The human salivary peptide histatin 5 exerts its antifungal activity through the formation of reactive oxygen species

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Previous studies have shown that the human salivary antifungal peptide histatin 5 is taken up by *Candida albicans* cells and associates intracellularly with mitochondria. The purpose of the present study was to investigate the biological consequence of this specific subcellular targeting. Histatin 5 inhibited respiration of isolated *C. albicans* mitochondria as well as the respiration of intact blastoconidia in a dose and time-dependent manner. A nearly perfect correlation was observed between histatin-induced inhibition of respiration and cell killing with either logarithmic- or stationary-phase cells, but stationary-phase cells were less sensitive. Because nonrespiring yeast cells are insensitive to histatin 5, the potential mechanistic relationship between histatin 5 interference with the respiratory apparatus and cell killing was explored by using an oxygen radical sensitive probe (dihydroethidium). Fluorimetric measurements showed that histatin 5 induced the formation of reactive oxygen species (ROS) in *C. albicans* cells as well as in isolated mitochondria and that ROS levels were highly correlated with cell death. In the presence of an oxygen scavenger (L-cysteine), cell killing and ROS formation were prevented. In addition, the membrane-permeant superoxide dismutase mimetic 2,2,6,6-tetramethylpiperidine-*N*-oxyl, abolished histatin-induced ROS formation in isolated mitochondria. In contrast to histatin 5, the conventional inhibitors of the respiratory chain, sodium cyanide or sodium azide, neither induced ROS nor killed yeast cells. These data provide strong evidence for a comprehensive mechanistic model of histatin-5-provoked yeast cell death in which oxygen radical formation is the ultimate and essential step.

Histatins constitute a distinct family of at least 12 low molecular weight, histidine-rich, cationic, salivary peptides, of which histatin 1, 3, and 5, containing 38, 32, and 24 amino acids, are the most abundant (1, 2). Major interest in histatins stems from the fact that they exhibit cidal activities against a broad range of pathogenic fungi, including *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (1, 3–5). In view of this *in vitro* activity and the relative abundance of histatins in parotid and submandibular/sublingual secretions (6), these proteins may represent major components of the nonimmune host defense system involved in the maintenance of oral health.

Understanding of the mechanism of antifungal action is of high significance when histatins are to be considered as potential candidates for drug therapy or as templates for drug design (7, 8). Structural analysis has revealed that histatins differ from amphipathic membrane-active peptides by virtue of their reluctance to rapidly adopt helical structures in moderately hydrophobic environments and the weak amphipathicity of their α-helical structures (8, 9). The interaction of histatin 5 with liposome vesicles, mitochondria, or *C. albicans* blastoconidia does not result in a massive disintegration and depolarization of the membranes such as is achieved with strongly amphipathic peptides (9–11). The general consensus therefore is that the ultimate events in histatin-provoked cell death are not restricted to direct membrane effects.

Interestingly, conditions that alter cell metabolism strongly influence cellular sensitivity to histatin 5. For example, low incubation temperatures and anaerobic incubation conditions prevent killing by histatin 5 (12–14). Protective effects have, furthermore, been reported with oxygen scavengers and with the cytochrome oxidase inhibitors cyanide and azide (13–15). The insensitivity of cells achieved by anaerobiosis or by respiratory chain inhibitors showed that cellular respiration is required for cell sensitivity to histatin 5. Indeed, the genetic modification of *C. albicans* into respiratory deficient "petite" mutants rendered the cells virtually insensitive to histatin 5 (16). Thus, cells with conditionally, chemically, or genetically impaired mitochondrial metabolism are protected against histatin 5 activity.

Localization studies have shown that histatins are able to translocate across the cytoplasmic membrane. The principles of the uptake process are as yet unknown, but internalization has now been reported for the 24-residue peptide histatin 5 (13, 14) as well as for its 32-residue precursor molecule histatin 3 (12). Once inside the cytosol of the yeast cell, histatin 5 reaches the mitochondria; these organelles seem to be the specific intracellular targets (13, 14). It is noteworthy that the mitochondrial targeting of histatin 5 is concomitant with the dissipation of the mitochondrial transmembrane potential, indicating detrimental effects on mitochondrial function at some stage in the fungicidal process by either a direct or an indirect mechanism.

Even though several phenomena that occur on exposure of yeast cells to histatin 5 have been described, it is unclear which of these events are tangential and which are central in the killing mechanism. As a consequence, the actual process that connects these phenomena to yeast cell death has not yet been established. The observations made with regard to respiration requirements prompted us to investigate in more detail the interaction of histatin 5 with mitochondria and the biological consequence of this interaction. This is of particular interest considering the fact that mitochondria are known to play a key role in apoptotic as well as necrotic cell death (17, 18). Data obtained in the present investigation indicate that the "point of no return" in the cascade of events that occur on exposure of *C. albicans* to histatin 5 is the generation of oxygen radicals.

