Study of Sterol Levels On The Transport Of Amino Acids

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ABSTRACT: Ergosterol and cholesterol supplementation resulted in a significant increase (1-5- fold) in the sterol content while phospholipid remained unaffected in *Microsporum gypseum.* The levels of phosphatidylethanolamine and phosphatidylcholine increased in ergosterol supplemented cells. However, a decrease in phosphatidylcholine and an increase in phosphatidylethanolamine was observed in cholesterol grown cells. The ratio of unsaturated to saturated fatty acids decreased on ergosterol/cholesterol supplementation. The uptake of amino acids (lysine, glycine and aspartic acid) decreased in sterol supplemented cells. Studies with fluorescent probe l-anilinonaphthalene-8-sulfonate showed structural changes in membrane organisation as evident by increased number of binding sites in such cells..

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1. INTRODUCTION

A nystatin-resistant mutant of Aspergillus niger has been isolated and used as a model system to study the effect of altered sterol levels on lipid composition, transport behavior and physical properties of membrane lipids. There is a decrease in the sterol to phospholipid ratio in the mutant compared to the wild type. Although there is no qualitative change in phospholipid composition, the mutant contains a higher amount of phosphatidylcholine and a lower amount of phosphatidylethanolamine compared to the wild type. The most significant change is the elevated level of linoleic acid in the mutant, concomitant with a decreased level of oleic acid. These adaptive changes to nystatin resistance are manifested in the altered thermotropic behavior of membrane lipids as studied by the steady-state fluorescence polarization technique. These changes are also associated with altered membrane permeability as evidenced by the change in Vmax values for uptake of some amino acids in the mutant compared to the wild type.

Sterols, one of the essential components of eukaryotes impart the mechanical strength to the membrane due to their ordered packing in phospholipid bilayer which is essential to maintain the structural integrity of the cell (Park, 1978). These components control the permeability of the membrane in conjuction with acyl groups of phospholipids (Bloch, 1983; Prasad, 1985). It has been demonstrated that sterols influence membrane function such as passive transport, carrier mediated transport and enzymatic activity of membrane bound enzymes (Demel and Kruyff, 1976). Polyene antibiotics have been extensively used to study the importance of sterols in fungal membranes as membrane sterols are known to interact with them (Gottlieb *et al.,* 1958; Lampen *et al.,*

1960). Lipid metabolism and influence of phospholipid polar head groups and fatty acyl chains on the permeability properties of membranes in dermatophytes have been studied (Larroya and Khuller, 1985,1986; Pandey *et al.,* 1987), whereas no information is available on the role of sterol on permeability behaviour of membranes of fungi except *Candida albicans* and *Aspergillus niger* (Singh *et al.,* 1979a, b; Mazumdar *et al.,* 1987). In this study *Microsporum gypseum,* a dermatophyte, was grown in the medium supplemented with ergosterol (a natural sterol) and cholesterol (a sterol foreign to fungus), in order to examine the sterol induced changes in membrane lipid composition and its impact on the structure and function of membrane of this fungus.

2. MATERIALS AND METHODS

Protection of 3-Hydroxy Group of 1-Substituted Glycerol: A mixture of 1-substituted glycerol and triphenyl chloride (1.5 equiv.) in anhydrous pyridine was stirred at 50° C. for 18 h under anhydrous condition. After cooling to room temperature (r.t., ca. 23° C.), the mixture was poured into ice-cold water, and extracted with 3 portions of hexane. Undissolved triphenylmethanol was removed by filtration. The filtrate was washed 3 times with water and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was dissolved in minimum amount of hexane. Additional triphenyl methanol was precipitated from the solution by standing overnight at 4° C. The solid was filtered off, and the filtrate was evaporated to dryness. The residue was dried over high vacuum and used directly for next step reaction. The reaction was monitored by TLC and yield was generally 80-90%.

Removal of Trityl Group from 1,2-Substituted-3-Trityl Glycerol: 1,2-substituted-3-trityl glycerol in chloroform was treated with boron trifluoride diethyl etherate (4 equiv.) at 0° C. for 3 h. The solution was washed with water/chloroform/methanol (2:2:1). The organic layer was dried over sodium sulfate and evaporated. The residue was dried and used directly for next step reaction. The reaction was monitored by TLC and yield was above 90%.

