Growth Phase in Relation to Ketoconazole and Miconazole Susceptibilities of Candida albicans

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The antifungal imidazoles miconazole and ketoconazole inhibit synthesis of essential cell membrane components. Furthermore, miconazole can exert direct physicochemical cell membrane damage at relatively high levels, but ketoconazole cannot. Experiments were designed to explain our previous observation that concentrations of miconazole capable of causing direct membrane damage were no more active against Candida albicans than equimolar levels of ketoconazole. When stationary-phase cells were inoculated into medium containing either drug at \(3.8 \times 10^{-3}\) M, fungicidal effects were indistinguishable. If, however, such cultures were incubated 3 h before drug addition, differences were remarkable. After 3 h, miconazole caused a 99% reduction in CFU per milliliter within 20 min, but ketoconazole again was only fungistatic. The immediate onset, rapidity, and magnitude of the miconazole effect were indicative of direct lethal cell damage. Miconazole concentrations as low as \(1.0 \times 10^{-3}\) M were similarly active. It was concluded that C. albicans undergoes phenotypic changes during the growth cycle that coincidentally confer susceptibility or resistance to the lethal direct membrane damage effect of miconazole. The fungistatic or metabolic effects of ketoconazole or low-level miconazole appeared to be independent of growth phase.

Antifungal imidazole-containing drugs can inhibit biosynthesis of essential fungal cell membrane components (10, 12, 15-17) and exert direct physicochemical cell membrane damage unrelated to any metabolic event (5, 8, 11, 14, 18). Each effect has been offered as the primary mechanism by which imidazoles disrupt membrane permeability. A possible reconciliation of these hypotheses is seen in some recent studies with Saccharomyces cerevisiae (12, 13). Low levels of miconazole (MCZ) inhibited synthesis of membrane components, and this was correlated with fungistatic activity. At relatively high concentrations the drug exerted direct membrane damage and was fungicidal. However, in parallel studies with ketoconazole (KCZ), the most promising of the newer imidazoles, a striking difference was noted (13). Although KCZ inhibited synthesis of membrane components and was fungistatic, it failed to show a capacity for direct membrane damage. Thus, at relatively high concentrations, MCZ exerted much greater antifungal activity than KCZ. From recent experiments with strains of Candida albicans and Candida parapsilosis, we obtained results supporting the existence of a basic difference between the drugs with respect to direct membrane damage potential (2, 3). However, we were unable to show that MCZ is more active than an equimolar concentration of KCZ against growing cultures (1, 3). In view of studies showing that in stationary phase C. albicans develops phenotypic resistance to the lethal action of KCZ (5, 9), the physiological state of our inoculum cells might explain the apparent inability of MCZ to cause direct membrane damage and death of C. albicans in a growth environment (1). This question was examined.

MATERIALS AND METHODS

Imidazole drugs. Janssen Pharmaceutica, New Brunswick, N.J., generously supplied us with miconazole nitrate (MCZ) and ketoconazole base (KCZ). Working solutions were prepared in dimethyl sulfoxide (Me2SO). The molar strengths of these solutions were such that in no instance was it necessary to add more than 1% (vol/vol) Me2SO to yeast cultures.

Organism and culture media. The 11651 strain of C. albicans used in this work was purchased from the American Type Culture Collection, Rockville, Md. It was maintained on a pH 5.5 synthetic semisolid medium composed of 6.7 g of yeast nitrogen base (Difco Laboratories, Detroit, Mich.), 1.5 g of L-asparagine, 10.0 g of dextrose, and 18.0 g of agar in 1 liter of deionized water. For experimental work, synthetic liquid medium was prepared with yeast nitrogen base, L-asparagine, and dextrose as described above, but agar was omitted. The broth was adjusted to pH 7 and sterilized by filtration.

Experimental. C. albicans was cultured in 20-ml volumes of medium contained in 50-ml Erlenmeyer flasks. All cultures were incubated at 37°C with rotary shaking (150 rpm). Drug or Me2SO was added to the medium either just before inoculation or after a 0.5- to 3-h incubation of the cells alone. The inoculum consisted of 0.04 ml of an 18-h culture grown as described above. This gave a time zero viable cell density of \(1.5 \times 10^{11}\) to \(2.5 \times 10^{11}\) CFU/ml. Viable counts were determined periodically during incubation by standard plate count techniques. Physiological saline containing 0.02% Tween 80 was used in the dilution blanks. Duplicate pour plates were prepared by mixing 1.0-ml volumes of diluted culture and 9- to 10-ml portions of melted Sabouraud dextrose agar (Difco) in standard petri dishes (100 by 15 mm). Plates were incubated for 24 to 48 h at 28 to 30°C and counted.

