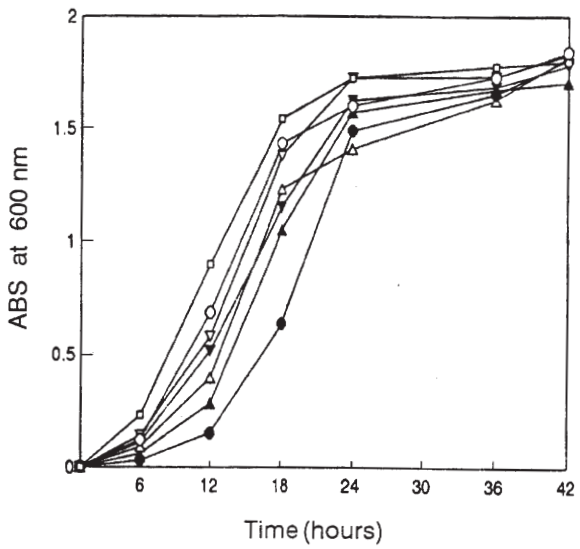

Fig. 1. Change of cell density during cultivation of *C. albicans* under normal and oxygen radical-stressed conditions. *C. albicans* was cultivated at 30°C with shaking. The aliquots taken out at the indicated time were used to measure the cell density at 600 nm. Normal (□), 0.5 mM H_2O_2 (○), 2.5 mM H_2O_2 (●), 2 mM pyrogallol (△), 10 mM pyrogallol (▲), 2 mM paraquat (▽), and 10 mM paraquat (▼).

Table 1. Effects of H_2O_2, pyrogallol, and paraquat on the production of CuZnSOD, MnSOD, and catalase

Treatment	Enzyme activity at stationary phase (U/mg protein)			
	CuZnSOD	MnSOD	Total SOD	Catalase
Control	45.3	20.4	65.7	30.6
0.5 mM H_2O_2	48.4	18.5	59.2	146.9
2 mM pyrogallol	66.3	27.8	94.1	20.3
2 mM paraquat	53.6	22.7	76.3	43.8

under oxygen-stressed conditions.

Effects of SOD and catalase on *C. albicans* growth under exogenous oxygen radical-generated conditions

To confirm the roles of SOD and catalase in defense against reactive oxygen radicals, experiments using oxygen radical-generating chemicals including pyrogallol, paraquat, hydrogen peroxide, and xanthine-xanthine oxidase were performed. The sensitivities of *C. albicans* to hydrogen peroxide and pyrogallol were reduced by the purified catalase and SOD (Table 2). Growth inhibition by hydrogen peroxide was markedly decreased by the purified candidal catalase in a dose-dependent manner. Growth inhibition by pyrogallol was also decreased by the purified candidal SOD in the same manner. However, there was no significant decrease of growth inhibition by the purified SOD in the case of paraquat. Interestingly, the growth of *C. albicans* was not affected by xanthine-xanthine oxidase.

Effects of SOD and catalase on the oxidative killing of *C. albicans* by macrophages

To investigate the roles of SOD and catalase as antioxidant enzymes which protect *C. albicans* from oxidative killing by macrophages, the death of *C. albicans* by macrophages in the presence or absence of the purified enzymes were determined (Table 3). Phagocytic rates after 1 and 4 h incubation were 64.6 ± 13% ($p<0.05$) and 89.5 ± 15% ($p<0.05$), respectively. From the phagocytic rate, the number of *C. albicans* engulfed in macrophages was estimated to 760 ± 24 and 994 ± 18 after 1 and 4 h incubation, respectively. Both SOD and catalase effectively inhibited the killing of *C. albicans* by macrophages. While SOD showed little effect on the protection of *C. albicans* from oxidative killing by macrophages, catalase greatly inhibited the killing of *C. albicans* by macrophages. This may be due to the fact that hydrogen peroxide shows a stronger candidacidal effect than the superoxide anion radical. The highly efficient fungicidal activity by macrophages would likely be exerted by oxygen-independent mechanisms as well as oxygen-dependent mechanisms. As shown in these data, the addition of oxygen radical scavengers (SOD and catalase) was not able to fully remove the candidacidal activity of macrophages. This may be consistent with the fact that oxygen-independent mechanisms are also responsible for the killing of *C. albicans* by activated macrophages.

