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Mechanism of Action of Miconazole: Labilization of Rat Liver Lysosomes In Vitro by Miconazole

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Miconazole, a potent antifungal agent, labilizes rat liver lysosomes. Its labilizing effect is followed by measuring the release of lysosomal hydrolases, namely, acid phosphatase, β-glucuronidase, and arylsulfatase A. The effect of miconazole is concentration dependent in the range of 10^{-5} to 1.2×10^{-4} M. However, at higher concentrations, miconazole inhibits enzyme release but does not inhibit enzyme activities per se. The effect of miconazole depends on the drug/lysosome ratio and is influenced by the pH of the incubation media, being minimal at alkaline pH. Membrane-active drugs such as nystatin, 2-phenethylalcohol, hexachlorophene, and digitonin have been compared with miconazole for their lysosome-labilizing action. The effect of miconazole on the lysosomal membrane is confirmed by a decrease in turbidity of the lysosomal suspension.

Miconazole {1-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)-phenethyl]imidazole nitrate} has a broad spectrum of antimicrobial activity against pathogenic and nonpathogenic yeasts, dermatophytes, numerous saprophytic fungi, and gram-positive bacteria (13, 25, 31). Chemotherapeutic activity of miconazole as a topical applicant is well documented in the treatment of skin and nail infections and vaginal candidiasis (2, 5, 14, 29, 32). Studies on the mechanism of biological action of miconazole have been recently reported (9, 10, 28, 33; K. H. Sreedhara Swamy, M. Sirsi, and G. Ramananda Rao, Biochem. Pharmacol., in press). The drug induces leakage of intracellular materials from cells of *Candida albicans* (28), and at low concentrations it selectively inhibits the uptake of purines and glutamine into these cells (33). Electron microscopic examination of cells of *C. albicans* exposed to miconazole revealed that the earliest drug-induced alterations are seen at the plasma membrane (9, 10). Further, miconazole has been shown to induce hemolysis of mammalian erythrocytes and binds strongly to erythrocyte membrane lipoproteins (Sreedhara Swamy et al., in press). These investigations clearly indicate that the paramount feature of the biological action of miconazole is its interaction with cell membrane of sensitive organisms, resulting in the impairment of membrane function and eventually cell death.

In an attempt to delineate the mode of action of miconazole on cellular and organellar membranes and to obtain a more comprehensive view of biological action, we have carried out studies on the interaction of miconazole with rat liver lysosomes. The present paper describes the effect of miconazole on the integrity of lysosomal membrane, providing further evidence that miconazole interacts with biological systems by impairing membrane function.

MATERIALS AND METHODS

Chemicals. Miconazole nitrate was a gift sample from Ethnor Ltd., Bombay, India. Hexachlorophene, digitonin, 2-phenethylalcohol, *p*-nitrophenylphosphate, *p*-nitrocatechol sulfate, phenolphthalein-β-D-glucuronide, Triton X-100, and tris(hydroxymethyl)-aminomethane (Trizma base) were purchased from Sigma Chemical Co., St. Louis, Mo. Nystatin was kindly donated by E. R. Squibb and Sons, Inc., Princeton, N.J. All other chemicals were of analytical reagent grade.

Preparation of rat liver lysosomes. Inbred Wistar A/Iisc rats weighing 100 to 120 g were killed by cervical dislocation, and the liver was quickly dissected out into ice-cold 0.15 M NaCl (isotonic saline). The liver was washed twice with 0.15 M NaCl, weighed, minced finely with scissors, and suspended in 0.25 M sucrose. The liver was homogenized in 0.25 M sucrose (5 ml of solution per g of liver) using a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle. The homogenate was first centrifuged at $1,500 \times g$ in a Sorvall centrifuge, model RC 2-B, for 10 min at 4 C to sediment unbroken cells and nuclei. The supernatant was then centrifuged at $20,000 \times g$ for 30 min, and the pellet containing the lysosomes was suspended gently in 0.25 M sucrose to give a final concentration of 10 mg of protein per ml.

