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Increased Sensitivity of *Candida albicans* Cells Accumulating 14α-Methylated Sterols to Active Oxygen: Possible Relevance to In Vivo Efficacies of Azole Antifungal Agents

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The sensitivity of *Candida albicans* cells to killing by hydrogen peroxide was found to increase markedly when they were grown in the presence of sub-growth-inhibitory concentrations of the azole drug clotrimazole (CTZ). A superoxide anion-generating system consisting of xanthine and xanthine oxidase also killed such CTZ-treated cells more efficiently than control cells, but this seemed to be accounted for by hydrogen peroxide secondarily formed from superoxide anion as judged by the effect of catalase and superoxide dismutase. The increased sensitivity to hydrogen peroxide was considered to be attributable to the inhibition of 14α-demethylation of ergosterol biosynthesis by CTZ, since a 14α-demethylation-deficient mutant of *C. albicans* exhibited a similar phenotype. It is suggested that the in vivo efficacy of azole antifungal agents against *C. albicans* infection is at least partially due to the sensitization of the fungal cells to the oxygen-dependent microbicidal system of the phagocyte.

Azole antifungal compounds are clinically useful for the treatment of systemic fungal infections, including candidosis (7). The best-characterized action of these drugs is interference with sterol 14α-demethylation, resulting in the accumulation of 14α-methylated sterols in the membrane (13, 14), which is manifest even at levels lower than the MIC (for example, see reference 9). Although this leads to suppression of hypha formation in *Candida albicans*, yeast cell proliferation is not much affected. There is evidence to suggest that an azole drug is efficacious in experimental candidosis with levels in serum much lower than the MIC (5). Thus, an intriguing possibility, among others, is that the inhibition of 14α-demethylation, despite the lack of effect on yeast cell growth, may account for the in vivo efficacies of azole antifungal agents against *C. albicans*. Conceptually, this should be possible if yeast cells accumulating 14α-methylated sterols are unusually susceptible to host defense mechanisms. In fact, De Brabander et al. (2) and Shigematsu et al. (8) noticed increased susceptibility of azole-treated *C. albicans* cells to the fungicidal action of leukocytes. On the other hand, we have previously shown that *C. albicans* cells treated with an azole, clotrimazole (CTZ), are unusually susceptible to a variety of antifungal drugs (10). Together, these observations immediately suggest the possibility that azole drugs may sensitize *C. albicans* cells to antimicrobial substances produced by the phagocyte.

In the present investigation, we tested this possibility with regard to active oxygen, one of the major microbicidal agents of the phagocyte. As anticipated, *C. albicans* cells grown in the presence of CTZ at sub-MIC were found to be strikingly more sensitive to hydrogen peroxide than control cells. Furthermore, experiments with a mutant strain defective in sterol 14α-demethylation showed that this increased sensitivity is actually associated with impaired 14α-demethylation.

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MATERIALS AND METHODS

***C. albicans* strains.** KD14 (serotype A, Lys^-) and its 14α-demethylation-deficient mutant, KD4900, have been described previously (9). Briefly, KD4900 is unable to form hyphae and grows as yeast cells considerably more slowly than its parent, their doubling times in YEPG medium being 110 and 72 min, respectively, at 37°C. The addition of ergosterol in the medium is without effect. KD4907, a spontaneous 14α-demethylation-proficient revertant of KD4900 with phenotypes indistinguishable from those of KD14, was obtained by a method previously described (9).

Culture media and cultural conditions. YEPG medium and YEPG agar have previously been described (11). CTZ was dissolved in dimethyl sulfoxide and added to sterile media; the final concentration of the solvent was 1% (vol/vol). All incubations were at 37°C.

Chemicals and enzymes. The reagents used and their sources were as follows: hydrogen peroxide, Mitsubishi Gasu Chemical Co. Ltd. (Tokyo, Japan); xanthine, Nacalai Tesque, Inc. (Kyoto, Japan); xanthine oxidase (from buttermilk, grade I), catalase (C-100), and superoxide dismutase (SOD) (from bovine erythrocytes), Sigma Chemical Co. (St. Louis, Mo.). CTZ was a gift from M. Niimi of Kagoshima University.

Determination of composition of cellular sterols. Determination of composition of cellular sterols was carried out as described previously (9).