Table 2. Effects of the purified SOD and catalase on the growth of *C. albicans* under exogenous oxygen radical-generated conditions

Chemical	Inhibition dia. (mm) [a]
Control	6.0 ± 0.00
30% H_2O_2 5 μl	24.6 ± 0.14
30% H_2O_2 5 μl + catalase 60 U	8.6 ± 0.17
30% H_2O_2 5 μl + catalase 120 U	6.2 ± 0.22
30% pyrogallol 10 μl	15.2 ± 0.26
30% pyrogallol 10 μl + SOD 150 U	10.9 ± 0.31
30% pyrogallol 10 μl + SOD 300 U	8.2 ± 0.23
10% paraquat 5 μl	27.5 ± 0.21
10% paraquat 5 μl + SOD 150 U	27.1 ± 0.27
10% paraquat 5 μl + SOD 300 U	26.8 ± 0.12

[a] size of clear zone

Table 3. Effects of the purified SOD and catalase on the survival of *C. albicans* within macrophages

Treatment	1 h incubation		4 h incubation	
	CFU[a] per well	Relative survival rate[b]	CFU[a] per well	Relative survival rate[b]
C. albicans+ Macrophage	253.8 ± 16	100.0%	300.2 ± 24	100.0%
Macrophage+SOD[c]	328.5 ± 34	129.4%	348.3 ± 12	116.0%
Macrophage+catalase[d]	462.0 ± 23	182.0%	579.8 ± 78*	193.1%

[a] CFU (colony forming unit): determined after 24 h of incubation at 30°C. Mean CFU ± standard deviation from the mean of triplicate samples in three independent experiments; [b] Survival rate in the experimental group of *C. albicans* plus macrophages was represented as 100%; [c] 1,000 U of the purified SOD; [d] 1,000 U of the purified catalase. Significance level was determined by chi-square analysis. $p<0.05$ except for *($p>0.05$).

Discussion

Phagocytosis and subsequent killing of pathogenic microorganisms represent one of the most important and early host defense mechanisms against infectious diseases. Thus far, a number of studies have emphasized the essential role of the phagocytic cells in the elimination of pathogens (9, 21, 26, 30, 31). The mechanism which phagocytic cells kill most pathogens includes conversion of oxy-

gen to reactive oxygens such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen ($\cdot O_2$) through respiratory burst of phagocytic cells. These reactive oxygens kill ingested microorganisms, presumably through oxidation reactions and in conjunction with the contents of lysosomal granules. Pathogenic microorganisms encounter these reactive oxygen species produced from the respiratory burst activity of phagocytic cells during the infection process. Consequently, many pathogenic microorganisms have developed antioxidant defense systems which aim to protect themselves from oxidative damages. These defense systems have been extensively studied in their contribution to the virulence of various pathogens (4, 5, 6, 7, 11)

In this study, we investigated the protective roles of SOD and catalase of *C. albicans* against environmental reactive oxygens and oxidative killing by macrophages. Growth of *C. albicans* under oxygen-stressed conditions made from oxygen-producing chemicals such as paraquat, pyrogallol, and hydrogen peroxide were inhibited in a dose-dependent manner. However, growth was restored as the production of antioxidant enzymes increased. In- duction of SOD and catalase under oxygen radical-stressed conditions suggest that these enzymes are important defense-oriented proteins to combat exogenous oxygen radicals and devote the ability of *C. albicans* to grow and survive even at high concentrations of exogenous oxygen radicals.

The growth of *C. albicans* under oxygen-stressed conditions produced by hydrogen peroxide and pyrogallol were increased in the presence of exogenous catalase and SOD in a dose-dependent manner, respectively. However, it was not increased by the addition of SOD in the case of paraquat. Paraquat is a redox cycling drug that produces the superoxide anion in the cytosol. Therefore, this may be due to the fact that exogenous SOD is not accessible to remove superoxide radicals formed by paraquat in the cytosol of *C. albicans*. Under xanthine-xanthine oxidase condition, there was no significant growth inhibition. The similar result that *Candida* species were not killed by the xanthine oxidase-mediated antimicrobial system was reported previously (32). This may be due to the fact that the amount of superoxide radicals produced from the xanthine-xanthine oxidase system was not sufficient to kill *C. albicans*. Stronger resistance of fungal cells to exogenous oxygen radicals than that of bacterial cells is thought to result from the presence of a more rigid cell wall.

Among the cells mediating natural candidacidal activity, polymorphonuclear leukocytes are doubtlessly the best known and the most studied (1, 3, 16, 17, 21, 24, 29). Although macrophages have been known to be capable of ingesting and killing *C. albicans* through oxygen-independent and oxygen-dependent mechanisms, it remains controversal. In this study, we investigated the candidacidal activity of macrophages and the protective roles of SOD and catalase of *C. albicans* against oxidative killing by macrophages. Killing of *C. albicans* by macrophages was inhibited effectively by SOD and catalase. The protection of *C. albicans* from death by SOD and catalase suggests that reactive oxygens are involved in macrophage-induced oxidative damage to *C. albicans* and that these antioxidant enzymes may function to detoxify the reactive oxygen species produced from the respiratory burst of macrophages.

Although considerable research has been conducted on the physiology of *Candida* and their mechanisms of pathogenesis, no single factor has been identified that consistently correlates with the pathogenesis by this fungus. Well known candidates for virulence factors of *C. albicans* are aspartic proteinase and phospholipase (2, 10, 12, 14, 20, 23). Our *in vitro* studies of antioxidant enzymes of *C. albicans* suggest that these enzymes are not only critical in defense against external reactive oxygen, but also provide a possibility as virulence factors which inhibit oxidative killing of *C. albicans* by oxidative burst of macrophages.

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