Materials and Methods

Antimicrobial Peptides. Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY) was obtained from American Peptide (Sunnyvale, CA). Analysis of this material by HPLC and mass spectroscopy revealed a purity of >98%. The peptide was dissolved in 10 mM

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Abbreviations: ROS, reactive oxygen species; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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potassium phosphate buffer (PPB; pH 7.0) to a final concentration of 10 mg/ml.

C. albicans Growth Conditions. *C. albicans* (ATCC no. 10231) cells were grown on Sabouraud dextrose agar (Difco) and transferred to 100 ml of Sabouraud dextrose broth (Difco) in a 250-ml of Erlenmeyer flask. After 16 h of incubation at 30°C, 300 μl from this suspension was subcultured for 5 h in 100 of ml Sabouraud dextrose broth to obtain logarithmic-phase cultures. Stationary-phase blastoconidia cultures were obtained after 48 h of incubation under the same conditions. Germ tube formation was induced by incubating early logarithmic-phase *C. albicans* cells for 3 h at 37°C in RMPI medium 1640 containing 25 mM Hepes and 2 mM L-glutamine (GIBCO/BRL), supplemented with 10 mM *N*-acetyl-D-glucosamine (Sigma; pH 7.4).

Isolation of _C. albicans_ Mitochondria. Mitochondria were isolated from *C. albicans* spheroplasts, essentially as described previously (19). Cells were grown to late logarithmic phase in 1 liter of Sabouraud dextrose broth (Difco), washed once in deionized water, and suspended in 1 ml of Zymolyase buffer containing 50 mM Tris, 10 mM $MgCl_2$, and 1.4 M sorbitol (pH 7.5) per g of yeast pellet. DTT (Sigma) was added to a final concentration of 30 mM, and the cells were incubated for 15 min at room temperature. Cells were collected by centrifugation and suspended in 3 ml of Zymolyase buffer containing 1 mM DDT and 2 mg of Zymolyase 100 T (Seikagaku America, Rockville, MD) per g of yeast pellet. After 30 min of incubation, cells were collected by centrifugation at 1,000 $\times$ *g* and washed twice in Zymolyase buffer. Cells were homogenized for 5 min on ice in 0.4 M sorbitol, 0.2% BSA (fraction V, Sigma), and 10 mM imidazole (Fisher Scientific; pH 6.4), by using a manual Potter–Elvehjem homogenizer. The homogenate was mixed with an equal volume of 1 M sorbitol, 25 mM KH_2PO_4, 4 mM EGTA (Sigma), 0.2% BSA, and 10 mM imidazole (pH 6.4) and centrifuged 5 min at 1,000 $\times$ *g* at 4°C. The supernatant was carefully removed and centrifuged for 10 min at 12,000 $\times$ *g* at 4°C. The reddish pellet containing the mitochondria was suspended in 0.6 M mannitol, 2 mM EGTA, 0.2% BSA, and 10 mM imidazole (pH 6.4), to an OD_{620} of $\approx$15, and kept on ice.

Measurement of _C. albicans_ Respiration and Viability. Oxygen consumption was measured by using a biological oxygen monitor model 5300 equipped with a 5331 standard oxygen probe (Yellow Springs Instruments). The apparatus consisted of a twin oxygen chamber, which enabled a control experiment to be conducted at the same time. Experiments with mitochondria were performed in 1.5 ml of air-saturated respiration buffer at 30°C containing 0.65 M mannitol, 2 mM $MgCl_2$, 16 mM KH_2PO_4, and 10 mM imidazole (pH 6.4). In all experiments, mitochondria were diluted from the stock suspension to a final OD_{620} of 0.15 $\pm$ 0.05. State 2, state 3, and state 4 respiration were determined as described previously (9), by using 1 mM NADH and 0.33 mM ADP as substrates. Polarographic measurements using *C. albicans* logarithmic- or stationary-phase blastoconidia were conducted in 1.5 ml of 1 mM PPB. Cells were added to the oxygen chambers from a concentrated stock suspension to a final OD_{620} of 1.7. The respiratory rates were determined graphically from the slope of the tangent line after various time intervals. In the same experimental setup, the viability of cells incubated with histatin 5 was determined after the same time intervals by removing a 15-μl aliquot from the chamber, diluting the cells in 9 ml of PBS and plating 15 μl of the suspension on Sabouraud dextrose agar. After 48 h of incubation at 30°C, the viability was determined by colony counting and compared with control experiments without histatin 5.