Effect of miconazole on rat liver lysosomes. The effect of miconazole on lysosomes was followed by measuring the release into the medium of lysosomal hydrolases. Rat liver lysosomes (0.5 mg of protein per ml) were incubated in 0.25 M sucrose containing

miconazole (dissolved in 50% ethanol) at various concentrations for 15 min at 37 C. All incubation mixtures, including controls, contained ethanol at a final concentration of 1%. After the incubation, the tubes were chilled in ice and centrifuged at 20,000 × *g* for 20 min, and the resulting supernatants were assayed for acid phosphatase, β-glucuronidase, and arylsulfatase A. The enzyme activity in the supernatant is expressed as percentage of total activity obtained in the presence of 0.1% Triton X-100. The data were corrected for the release of enzymes in control samples.

Enzyme assays. Acid phosphatase activity was determined by the method of Igarashi and Hollander (17), using *p*-nitrophenyl phosphate as substrate.

The reaction mixture for β-glucuronidase assay in 1 ml contained 30 mM acetate buffer, pH 4.5, 0.5 ml of the supernatant, and 0.4 mM phenolphthalein-β-D-glucuronide (sodium salt). The reaction mixture was incubated at 37 C for 30 min, and the reaction was stopped by adding 5 ml of 0.2 M glycine-NaOH buffer, pH 10.4. The absorbancy of the color was measured at 540 nm.

Arylsulfatase A was estimated by the method of Jerfy and Roy (18), using *p*-nitrocatechol sulfate as substrate.

Protein was estimated by the method of Lowry et al. (22).

RESULTS

Effect of miconazole on rat liver lysosomes. The time course of miconazole-induced release of lysosomal enzymes is shown in Fig. 1. At a miconazole concentration of 5×10^{-5} M, the rate of release of acid phosphatase and arylsulfatase A showed an increase up to 40 min and thereafter remained constant. On the other hand, the release of β-glucuronidase reached maximum by 30 min and showed no further significant increase up to 60 min of incubation. The release of acid phosphatase and arylsulfatase A by miconazole at 60 min is about 44%, and that of β-glucuronidase is 32% of the total enzyme activity present in the lysosomes.

The effect of increasing concentrations of miconazole on the release of acid phosphatase, arylsulfatase A, and β-glucuronidase from lysosomes is shown in Fig. 2. The lysosomes were incubated with various concentrations of miconazole in 0.25 M sucrose for 15 min. Miconazole caused an increased release of all three lysosomal enzymes up to a concentration of 1.2×10^{-4} M, and further increase in the drug concentration showed decreasing enzyme activities in the supernatant.

The labilization of lysosomes is dependent not only on the concentration of miconazole, but also on the amount of lysosomes in the incubation medium. Increase in the lysosomal protein concentration (number of lysosomes) per unit volume of suspending medium at a constant miconazole concentration caused a progressive decrease in the release of enzymes from lysosomes (data not shown).

Influence of pH on the miconazole-induced labilization of lysosomes. The effect of miconazole on lysosomes is dependent on the pH of the incubation media (Table 1). The release of lysosomal enzymes by miconazole was equally effective at pH 5.0 (0.25 M sucrose-0.01 M acetate) and pH 6.8 (unbuffered 0.25 M su-

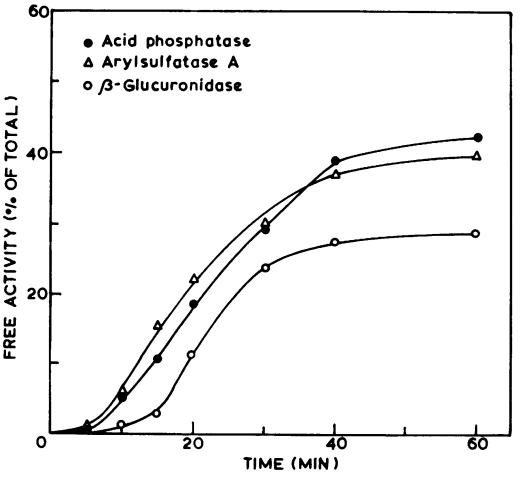

FIG. 1. *Time course of miconazole-induced lysis of rat liver lysosomes. Miconazole concentration, 5 × 10^{-5} M.*