Phosphorylation of 1,2-Substituted-Glycerol: A solution of 1,2-substituted glycerol and anhydrous pyridine (2 equiv.) in anhydrous tetrahydrofuran (THF) was added dropwise to the freshly distilled phosphorus oxychloride (1.1 equiv.) in THF with stirring at 0° C. Stirring was continued for 2-3 h at 0° C. Then 10% sodium bicarbonate (ca. 5 equiv.) was added, and the mixture was stirred for 15 min at 0° C. The solution was then poured on ice water, acidified with HCl (pH ca. 2), and extracted with diethyl ether. The product in aqueous layer was precipitated by adding acetone into water. The precipitate was combined with product from the ether extract, azeotropically dried with toluene, and used directly for next step reaction. Yield was generally above 90%. 1,2-Substituted-Glycero-Phosphocholine: 1,2-Substituted-glycero phosphate, choline tetraphenyl borate (2 equiv.) and 2,4,6-triisoproylbenzene sulfonyl chloride (TPS) (2.5 equiv.) were dissolved in anhydrous pyridine with brief warming, then stirred for 1 h at 70° C. and 3 h at room temperature. After the addition of water, the solvents are removed by rotary evaporation. The residue was extracted with diethyl ether twice. The extract was combined and evaporated. The crude product was purified by HPFC. Yield of this step is generally 80-90%.

Radiolabelled [U-^{14}C]-L -aspartic acid, [U-^{14}C]-L-lysine and [U-^{14}C]-glycine were procured from Bhabha Atomic Research Centre, Bombay. Cholesterol, ergosterol, L-lysine, glycine and L-aspartic acid were obtained from Sigma Chemical Co., St. Louis, Missouri, USA, Novozyme, 234, was procured from Novo-industries, Baegsvaerd, Denmark. Cellulase 'CP' was obtained from John and E.

Sturge, North Yorkshire England. l-Anilinonaphthalene-8-sulfonate (ANS) was a product of Fluka, Switzerland.

Growth of culture

M. gypseum, obtained from the Mycological Reference Laboratory, School of Hygiene and Tropical Medicine, London was grown as shaking cultures in Sabouraud's broth (4% glucose, 1 % peptone, pH 5-4-5-6) at 27°C. Varied concentrations of ergosterol/cholesterol in ethanol (0-1%) were added to the growth medium before inoculation. Cells were harvested in the mid log phase (4 days) and processed further as per the following methods.

Quantitation off lipids

Lipids were extracted by the method of Folch *et al.* (1957). Phospholipids were quantitated by the method of Marinetti (1962). Individual phospholipids were separated by single dimensional thin-layer chromatography in chloroform: methanol: 7N ammonia (65:25:4, v/v). Methyl esters of phospholipid fatty acids (separated from the neutral lipids by acetone precipitation) were prepared by transesterification with methanol in the presence of thionyl chloride (Khuller *et. al.,* 1981) and were resolved on a Perkin Elmer gas liquid Chromatograph fitted with a 0 V-225 column at 190°C using nitrogen as a carrier gas (the flow rate was 40 ml/min). Fatty acid methylesters were identified by comparison of their retention times with that of authentic standards. Fatty acids were quantitated by triangulation of peak area.

Quantitation of sterols

Sterols were extracted by boiling the cells in alcoholic KOH for 1 h (Singh *et al.,* 1979).The hydrolysates were cooled and extracted thrice with petroleum ether and quantitated by the method of Zlatkis *et al.* (1953).

Uptake studies of amino acids

This was examined by incubating 1 ml of cell suspension (40-50 mg fresh weight of cells in 1 ml of citrate phosphate buffer, pH 6-5) at 27°C for different time periods. The reaction was initiated by addition of labelled amino acid 5 mM (specific acitivity 120 ^Ci/mmol) and was stopped by diluting it with chilled normal saline and filtering through 0-45 *fi*M millipore membrane filters. After washing 2-3 times with chilled normal saline (0-85% NaCl), filters were dried, weighed and counted in toluene based scintillation fluid.