Measurement of _C. albicans_ Reactive Oxygen Species (ROS) Formation and Viability. The formation of ROS in *C. albicans* by histatin 5 was determined by using dihydroethidium (Molecular Probes), which is rapidly oxidized by ROS into its fluorescent derivative. Dihydroethidium was added from a stock solution of 2.5 mg/ml in DMSO to a suspension of logarithmic-phase *C. albicans* blastoconidia or germinated cells in 1 mM PPB to a final concentration of 6.7 μg/ml. After 10 min of incubation at 30°C the cells were collected by centrifugation, and suspended in 1 mM PPB to OD_{620} values between 2.0 and 4.0. From these suspensions, 100 μl was added to 100 μl of a dilution series of histatin 5, peptide with N-terminal glycine and C-terminal leucine amide (PGLa), sodium cyanide or sodium azide in 1 mM PPB in a Microfluor black microtiterplate (Dynex, Chantilly, VA). In control experiments without cells, 6.7 μg/ml dihydroethidium was added to a dilution series of histatin 5 in the presence or absence of 5 μg/ml herring sperm DNA (Sigma). Oxidation of the probe was followed at 2.5-min intervals for 15 min at 30°C at λ_{ex} 485 nm and λ_{em} 595 nm, and the kinetics of dye oxidation were calculated for each concentration of histatin 5 (TECAN Spectrofluor Plus fluorimeter and DELTASOFT 3V2.199SPFL software packet, TECAN, Männedorf, Switzerland). In addition to kinetic measurements, endpoint readings were performed after 1 h of incubation of cells with a dilution series of histatin 5, and the calculation of the percent of ROS formed was based on the maximum fluorescence intensity observed. These experiments were also conducted in the presence of 5 mM of the oxygen scavenger L-cysteine (Sigma). Immediately after the fluorescence reading, cells were diluted in PBS and plated on Sabouraud dextrose agar to be able to compare ROS formation with cell killing.

Measurement of ROS Formation in Isolated Mitochondria. Mitochondria were diluted to an OD_{620} of 1.7 in respiration buffer supplemented with 1 mM NADH. In a black microtiter plate, a dilution series of histatin 5 was prepared in respiration buffer, in respiration buffer supplemented with 3 mM of the membrane-permeant superoxide dismutase mimetic 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), and in respiration buffer supplemented with 10 mM L-cysteine (50 μl per well). A total of 50 μl of mitochondria suspension was added to each well, immediately followed by the addition of 5 μl of dihydroethidium to a final concentration of 6.7 μg/ml. Plates were incubated for 30 min at 30°C, after which the fluorescence intensity was determined as described above.

Results

Histatin 5 Effect on Mitochondrial Respiration. Mitochondria were isolated from *C. albicans* by spheroplast formation and a mild homogenization procedure. To verify the integrity of the mitochondrial inner membrane, the "respiratory control rate" and the uncoupling activity of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were determined for each mitochondrial preparation; these values ranged between 2.5 and 2.7 (9), which are normal values for yeast mitochondria (19). As described previously (9), histatin 5 at a concentration of 33 μM inhibits state 2 respiration. Data obtained with six independent mitochondrial preparations showed that histatin 5 also inhibits state 3 respiration (in the presence of ADP) and the respiration of uncoupled mitochondria (i.e., in the presence of CCCP; Table 1). A synthetic control peptide derived from cystatin SA at the same concentration inhibited respiration by $<$5% (data not shown). These results indicate that histatin 5 is an inhibitor of mitochondrial respiration.

Histatin 5 Effect on _C. albicans_ Respiration. To assess the biological significance of the observed inhibitory activity of histatin 5 on isolated mitochondria, similar experiments were conducted with

Table 1. Effect of histatin 5 (33 μM) on mitochondrial respiration

Respiratory state	Inhibition, %
State 2	60.3 ± 8.2
State 3	86.0 ± 5.4
CCCP-uncoupled	83.0 ± 3.0