Determination of sensitivity to active oxygen. Cells were grown in YEPG medium containing or not containing CTZ to late log phase with gentle shaking, collected by low-speed centrifugation, washed twice with distilled water, and suspended in 10 mM potassium phosphate buffer (pH 7.2) at a cell density of approximately 2×10^6 CFU/ml. In such a suspension, cells showed no loss of viability during the experimental period of 60 min. For determination of sensitivity to hydrogen peroxide, 10 μl of an appropriate dilution of a 31% (wt/wt) solution was mixed with 10 μl of the cell suspension and 30 μl of distilled water in an Eppendorf tube (1.5 ml), and the mixture was incubated at 37°C. One-

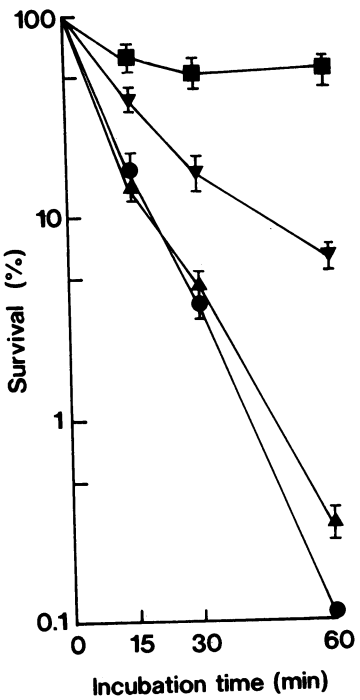

FIG. 1. Hydrogen peroxide killing of KD14 cells treated with different concentrations of CTZ. The results are shown as means ± standard deviations of triplicate experiments. Cells were grown at 37°C in YEPG medium to late log phase in the absence (■) or presence of CTZ at 0.1 (▼), 1 (▲), or 5 (●) μg/ml and then exposed to a fixed concentration (3.5 mM) of H_2O_2 at 37°C for various lengths of time.

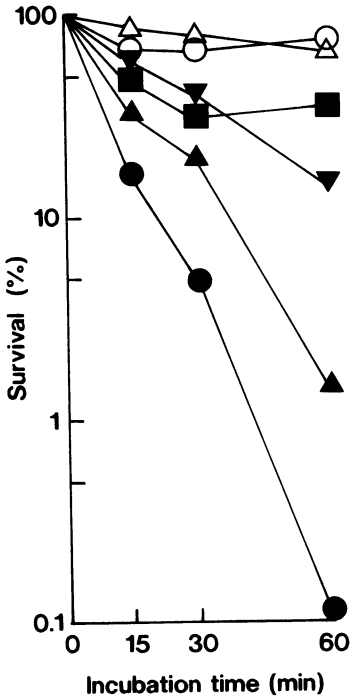

FIG. 2. Killing of CTZ-treated KD14 cells by different concentrations of hydrogen peroxide. The results of a typical experiment are shown. Cells were grown in YEPG medium at 37°C to late log phase in the absence (open symbols) or presence of CTZ at 1 μg/ml (closed symbols) and then exposed to H_2O_2 at 37°C for various lengths of time at a concentration of 0.44 mM (■), 0.88 mM (▼), 1.8 mM (△,▲), or 3.5 mM (○,●).

microliter portions were removed at 15, 30, and 60 min and spread with a glass spreader on YEPG agar plates. The plates were incubated at 37°C.

To test the effect of enzymatically generated superoxide anion, we employed a system consisting of xanthine and xanthine oxidase (4). Ten microliters of the cell suspension was added to 39 μl of the potassium phosphate buffer containing 0.78 mM xanthine, and treatment was started by adding 1 μl of a xanthine oxidase solution containing 0.8 U of the enzyme in the same buffer. Viability was determined as described above. When catalase or SOD was used, 1 μl of a solution of each enzyme in the potassium phosphate buffer was added before the reaction was started.

RESULTS

Effect of hydrogen peroxide on the viability of *C. albicans* cells. KD14 cells were grown in the absence or presence of CTZ at concentrations of 0.1 to 5 μg/ml. The drug inhibited sterol 14α-demethylation by about 95% at 0.1 μg/ml and almost totally at 1 μg/ml (data not shown), whereas its effect on the yeast cell growth was relatively small, the doubling time in YEPG medium being 84 min at the latter concentration, in contrast with the control value of 72 min. The cells grown with various concentrations of CTZ in the above range were found to show increased sensitivity to 3.5 mM hydrogen peroxide (Fig. 1). Experiments performed with various concentrations of hydrogen peroxide and a fixed CTZ concentration of 1 μg/ml confirmed the increased sensitivity (Fig. 2).