Preparation of spheroplasts

Spheroplasts were prepared according to the method of Larroya *et al.* (1984). Cultures were incubated under sterile conditions with 30 mg of each Novozym '234' and cellulase 'CP' in 10 mM citrate-phosphate buffer (pH 6-5) containing 0-7 M NaCl for 18 h at 30°C. Formation of spheroplasts was monitored microscopically. The incubation mixture was centrifuged at 1000 g for 10 min and the supernatant containing the cell wall degrading enzymes was discarded. Pellet was washed twice with the buffer and the spheroplasts were purified by centrifugation on a Ficoll-paque gradient at 400 g for 15 min. The purified spheroplasts free of cell debris recovered from the supernatant were used for further studies.

Structural studies

A fluorescent probe, ANS was used for structural studies. The basic assay mixture consisted of a total volume of 2 ml in 10 mM citrate phosphate buffer, pH 6-5 containing 0-7 M sodium chloride, 10 *f*M ANS and spheroplast protein ranging from 50-200 fg. The fluorescence emission was recorded on a Kontron SFM-25 spectrofluorimeter. The number of binding sites were calculated from Scatchard plot analysis (Azzi, 1974). Spheroplast protein was

estimated by the method of Lowry *et al.* (1951). The statistical significance of the results was tested by Student's *t* test.

3. RESULTS AND DISCUSSION

Changes in the levels of sterols are likely to alter the structural and functional aspects of membranes. In this study, *M. gypseum* was grown in the medium supplemented with different concentrations of cholesterol and ergosterol. Optimum concentrations of cholesterol and ergosterol were determined by measuring the levels of total sterols and phospholipids in supplemented cells and the concentration which induced maximum alteration in sterol levels with marginal changes in phospholipids was selected. Figure 1 shows that 50 and 80 fg/ml of ergosterol and cholesterol, respectively were the optimum concentrations as maximum changes in sterols were seen at these concentrations.

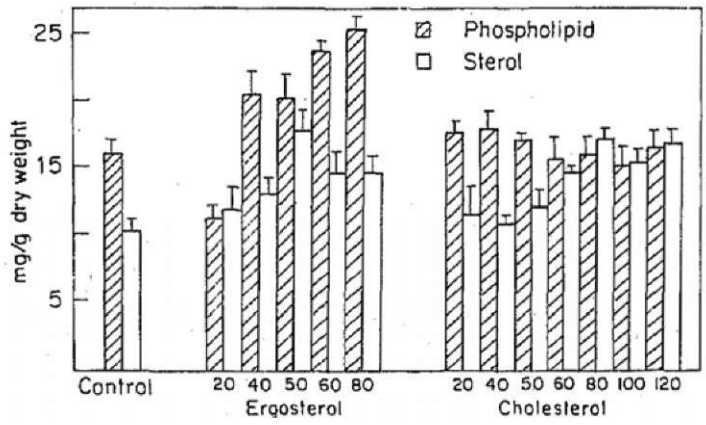

Figure 1. Effect of different concentrations of ergosterol (50 μg/ml) and cholesterol (80 μg/ml) on total phospholipid and sterol content of *M. gypseum.* Values are mean ± SD of 3 batches.

The optimum concentration determined for cholesterol was much higher as compared to ergosterol which is probably due to the different behaviour of ergosterol and cholesterol. In an earlier study, Pinto *et al.* (1985), have reported that ergosterol is taken up by *Saccharomyces cerevisiae* cells up to a certain concentration of exogenous sterol, while cholesterol content plateau at a much higher concentration of sterol in the medium. In addition, uptake of sterol from the medium through the plasma membrane and cell wall need not be equivalent for all sterols as ergosterol partitions through phospholipid bilayer with great difficulty in comparison with cholesterol (Park, 1978). Both these sterols at optimum concentration had a stimulatory effect on growth of *M. gypseum* which is unexplainable. However, the stimulatory effect was more with cholesterol which is similar to the observation of Wright *et al.* (1983), where ergosterol when added alone or in combination with fatty acids had a slight but consistant stimulatory effect on the growth of *Talaromyces thermophilus.*

Total sterols increased (1-5-fold) significantly on supplementation of ergosterol and cholesterol while no change was seen in total phospholipids (table 1). These results indicate the capacity of *M. gypseum* to take up sterols from the medium. Alterations were also observed in the individual phospholipid composition (table 2).