C. albicans blastoconidia, collected in the logarithmic growth phase. In accordance with the effects of histatin 5 on isolated mitochondria, histatin 5 inhibited cellular respiration. The inhibitory effect was concentration dependent and with higher histatin concentrations (33 μM) complete within 5 min of incubation (Fig. 1). To investigate whether there is an interrelationship between inhibition of cell respiration and killing, a polarographic experiment was conducted in parallel with a colony counting assay in which the percentage inhibition of respiration and the percentage loss in cell viability were determined in the same experimental setup at different time intervals. A nearly perfect correlation between the two parameters was found (Fig. 2). When logarithmic-phase cells were used, the detrimental effect of histatin 5 on both cell viability and respiration was rapid (i.e., minutes) and reached >95%. When stationary-phase cells were used, also an excellent correlation between the percent inhibition of respiration and killing was found, but the stationary-phase cells were overall less sensitive to histatin 5 than were logarithmic-phase cells. With respect to the reduced sensitivity of stationary-phase cells compared with log-phase cells, it is of importance to note that the intrinsic respiratory rate (i.e., before addition of histatin 5) of stationary-phase cells was approximately 1/4 of that of logarithmic-phase cells (Table 2). Although there are many differences between logarithmic- and stationary-phase cells that may explain their different susceptibilities to histatin 5, the influence of the intrinsic respiratory rate may be of crucial importance, as previous studies have shown that cells that do not respire at all are almost completely protected against histatin 5 killing activity (14, 16). In other words, the sensitivity of the cells to histatin 5 decreases with a decreasing respiratory rate. Taken together, these observations point toward the intriguing possibility that the respiratory apparatus itself may be actively involved in initiating cell death. Therefore, we hypothesized that histatin 5 exerts its antifungal activity through the formation of ROS.

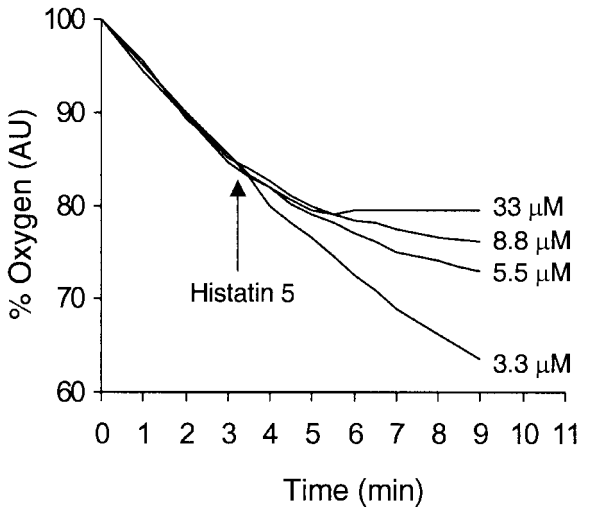

Fig. 1. Concentration-dependent effect of histatin 5 on *C. albicans* respiration. To a suspension of actively respiring logarithmic-phase *C. albicans* blastoconidia in 1 mM PPB, histatin 5 was added to final concentrations of 3.3 μM, 5.5 μM, 8.8 μM, and 33 μM, and the effect on cellular oxygen consumption was monitored. AU, arbitrary units. Curves are representative of four independent experiments performed on different days.

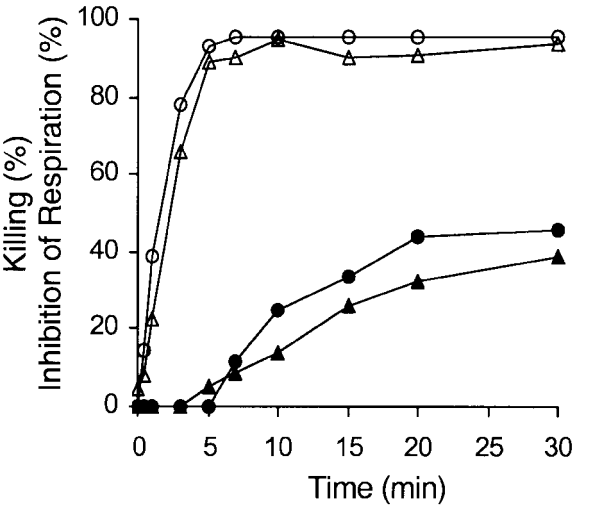

Fig. 2. Histatin 5 effect on logarithmic- and stationary-phase *C. albicans* respiration and viability. Polarographic and viability experiments were performed in a twin oxygen chamber device. Histatin 5 (33 μM) was added to *C. albicans* cells in 1 mM PPB, OD_{620} = 1.7, at time point 0. At various time intervals, the percent inhibition of respiration (circles) and the percent killing (triangles) were determined. Logarithmic-phase cells, open symbols; stationary-phase cells, filled symbols. Represented are the mean of two experiments performed in duplicate.