RESULTS

At a concentration of \(3.8 \times 10^{-3}\) M (representing 20 \(\mu\)g of KCZ or 16 \(\mu\)g of MCZ per ml), MCZ can exert direct cell membrane damage against C. albicans 11651, but KCZ apparently cannot (2, 3). Note in Fig. 1, however, that when drug and cells were both added to growth medium at time zero, the activities of MCZ and KCZ were nearly identical.
and characterized as strong fungistasis. An 18-h culture in early stationary phase was used as the inoculum for the type of experiment shown in Fig. 1. Cells in this physiological state might have been phenotypically resistant to the lethal action of MCZ (5, 9). Therefore, drug effects were tested and compared under conditions in which MCZ and KCZ were added after a 3-h incubation of the inoculum cells alone. That 3 h represented approximately the end of the lag phase in these studies is shown by the controls (no drug) in Fig. 2 and 3. KCZ was only fungistatic whether added at time zero or at 3 h (Fig. 2). As expected, the addition of MCZ at time zero resulted in fungistasis similar to that seen with KCZ. However, when MCZ was added at 3 h, the number of CFU per milliliter was reduced by about 99% in 20 min. Nearly identical results were obtained when the MCZ concentration was halved (Fig. 2). When the concentration was reduced further to $1.0 \times 10^{-5}$ M, MCZ still caused a pronounced reduction in CFU per milliliter within several hours (Fig. 3). However, no reduction was observed when the drug was added at $5.0 \times 10^{-6}$ M to a 3-h culture. The rapid rate at which increased susceptibility to MCZ developed when stationary-phase cells were inoculated and incubated in fresh medium is seen in Fig. 4.

**DISCUSSION**

Experiments presented in this study support earlier reports that MCZ, in contrast to KCZ, can exert direct physicochemical fungal cell membrane damage (2, 3, 13) and that *C. albicans* undergoes marked growth phase-dependent phenotypic changes in its susceptibility to the direct membrane damage activity of MCZ (5, 9). The immediate onset and rapidity of the two-log reductions in CFU observed when 3-h cells were treated with $1.9 \times 10^{-5}$ or $3.8 \times 10^{-5}$ M MCZ are consistent with a direct physicochemical drug-cell interaction that is lethal and unrelated to any metabolic function (Fig. 2). KCZ failed to show such activity. In our system, 18-h stationary-phase *C. albicans* cells showed marked resistance to the direct effect of MCZ. Susceptibility, however, was very quickly regained when these cells

**FIG. 1.** Comparison between MCZ, at a concentration that can cause direct membrane damage, and KCZ, at the same molarity, for their activities against *C. albicans* when stationary-phase inoculum cells and drug were added to fresh medium at time zero.

**FIG. 2.** Comparison between MCZ, at a concentration that can cause direct membrane damage, and KCZ, at the same molarity, for their activities against cultures of *C. albicans*. Drugs were added either just before inoculation with stationary-phase cells or after inoculation and a 3-h incubation in the absence of drugs. *, $<5 \times 10^2$ CFU/ml.

**FIG. 3.** Estimation of the minimal concentration of MCZ that can cause a marked reduction in CFU per milliliter when tested against a 3-h culture of *C. albicans*. *, $<5 \times 10^3$ CFU/ml.
FIG. 4. Development of increased MCZ susceptibility of C. albicans during the first 3 h of incubation after inoculation of stationary-phase cells into fresh medium.

were inoculated into fresh medium (Fig. 4). This was indicative of a phenotypic change in the-resistance characteristic.

The results of this study show that the addition of a direct membrane-damaging concentration of MCZ to phenotypically resistant cells prevented the cellular changes necessary to restore direct membrane damage susceptibility (Fig. 1). It is reasonable to suppose that MCZ accomplished this by virtue of its ability to inhibit metabolic reactions (10, 15–17). In this regard, Cassone et al. (4) observed subtle structural, organizational, and chemical changes in the cell walls of C. albicans cells as they passed from exponential- to mid-stationary-phase growth. Recent studies reported by Cope (6) indicated that such changes account for phenotypic resistance to MCZ. It would appear, therefore, that in preparation for a round of active growth and multiplication, cell walls must undergo certain modifications, which coincidentally confer direct membrane damage susceptibility, and that such changes cannot occur in the presence of MCZ. Thus, under the conditions of the experiment shown in Fig. 1, it can be concluded that the direct membrane damage effect of MCZ was not expressed and the nearly identical patterns of fungistasis seen with 3.8 x 10^-5 M concentrations of MCZ and KCZ reflected the same type of inhibitory action on some aspect of cellular metabolism. With respect to the strictly metabolic effect of imidazoles, data presented in Fig. 2 suggest that such action is independent of growth phase. A 3.8 x 10^-5 M concentration of KCZ exerted essentially the same degree of fungistasis whether added at time zero or after 3 h of incubation.

There were two points of potential clinical relevance raised in these studies that deserve comment. The data suggest that direct membrane-damaging action requires only 1.0 x 10^-3 to 1.9 x 10^-3 M MCZ (i.e., 4 to 8 M) MCZ. Such concentrations are reportedly achievable in human serum (7), and, depending on the state of the organism, direct membrane damage could be an operative mechanism in vivo. Second, the growth phase of C. albicans isolates could be a highly important consideration in clinical laboratory susceptibility tests involving MCZ.

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LITERATURE CITED