	Total phospholipids	Total sterol
	mg/g dry wt.	
Control	16·14 ± 1·21	10·35 ± 1·39
Cholesterol[a]	16·30 ± 1·49[NS]	16·69 ± 0·83[b]
Ergosterol[a]	20·48 ± 2·48[NS]	17·79 ± 1·78[a]

Table 1. Effect of sterol supplementation on phospholipid and sterol content of *M. gypseum.*

Values are meanSD of 4 independent batches. ± NS, Not significant. [a]Optimum concentrations (50 and 80 μg/ml of

ergosterol and cholesterol, respectively were used). $^{b}P \leqslant 0{\cdot}01$.

Phospholipid fraction	Control	Ergosterol[a] (mg phospholipids/g dry wt.)	Cholesterol[a]
LPC	2·83 ± 0·15	2·89 ± 0·18^{NS}	3·24 ± 0·73^{NS}
PS + PI	4·28 ± 0·82	4·5 ± 0·38^{NS}	3·76 ± 0·55^{NS}
PC	6·23 ± 0·12	8·15 ± 0·90^{b}	5·03 ± 0·36^{c}
PE	2·22 ± 0·35	3·70 ± 0·56^{b}	3·22 ± 0·46^{b}
Unknown PL	1·15 ± 0·07	0·38 ± 0·05^{c}	1·07 ± 0·09^{NS}
LPC + PC + PE/PS + PI	2·64	3·28	3·05

Table 2. Effect of sterol supplementation on individual phospholipid composition of *M. gypseum*

Values are mean ± SD of 4 independent batches. NS, Not significant. [a]Optimum concentrations (50 and 80 *f*g/ml of ergosterol and cholesterol, respectively were used). $^{b}P \leqslant 0{\cdot}01$; $^{c}P \leqslant 0{\cdot}001$.

The levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) increased significantly while uncharacterized phospholipid decreased on ergosterol supplementation. The increase in PC level on ergosterol supplementation is similar to the observations of Kawasaki *et al.* (1985) where ergosterol has been found to stimulate the methylation of PE to PC in yeast mutant GL 7. On the other hand, a decrease in PC and an increase in PE levels were observed on cholesterol supplementation. Sterol supplementation also induced changes in the membrane surface charge as observed by alterations in ratio of zwitterionic to anionic phospholipids. Alterations observed in individual phospholipid components as well as their acyl group composition indicate the capacity of the cell to adjust its phospholipid composition in such a manner so as to maintain the normal functioning of the cell.

Phospholipid fatty acid composition did not alter on sterol supplementation. However, a significant decrease in the ratio of unsaturated to saturated fatty acids was seen (table 3), which was mainly accomplished by increased amount of palmitic acid (35-43%) with a decrease in linoleate (18:2) in sterol supplemented cells as compared to control cells. A decrease in the ratio of unsaturated/saturated fatty acids on supplementation of sterols indicates a rigidifying effect of sterols on the membrane. Buttke *et al.* (1980) also observed invariably high percentage (40-45%) of palmitic acid and stearic acid in both the major phospholipids, PC and PE with ergosterol as the sterol source as compared to the cells supplied with 7-dehydrocholesterol in yeast.

Fatty acids	Control	Ergosterol (relative percentage)	Cholesterol
$C_{12:0}$	2·313	2·015	2·588
$C_{14:0}$	1·813	2·506	2·638
$C_{16:0}$	21·807	29·525	31·207
$C_{16:1}$	2·648	1·545	1·152
$C_{18:0}$	2·763	2·819	0·852
$C_{18:1}$	32·630	28·387	31·498
$C_{18:2}$	36·020	33·201	30·063
U/S	2·485	1·712	1·682

Table 3. Effect of sterol supplementation at optimum concentration on phospholipid fatty acid composition of *M. gypseum.* Values of average of two independent runs.