ROS Formation in *C. albicans* Cells. The fluorescent probe dihydroethidium, suitable for measurement of ROS formation in yeast cells (17) was used to monitor ROS formation in *C. albicans* blastoconidia and germinated cells. Fig. 3*A* shows that histatin 5 causes a concentration-dependent and cell-density-dependent increase in the fluorescence intensity of *C. albicans* blastoconidia as well as of germinated *C. albicans* cells loaded with dihydroethidium. Histatin 5 alone had no effect on the fluorescence properties of the probe (data not shown). These data indicate that histatin induces cell-mediated oxidation of the probe by ROS. ROS formation in both the blastoconidial and the germinated growth form of *C. albicans* is consistent with earlier reports that both forms are sensitive to histatin 5 killing activity (20). In contrast to histatin 5, PGLa, a highly fungicidal peptide that acts by forming membrane-spanning pore complexes (21), did not induce ROS formation (data not shown). Importantly, two conventional inhibitors of the respiratory chain, sodium cyanide and sodium azide, did not induce ROS formation in *C. albicans* (Fig. 3*B*). These data indicated that neither cell death (i.e., PGLa killing) nor inhibition of respiration (i.e., cyanide and

Table 2. Candidacidal activity and inhibition of respiration by histatin 5

	O_2 consumption, nmol/min/OD_{620}		Viable count, × 10^5 per ml	
Cells	Before histatin 5	After histatin 5	Before histatin 5	After histatin 5
Logarithmic phase	21.7 ± 1.6	1.48 ± 0.09	138 ± 4	14 ± 2
Stationary phase	5.75 ± 0.03	5.40 ± 0.03	127 ± 1	122 ± 7

O_2 consumption and viable counts were determined before and after 5 min of incubation with 33 μM histatin 5. Results are presented as mean ± SD.

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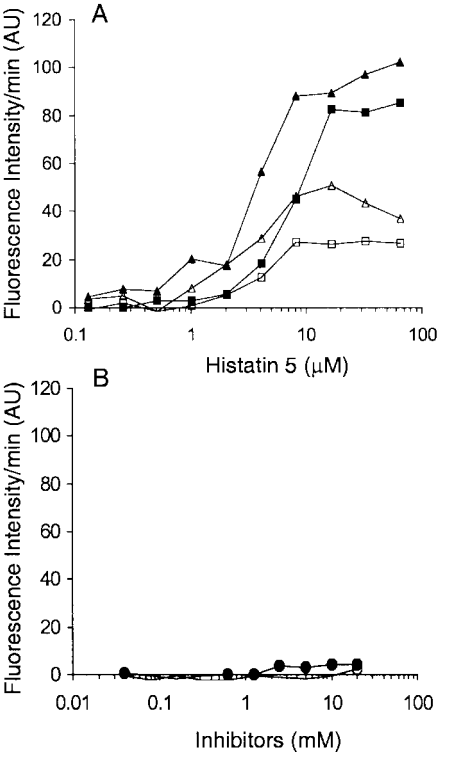

Fig. 3. The formation of ROS in *C. albicans* blastoconidia and germinated *C. albicans* cells by histatin 5 (*A*) and by respiratory inhibitors (*B*). (*A*) Logarithmic-phase *C. albicans* blastoconidia or germinated *C. albicans* cells were loaded with the ROS-sensitive dye dihydroethidium and subsequently exposed to a dilution series of histatin 5 in 1 mM PPB. The kinetics of the oxidation of the probe, indicating the formation of ROS, was followed fluorimetrically and averaged over a 15-min time interval. AU, arbitrary units; squares, *C. albicans* blastoconidia; triangles, germinated *C. albicans* cells; open symbols, cell density OD_{620} = 1.0; filled symbols, cell density OD_{620} = 2.0. (*B*) ROS formation in *C. albicans* blastoconidia, OD_{620} = 1.7, by sodium cyanide (○) and sodium azide (●). Curves represent the mean of two experiments performed in duplicate.

azide), *per se*, leads to the production of ROS and that the effect observed with histatin 5 was specific.

ROS Formation in *C. albicans* Mitochondria. To investigate whether the histatin induced promotion of oxygen radical formation in *C. albicans* blastoconidia occurs at the mitochondrial level, similar experiments were carried out with mitochondria in their isolated form. Histatin 5 added to a suspension of isolated mitochondria in the presence of dihydroethidium caused a concentration dependent increase in its fluorescence, consistent with ROS formation. This effect was abolished in the presence of the oxygen scavenger L-cysteine or the membrane-permeant mimetic of superoxide dismutase 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) indicating that the increase in fluorescence was indeed because of ROS formation (Fig. 4).

