J. Biosci., Vol. 12, Number 3, September 1987, pp. 297–302. © Printed in India.

Effect of environmental factors on the limpid composition, membrane structure and permeability of *Microsporum* gypseum

DIPAK KAPUR, RAKESH PANDEY, SEEMA VAIDYA and G. K. KHULLER*
Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India

MS received 30 April 1987; revised 4 July 1987

Abstract. Supplementation with unsaturated fatty acids, substitution of glucose by glycerol as carbon source and lowered growth temperature (20°C) increased the total phospholipid content of *Microsporum gypseum* spheroplasts. Levels of sterols increased with glycerol substitution and decreased in other growth conditions. Substantial changes were seen in the ratios of unsaturated to saturated fatty acids and phosphatidylcholine to phosphatidylethanolafne under all the experimental conditions. Changed lipid composition resulted in altered uptake of amino acids (L-lysine, L-aspartic acid and L-glycine) and increased number of binding sites for a fluorescent probe, 1-anilinonaphthalene-8-sulfonate.

Keywords. Environmental factor; lipid composition; membrane structure; permeability; *Microsporum gypseum.*

Introduction

Microorganisms survive under varied growth conditions by manipulating their phospholipid composition as these components are almost exclusively present in cellular membranes. This characteristic property of microorganisms has been exploited by several workers to elucidate the role of lipids in influencing membrane properties (Prasad, 1985). Transport of different solutes across the membrane bilayer was found to be affected by the lipid environment in *Candida albicans* and *Saccharomyces ceremsiae* (Singh *et al.,* 1978; Trivedi *et al.,* 1982; Basu *et al.,* 1986). Changes in temperature and of carbon source (Larroya and Khuller, 1985, 1986) and supplementation of growth media with fatty acids have been reported to alter lipid composition in the dermatophytes *Microsporum gypseum* (Bansal and Khuller, 1980) and *Microsporum cookei* (Bansal and Khuller, 1981). However, those studies were carried out with the mycelium and membrane lipid composition has not been examined to correlate membrane structure and function with lipid composition. Spheroplasts (protoplasmic bodies devoid of cell wall and having a membrane covering) were prepared from *M. gypseum* exposed to different, environmental conditions used to study the influence of membrane lipids on membrane properties of *M. gypseum.*

Materials and method

Materials

Palmitoleic acid, oleic acid and linoleic acid were obtained from Nu-check Prep,

*To whom all correspondence should be addressed.

Abbreviations used: ANS, Anilinonaphthalene-8-sulfonate; TPL, total phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolarnine.

Minneapolis, USA. Labelled amino acids [l-^{14}C]-glycine, [U-^{14}C]-lysine and [U-^{14}C]-aspartic acid were obtained from Bhabha Atomic Research Centre, Bombay. Glycine, aspartic acid and lysine were the products of Sigma Chemical Company, St. Louis, Missouri, USA. Novozym '234' and Cellulase 'CP' were obtained from M/s Novo Industries, Bagsvaerd, Denmark, and John and E. Sturge Ltd., Selby, North Yorkshire, England, respectively. Ficoll-paque was purchased from Pharmacia Fine Chemical Co., Uppsala, Sweden.

Growth of culture

M. gypseum, obtained from the Mycological Reference Laboratory, School of Hygiene and Tropical Medicine, London, was grown as a stationary culture in Sabouraud's broth (4% glucose, 1% peptone, pH 5·4–5·6) at 27 or 20°C. Ethanolic solutions of the fatty acids were added to the growth medium (at a concentration of 10 μg/ml) before inoculation where indicated. Ethanol was added to the autoclaved medium (0·1%, v/v) under sterile conditions to serve as control. Where indicated, glucose was replaced with glycerol (4%, w/v) in the Sabouraud's medium. Cells were harvested in the early stationary phase (21 days for 27°C cells and 30 days for 20°C) and processed further as follows.