Although the main action of azole antifungal agents, including CTZ, is interference with microsomal cytochrome P-450 to inhibit sterol 14α-demethylation, the possibilities of other effects being involved in the sensitization of cells against hydrogen peroxide may also exist. For instance, it has been shown that the agents directly attack the cytoplasmic membrane of the fungus at higher concentrations (13). To confirm whether the increased sensitivity described above was attributable to the inhibition of 14α-demethylation, we examined the sterol 14α-demethylation-deficient mutant KD4900 and its revertant, KD4907, for sensitivity to hydrogen peroxide. The results clearly showed that KD4900 was as sensitive to hydrogen peroxide as the KD14 treated with CTZ at 1 μg/ml, while KD4907 was nearly as resistant as nontreated KD14 (Fig. 3).

Effect of a superoxide anion-generating system on the viability of *C. albicans* cells. In the phagocyte, the primary product in the chain of active-oxygen-generating reactions is superoxide anion (1). In an attempt to mimic the phagocyte-mediated killing of fungus cells, we used the xanthine–xanthine oxidase system, which gives rise to this species of active oxygen. It was demonstrated that KD14 cells grown in the presence of 1 μg of CTZ per ml were significantly more sensitive to this system than control cells (Fig. 4). The killing kinetics for KD4900 was almost the same as that for the CTZ-treated KD14, whereas KD4907, like untreated KD14, was not affected at all (Fig. 5). These results indicate that substantial killing of 14α-demethylation-inhibited cells can be effected once superoxide anion is generated in the system.

The observed effect of the superoxide anion-producing system does not necessarily implicate superoxide anion per

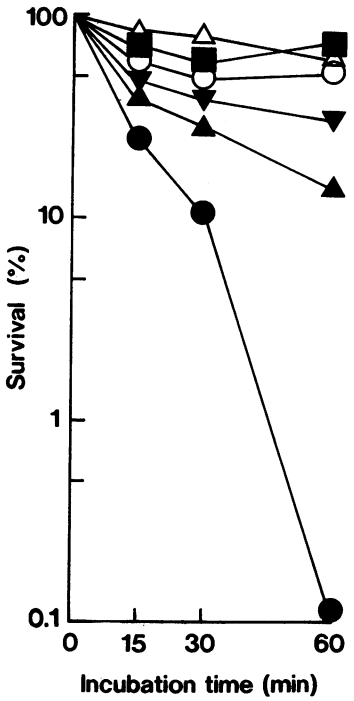

FIG. 3. Killing of KD4900 and KD4907 cells by hydrogen peroxide. The results of a typical experiment are shown. KD4900 (closed symbols) or KD4907 (open symbols) cells were grown in YEPG medium at 37°C to late log phase and then exposed to H_2O_2 at 37°C for various lengths of time at a concentration of 0.44 mM (■), 0.88 mM (▼), 1.8 mM (△,▲), or 3.5 mM (○,●).

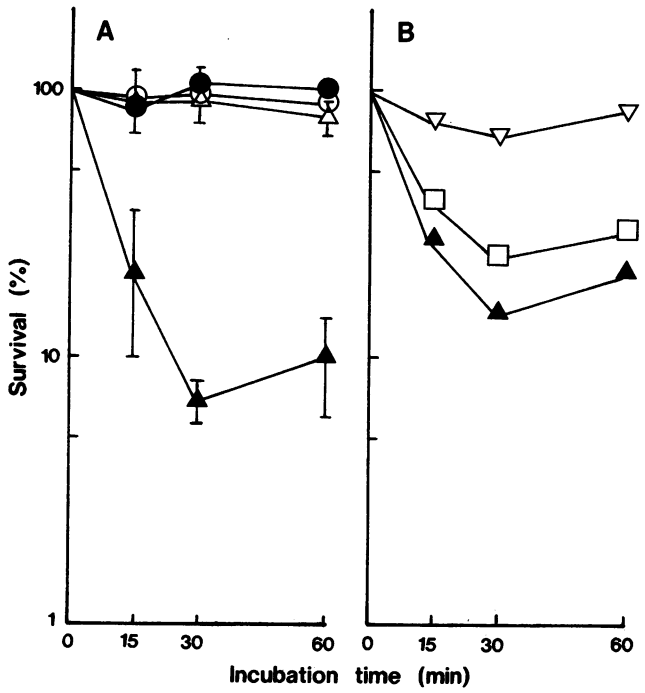

FIG. 4. Killing of CTZ-treated KD14 cells by the xanthine–xanthine oxidase system and effect of SOD and catalase on the killing. (A) Cells were grown at 37°C in YEPG medium to late log phase in the absence (open symbols) or presence of CTZ at 1 μg/ml (closed symbols). The cells were then incubated with (△,▲) or without (○,●) xanthine and xanthine oxidase. The experiment was run in triplicate, and the results are shown as means ± standard deviations. (B) Cells treated with CTZ as described for panel A were subjected to incubation with xanthine and xanthine oxidase alone (▲), xanthine and xanthine oxidase plus SOD (300 U/ml) (□), or xanthine and xanthine oxidase plus catalase (300 U/ml) (▽).