Correlation Between ROS Formation and *C. albicans* Killing. The results above suggest that killing of *C. albicans* by histatin is induced by ROS. To further investigate such a histatin-induced cell suicide mechanism, we carried out additional experiments to assess the interrelationship between cell killing and the intracellular formation of ROS. Cells were loaded with dihydroethidium and incubated for 1 h with various concentrations of histatin 5, after which the fluorescence intensity and cell viability of the same inoculum were determined. It was found that the amount of ROS produced, expressed as a percentage of the maximum fluorescence intensity observed after that incubation

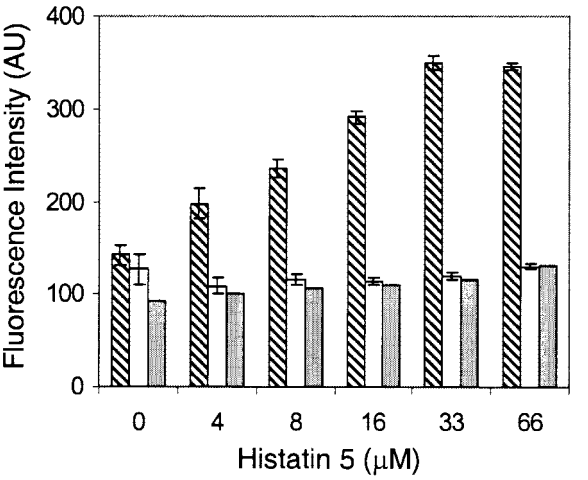

Fig. 4. Mitochondrial ROS formation by histatin 5. Isolated mitochondria respiring in state 2 were added to a dilution series of histatin 5 in the absence or presence of 1.5 mM TEMPO or 5 mM cysteine, immediately followed by the addition of the ROS-sensitive probe dihydroethidium. ROS formation was assessed by a spectrofluorimetric endpoint reading, after 30 min of incubation. Hatched bars, mitochondria exposed to histatin 5 only; white bars, mitochondria exposed to histatin 5 in the presence of TEMPO; gray bars, mitochondria exposed to histatin 5 in the presence of L-cysteine.

period, was highly correlated with cell killing (Fig. 5*A*). When experiments were conducted in the presence of L-cysteine, neither ROS formation nor cell killing occurred (Fig. 5*B*). These data strongly support the hypothesis that ROS formation is the key event in histatin-induced killing of *C. albicans*.

Discussion

Earlier studies have shown that the interaction of histatin 5 with *C. albicans* leads to cellular uptake and mitochondrial targeting of this peptide, ultimately resulting in the loss of cell viability. In the present investigation, the relationship between this specific subcellular targeting and cell death was established. Evidence is presented showing that the respiratory apparatus is the target for histatin 5 and that the derangement of respiratory activity, leading to the formation of oxygen radicals, is the cause of cell death. We therefore propose a histatin-triggered, self-inflicted cell suicide mechanism in which ROS formation is the key element of the histatin 5 killing mechanism (Fig. 6). This model involves the following steps: (*i*) uptake of histatin 5 by means of a receptor-mediated or transmembrane-potential-driven mechanism, (*ii*) targeting of histatin 5 to the mitochondria, (*iii*) inhibition of mitochondrial respiration, (*iv*) generation of ROS by means of out-of-sequence electron transfer to molecular oxygen, and (*v*) cell death because of the oxidation of biological macromolecules and the loss of cellular integrity.

The results of the present investigation identified histatin 5 as an inhibitor of respiration. Our respiratory measurements contrast with previous publications by Edgerton and coworkers, who reported that histatin 5 had virtually no effect on yeast cellular respiration (15, 22). Surprisingly, data generated by this group suggested that cells exposed to histatin were dead and had released all of their ATP, but continued to respire for 1.5 h. Although these observations remain unexplained, the results reported here show that there is an almost perfect correlation between inhibition of respiration and cell killing when either logarithmic-phase or stationary-phase cells were used. It was not clear at first sight, however, how blockage of the respiratory chain alone would lead to cell death, because *Candida*, like most yeasts, is fully capable of fermentation. For example, high concentrations of respiratory inhibitors (azide, cyanide, antimy-