The lipid composition of cell membrane plays an important role in regulating the functions of cells. Therefore permeability properties of intact cells were examined by monitoring the uptake of amino acids (lysine, glycine and aspartic acid) for different time periods. Maximum uptake was seen after 5 min of incubation, hence the transport studies were carried out at this time point. A significant reduction in the uptake of lysine, glycine and aspartic acid was observed in both cholesterol and ergosterol supplemented cells as compared to control cells (table 4). Elevated levels of sterols and increased saturation of phospholipid fatty acids (tables 1 and 3) are probably responsible for decreased amino acid uptake as these

components are known to reduce membrane permeability (Demel *et al.,* 1972; Prasad, 1985). Singh *et al.* (1979) also reported a significant reduction in the uptake of lysine, glycine, proline and glutamic acid in *Candida albican* enriched with ergosterol when grown on hydroquinone supplemented medium.

	Lysine	Glycine (n mol/100 mg dry wt./5 min)	Aspartic acid
Control	8751 ± 120·8	8002 ± 128·0	1502 ± 125·6
Ergosterol[a]	7752 ± 135·0[b]	4750 ± 130·8[c]	1374 ± 130·4[NS]
Cholesterol[a]	6750 ± 105·2[c]	6003 ± 135·8[c]	1173 ± 121·2[b]

Table 4. Uptake of amino acids by *M. gypseum* cells grown in the presence of cholesterol and ergosterol.

Values are mean±SD of 3 independent batches. NS, Not significant. [a]Optimum concentrations (50 and 80 *f*g/ml of ergosterol and cholesterol, respectively were used). $^{b}P \leqslant 0{\cdot}05$; $^{c}P \leqslant 0{\cdot}01$.

Structural changes occurring due to the alterations in the membrane lipid composition were examined using ANS, a membrane probe. ANS binds non-covalently to proteins and lipids of the membrane and its binding to the phospholipids is in the region of their polar headgroups. Hence it can provide information regarding membrane surface charge and the microenvironment of the bound dye (Azzi *et al.,* 1969; Brocklehurt *et al.,* 1978). The number of binding sites, as calculated from the Scatchard plots showed a significant increase in spheroplasts prepared from ergosterol and cholesterol supplemented cells (figure 2). Though the number of binding sites for proteins are less than those for the lipids, yet due to their very high affinity for binding to ANS, the protein binding sites remain saturated at low concentration of dye. Therefore increasing the dye concentration affects lipid binding sites more efficiently, hence changes due to alteration of phospholipid composition can be studied with ANS (Zierler and Rogus, 1978). Increased binding sites might be due to changes in the membrane surface charge resulting from changed ratio of zwitterionic to anionic phospholipids as suggested by Au *et al.* (1986) as well as altered ratio of unsaturated/saturated fatty acids of phospholipids as seen from tables 2 and 3. The changes observed in ANS binding may also be due to changed hydrophobic environment around the embedded dye, due to integral membrane proteins (Slavik, 1982). Since the dye binds to the membrane lipid and proteins,

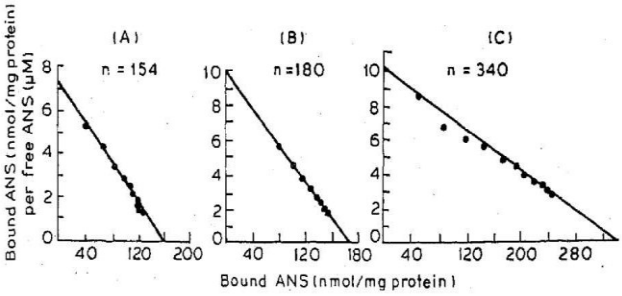

Figure 2. Scatchard plot of ANS binding (based on one batch run in duplicate) with *M. gypseum* spheroplasts. Spheroplasts were isolated from control and sterol supplemented cultures, (a), Control; (b), ergosterol; (c), cholesterol.

Alterations observed in the membrane components are responsible for the changes in ANS binding and indicate structural and conformational changes in the membrane.

In brief the results of this study suggest that sterols alongwith phospholipid fatty acids have a role in regulating the permeability of the cell as well as the structural aspects of membrane in *M. gypseum.*

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