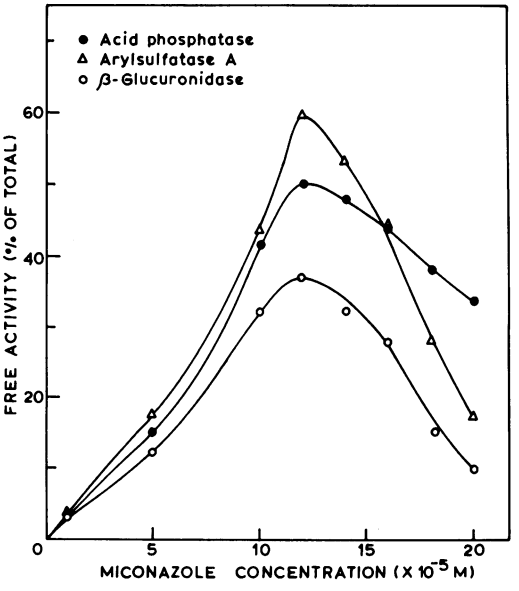

FIG. 2. *Effect of miconazole concentration on the release of lysosomal enzymes. Incubation time, 15 min at 37 C.*

TABLE 1. *Influence of pH of the incubation medium on the release of lysosomal enzymes by miconazole*[a]

Incubation medium	Free activity (% of total)					
	Acid phosphatase		Arylsulfatase A		β-Glucuronidase	
	5×10^{-5} M[b]	10^{-4} M	5×10^{-5} M	10^{-4} M	5×10^{-5} M	10^{-4} M
0.25 M sucrose-0.01 M acetate (pH 5.0)	11.6	30.8	15.8	46.2	16.0	35.7
0.25 M sucrose (pH 6.8)	17.0	44.0	17.0	43.8	12.0	32.0
0.25 M sucrose-0.01 M Tris-hydrochloride (pH 8.0)	5.0	16.5	10.3	20.9	5.5	19.4

[a] Lysosomes (0.5 mg of protein per ml) were incubated at different pH values with the indicated concentration of miconazole for 15 min at 37 C. After centrifugation at 20,000 × *g* for 20 min at 4 C, the enzyme activities released into the supernatants were determined. Total activity in each sample was measured by incubation of lysosomes with 0.1% Triton X-100. After correction for release of enzymes in controls containing 1% ethanol, the data were expressed as percentage of total enzyme activity. Tris, Tris(hydroxymethyl)aminomethane.

[b] Miconazole concentration.

crose), but the extent of release was reduced at pH 8.0 [0.25 M sucrose-0.01 M tris(hydroxymethyl)aminomethane-hydrochloride].

Decrease in turbidity of lysosomal suspension caused by miconazole. Incubation of lysosomes with miconazole in 0.25 M sucrose resulted in a decrease in the lysosomal turbidity. It was measured at 25 C by adding lysosomes to 0.25 M sucrose containing miconazole, and the absorbancy of the suspension was measured at 520 nm in a Carl-Zeiss spectrophotometer at different time intervals. The decrease in turbidity of the lysosomal suspension after 2 min was about 12 and 26% at miconazole concentrations of 5×10^{-5} M and 10^{-4} M, respectively. Under similar conditions, 0.1% Triton X-100 decreased the turbidity of lysosomal suspension by about 71%.

Lysosome labilizing action of miconazole as compared with other membrane-active drugs. For comparison, the effect of some membrane-active drugs such as nystatin, 2-phenethylalcohol, hexachlorophene, and digitonin on rat liver lysosomes was studied (Table 2). Nystatin was relatively ineffective in releasing enzymes from lysosomes. 2-Phenethylalcohol required a very high concentration (5×10^{-2} M) to induce drastic changes in lysosomal integrity, resulting in the release of lysosomal enzymes. Both hexachlorophene and digitonin disrupted lysosomes, and at 10^{-4} M the lysosome labilization brought about by these drugs and miconazole was quite similar.