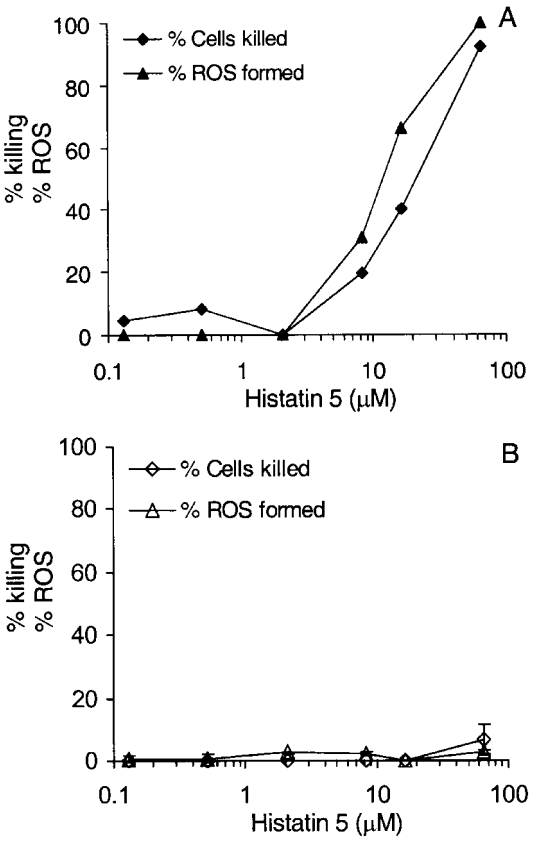

Fig. 5. Comparison of histatin-induced killing and ROS formation in *C. albicans*. *C. albicans* blastoconidia were preincubated with dihydroethidium and subsequently exposed to a dilution series of histatin 5 in 1 mM PPB in the absence (*A*) and presence (*B*) of 5 mM L-cysteine. The total amount of intracellular ROS, formed after 1 h of incubation, was determined spectrofluorimetrically for each histatin 5 concentration. Values are expressed as percentages of the maximum fluorescence intensity observed. The percent killing of the same inoculum of cells was determined in a colony-counting assay. Values represent the mean of two experiments performed in duplicate.

cin, or combinations of these agents) are not fungicidal (23). The observation that histatin 5, but neither sodium azide nor sodium cyanide, generates ROS is consistent with the fact that histatin 5 is fungicidal, whereas these conventional respiratory inhibitors are not. Therefore, the inhibition of respiration afforded by histatin 5 should be considered different in principle from that caused by cyanide and azide. Interestingly, the cationic pore-forming antifungal peptide PGLa did not induce ROS, further supporting that different mechanisms of action underlie the fungicidal activities of PGLa and histatin 5 (9).

The interference of histatin 5 with the respiratory chain and the formation of ROS indicate the out-of-sequence electron transfer from some carrier in the respiratory chain to molecular oxygen. The specific site of inhibition of histatin 5 has yet to be identified. In general, it has been shown that mitochondrial ROS is formed both at the NADH:ubiquinone reductase level (complex I) and the ubiquinol:cytochrome *c* reductase level (complex III) (24, 25). Under normal physiological conditions, the highly reactive CoQ semiquinone radical ($CoQ^{\bullet -}$) is rapidly neutralized when a second electron is transferred to cytochromes b_{566} and b_{562}, thereby regenerating the oxidized form of CoQ (the Q cycle). In mammalian cells, inhibitors that bind to cytochrome b_{562} and prevent cytochrome *b* reoxidation, such as antimycin A, lead to the accumulation of the unstable $CoQ^{\bullet -}$ radical, and subsequent random collisions of this radical with molecular oxygen will form various ROS (24, 25). It is feasible that histatin 5 generates ROS by means of a similar mechanism. A highly conserved target such as coenzyme Q, however, would not explain the apparent specificity of histatins 5 for yeast-cell killing and absence of mammalian-cell killing. It is possible that such specificity is accomplished at a different level, e.g., by species-specific cell surface receptors or specific peptide translocation mechanisms. Additional studies are required to elucidate the basis for this histatin 5 specificity.

We previously demonstrated that addition of histatin 5 to *C. albicans* cells leads to the release of rhodamine 123 from mitochondria, indicating the dissipation of the mitochondrial transmembrane potential, $\Delta\Psi_{mito}$ (14). Here we present evidence that histatin 5 has no direct membrane-permeablizing