se in the fungicidal action. Since superoxide anion is known to be subject to spontaneous dismutation to yield hydrogen peroxide, there is the possibility that the hydrogen peroxide thus formed rather than superoxide anion accounts for the killing effect. In support of this possibility, catalase abolished the fungicidal effect of the xanthine–xanthine oxidase system almost completely while SOD showed little, if any, effect (Fig. 4). Theoretically, the xanthine–xanthine oxidase system used here can generate 0.62 mM hydrogen peroxide. The degree of killing observed with this system appears to be consistent with the results shown in Fig. 2 and 3.

DISCUSSION

Our results presented above indicate that *C. albicans* cells become highly sensitive to hydrogen peroxide when grown under the conditions of impaired sterol 14α-demethylation caused either by CTZ or by a mutation. Because CTZ-treated KD14 cells and KD4900 cells show different patterns of 14α-methylated sterols (9), the increased sensitivity is likely to be directly related to the 14α-methyl group of cellular sterols and not to other structural features of 14α-methylated sterols. We hence anticipate that this effect should be shared by all azole drugs that are capable of inhibiting 14α-demethylation.

Not only hydrogen peroxide per se but also a superoxide anion-generating system that gives rise to hydrogen peroxide by spontaneous dismutation (3) was capable of killing such cells efficiently. It therefore seems reasonable to postulate that 14α-demethylation-inhibited *C. albicans* cells are especially vulnerable to the oxygen-dependent microbicidal system of the phagocyte, in which the initial active oxygen product is superoxide anion (1). Furthermore, judging from the general increase in susceptibility to antifungal chemicals (10), it is possible that 14α-demethylation-inhibited cells may also be susceptible to some of the oxygen-independent microbicidal mechanisms of the phagocyte such as defensins, a family of antimicrobial peptides. In fact, our preliminary experiments have demonstrated that 14α-demethylation-inhibited cells of *C. albicans* are more sensitive than control cells to tachyplesin, an antimicrobial peptide from the horseshoe crab (6). Taken together, these findings seem to support the notion that azole antifungal drugs sensitize *C. albicans* cells to the phagocytic defense system of humans through inhibition of sterol 14α-demethylation. This idea is consistent with the increased sensitivity of azole-treated *C. albicans* cells to killing by the phagocyte (2, 8).

The suggested vulnerability of 14α-demethylation-inhibited cells to the phagocytic defense mechanism, if proven, may account at least partially for the in vivo efficacy of azole antifungal drugs against *C. albicans* infection that is manifest at concentrations below the MIC (2). It must be kept in mind, however, that other factors may also be relevant to the apparent discrepancy between the in vitro and in vivo effectiveness of those drugs. For instance, the inability of 14α-demethylation-deficient cells to form hyphae (9) might render the fungus easy to phagocytize. It is also possible that the reportedly higher susceptibility of the hyphal form compared with that of the yeast form to azoles (2) may account

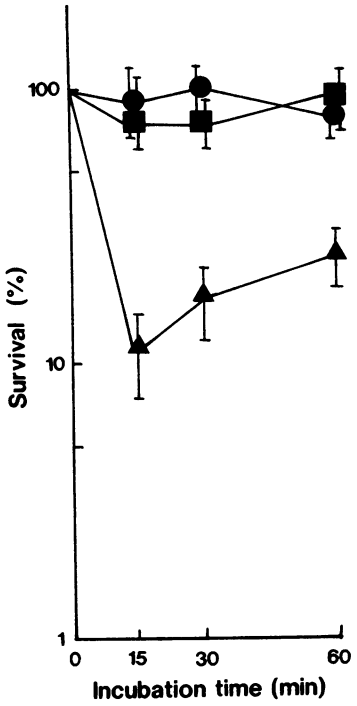

FIG. 5. Effect of the 14α-demethylation-defective mutation on killing by the xanthine–xanthine oxidase system. The experiment was run in triplicate with KD14 (●), KD4900 (▲), and KD4907 (■), and the results are shown as means ± standard deviations.

for the in vivo efficacy. These two possibilities are attractive in view of the well-documented observation that the hyphal form predominates in the lesions of *C. albicans* infection. Still another possibility is that some metabolic changes resulting from the inhibition of 14α-demethylation, exemplified by impaired utilization of certain substrates (12), may hinder the in vivo growth of the fungus. The present results do not rule out these and other possibilities.

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