TABLE 2. *Comparative effect of miconazole with some membrane-active drugs on labilization of rat liver lysosomes*[a]

Drugs	Concn (M)	Free enzyme activity (% of total)		
		Acid phosphatase	Arylsulfatase A	β-Glucuronidase
Nystatin	1×10^{-4}	1.70	0.97	2.40
2-Phenethylalcohol	5×10^{-4}	5.30	2.90	5.80
	1×10^{-4}	2.40	1.10	2.90
	5×10^{-3}	5.40	1.60	11.70
	5×10^{-2}	59.20	38.20	25.40
Hexachlorophene	1×10^{-5}	8.10	7.40	7.80
	1×10^{-4}	35.60	34.80	58.80
Digitonin	1×10^{-5}	2.00	1.00	11.70
	1×10^{-4}	46.70	40.00	63.70
Miconazole	1×10^{-5}	4.20	4.40	3.40
	1×10^{-4}	44.00	43.80	32.00

[a] Lysosomes (0.5 mg/ml) were incubated for 15 min in 0.25 M sucrose containing different concentrations of drugs (as indicated in the table) and centrifuged at 20,000 × *g* for 20 min at 4 C. The enzyme activities in the supernatant were determined as described in Materials and Methods. Total enzyme activity was measured by incubation of lysosomes with 0.1% Triton X-100. Nystatin was dissolved in dimethylformamide. Digitonin, hexachlorophene, and miconazole were dissolved in 50% ethanol, and dilutions of 2-phenethylalcohol were made in 30% ethanol. The control samples contained solvents at concentrations present in experimental tubes.

DISCUSSION

The data presented in this paper clearly reveal that miconazole has a profound effect on lysosomal membrane and causes release of acid phosphatase, β-glucuronidase, and arylsulfatase A from lysosomes. Its effect is concentration dependent, and, when lysosomes are exposed to different concentrations of miconazole, an opti-

mum concentration for lysis is reached (1.2×10^{-4} M) instead of a saturation response (Fig. 2). The release of enzymes is reduced beyond this optimum concentration. The decreased enzyme activities in the supernatant at higher concentrations of miconazole is apparently due to its interference with release of enzymes, since the drug failed to inhibit enzyme activities per se (data not shown).

Turbidity of lysosomes often serves as an indication of their structural integrity. Miconazole decreased the turbidity of lysosomal suspension, thus providing evidence for its effect on lysosomal membrane structure.

The lysosome-labilizing effect of miconazole was compared with that of nystatin, 2-phenethylalcohol, hexachlorophene, and digitonin. Nystatin, a polyene antibiotic, impairs cell membrane function by binding to sterols in the membrane of susceptible organisms (15, 20). Nystatin is relatively ineffective in releasing enzymes from lysosomes. The data is consistent with the previous finding (34) that the high-molecular-weight group of polyenes (nystatin and amphotericin B) are least effective in disrupting lysosomes. 2-Phenethylalcohol, which is known to interact with the cell membrane of bacteria (26, 30), yeasts (6; T. K. Narayanan, Ph.D. thesis, Indian Institute of Science, Bangalore, India, 1975), fungi (21), tumor cells (4), and mammalian erythrocytes (3; Sreedhara Swamy et al., in press), requires very high concentrations to disrupt lysosomes. Earlier, 2-phenethylalcohol was shown to release acid phosphatase from chicken liver lysosomes at high concentrations (16).

Hexachlorophene and digitonin are included in the present studies because they have been shown to interact with various biological systems by impairing cell membrane function. Hexachlorophene has been shown to alter the permeability of plant (24), bacterial (8, 19, 27), and mammalian erythrocyte membranes (7, 12, 23). Digitonin, a plant saponin, induces membrane damage by binding to cholesterol in the membrane (1, 11). The results presented in this paper clearly show that both hexachlorophene and digitonin disrupt rat liver lysosomes and release enzymes. These compounds both exert a maximum release of β-glucuronidase when compared with the release of acid phosphatase and arylsulfatase A. In contrast, miconazole and 2-phenethylalcohol released β-glucuronidase to a lesser extent than did the other two enzymes. Thus, the differential effects showed by these drugs on lysosomes appear to be drug specific.

In conclusion, the present findings and the earlier studies on the action of miconazole on membranes of yeasts (9, 10, 28, 33) and mammalian erythrocytes (Sreedhara Swamy et al., in press) show that the drug interacts with both cellular and subcellular membranes.

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