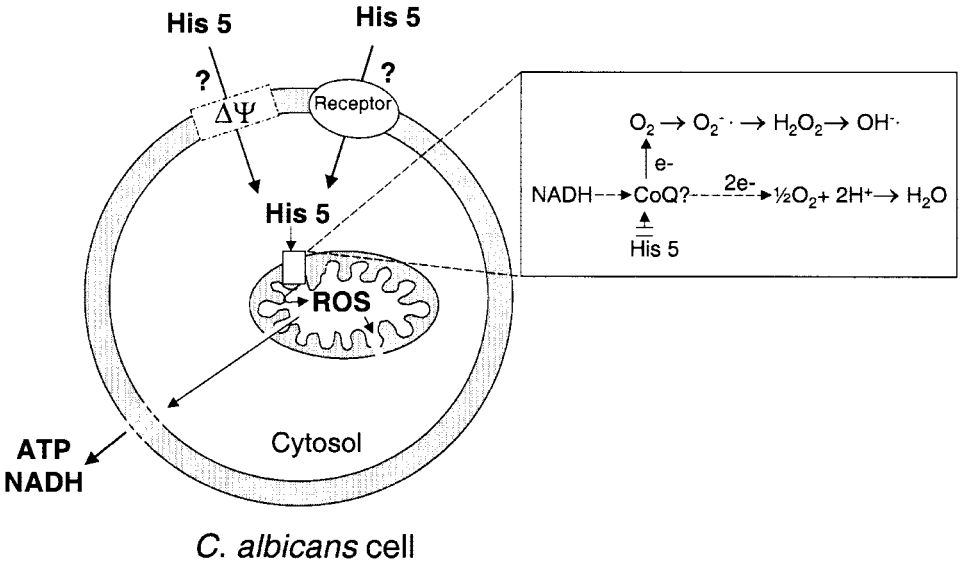

Fig. 6. Mechanistic model for the fungicidal activity of histatin 5. The cationic peptide histatin 5 is internalized by *C. albicans* (13, 14), a process that either may involve a nonselective histatin 5-binding cell surface receptor protein (22) or may be driven by the cytoplasmic, inside negative transmembrane potential ($\Delta\psi$). Once inside the cell, histatin 5 targets the mitochondria (14), inhibits respiration, possibly at the coenzyme Q level, and induces the formation of ROS. The oxidation of phospholipids and other macromolecules by these radicals leads in rapid succession to mitochondrial membrane damage, cytoplasmic membrane damage (14), efflux of various nucleotides (15, 22), and cell death.

effect (no uncoupling effect) on yeast mitochondria in their isolated form (Fig. 1 and ref. 9). It is possible that the dissipation of $\Delta\Psi_{mito}$ in cells is a result of the inhibition of electron transport. Alternatively, the dissipation of $\Delta\Psi_{mito}$ is due to mitochondrial membrane permeabilization, but occurs as a secondary event after histatin 5 exposure. Such membrane-permeabilizing effects are consistent with the fact that ROS are formed because it is well recognized that a local accumulation of such radicals will rapidly lead to disintegration of biological membranes. With respect to the above-mentioned membrane effects, it should be noted that the primary site of action of ROS will be in close proximity to their site of generation, the mitochondrion, because of the high reactivity of ROS (26). Indeed, the cell cytoplasmic membrane, distant from the site of oxygen radical generation, is only moderately affected shortly after exposure of cells to histatin 5 (9–11). These minor cytoplasmic membrane permeability changes are reflected by the influx of the cell necrosis marker propidium iodide (14) and the efflux of intracellular ATP (15, 22). The close correlation between the release of these intracellular constituents and cell death (22) is an indication that the efflux of such molecules is not compatible with cell viability.

It has become evident that histatin 5 causes the release of not only ATP but of a variety of nucleotides (C. Gyurko, E.J.H., R.F.T., and F.G.O., unpublished data). It has been suggested that one of these nucleotides, ATP, is not only released from the cell, but actually serves as an effector molecule by binding to purinergic receptors, and that this event in some way is responsible for causing cell death (15). Although the release of such nucleotides, including ATP, is likely part of a cascade of events that occur during the killing process, the purinergic receptor model for histatin killing does not adequately explain the requirement for cellular respiration in the sensitivity of the cells to histatin 5. The ROS model for histatin killing as outlined in Fig. 6 is consistent with the protective effects of anaerobiosis and "petite" mutations of *C. albicans* (16, 18), and has become attractive because the present weight of evidence from studies by us (9, 13, 14, 16) and others (12, 15, 22) are consistent with this model.

It is of great interest that the restricted conditions under which histatin 5 is active are not unique to this peptide. A number of reports have appeared showing that the activity of a number of toxins from either plant, bacterial, or human origin, are dependent on active participation by the target cell. For example, studies on human defensins (HNP1, -2, and -3)-mediated cytotoxicity showed the requirement for peptide internalization and for target cell metabolic processes (27). Other examples are the candidacidal activity of HNP-1, which is negatively affected in the presence of respiratory inhibitors (28), and the activity of killer toxin from *Pichia kluyveri* against *Saccharomyces cerevisiae* cells that is highly dependent on the physiological state of cells (29) in a very similar fashion as that described for histatin 5. It is therefore feasible that the present discovery that ROS formation is a quintessential step in the killing process provoked by histatin 5 is not restricted to histatin 5 activity, but may represent a common mechanism of target-cell killing afforded by agents that are dependent on active cell metabolism.

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