Preparation of spheroplasts

Spheroplasts were prepared by the procedure of Larroya *et al.* (1984) with some modifications. Mycelia were incubated under sterile conditions with Novozym '234' and Cellulase 'CP' (30 mg each/g wet weight of mycelium) in a conical flask in 10 mM citrate-phosphate buffer (pH 6·5) containing 0·7 M NaCl (10–15 ml of buffer/g wet weight of mycelium) at 30°C for 20 h in a shaking water bath. Formation of spheroplasts was monitored microscopically. At the end of 20 h the incubation mixture was centrifuged at 1000 *g* for 10 min to recover the cells. The pellet was washed twice with buffer to remove traces of cell wall degrading enzymes. The spheroplasts were purified by centrifugation on a Ficoll-paque density gradient at 400 *g* for 15 min. The spheroplast fraction free of cell debris was used for further studies.

Quantitation of lipids

Spheroplast lipids were extracted by the method of Bligh and Dyer (1959). Phospholipids and sterols were quantitated by the method of Marinetti (1962) and Zlatkis *et al.* (1953), respectively. Individual phospholipids were separated by one-dimensional thin-layer chromatography in chloroform: methanol: 7 N ammonia (65:25:4, v/v). Total lipids were repeatedly triturated with acetone and the acetone-soluble neutral lipids and glycolipids were discarded. The acetone-insoluble phospholipids were used for fatty acid analysis (Khuller and Brennan, 1972). Methylesters of phospholipid fatty acids 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 using a flame ionization detector and column containing OV-225 at 190°C with nitrogen as a carrier gas at a flow rate of

40 ml/min. Fatty acid methylesters were identified by comparison of their retention times with those of authentic standards. Fatty acids were quantitated by triangulartion of peak area.

Amino acid uptake studies

Spheroplasts (100–150 μg protein) were incubated with the amino acid in 0·5 ml 10 mM citrate-phosphate buffer (pH 6·5) containing 0·7 M NaCl. The reaction was initiated by the addition of labelled amino acid (specific activity 125 μCi/mmol) and was stopped after 7 min (the optimum time) of incubation at 28°C by diluting with chilled normal saline and filtering through 0·45 μm Millipore membrane filters. After washing 2-3 times with chilled normal saline, filters were air dried and counted in toluene-based scintillation fluid.

Structural studies

A fluorescent probe, l-anilinonaphthalene-8-sulfonate (ANS) was used for structural studies. The assay mixture consisted of 10 mM citrate-phosphate bufer (pH 6·5) containing 0·7 M NaCl, 10 μM ANS and spheroplasts (50–200 μg protein) in a total volume of 2 ml. The fluorescence emission was recorded on a Kontron SFM-25 spectrofluorimeter. The number of binding sites was calculated from the Scatchard plot (Azzi, 1974). Protein was estimated by the method of Lowry *et al.* (1951).

Results and discussion

As lipids constitute a major portion of plasma membranes, an attempt was made to study their role in regulating the membrane properties of *M. gypseum* grown under various environmental conditions. Spheroplasts prepared from *M. gypseum* cells grown in media supplemented with palmitoleic and linoleic acid had significantly increased total phospholipids (TPL), whereas no change was observed with oleic acid (table 1). These observations are in accordance with our earlier report (Bansal and Khuller, 1980) where oleic acid was the least effective in enhancing the TPL content of *M. gypseum* mycelium. Sterols, another important membrane constituent of fungi, have been known to act as buffering molecules in membrane organization (Bloch, 1983). Levels of these components did not change on oleic and linoleic acid supplementation, while a significant decrease was seen with palmitoleic acid (table 1). Koh *et al.* (1977) also failed to observe any significant change in the sterol content on supplementation of growth medium of *C. albicans* with fatty acids. Besides the changes in TPL and sterol, the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) also increased/decreased as a result of altered polar phospholipid composition (data not shown). Supplementation with palmitoleic acid resulted in 65% increase in the PC/PE ratio; however, it decreased to the same extent in linoleic acid supplemented cells (table 1). The relative proportions of phospholipid fatty acids changed on supplementation with fatty acids resulting in altered unsaturation of phospholipids. Spheroplasts prepared from oleic and linoleic acid grown cells showed an increased (40%) ratio of unsaturated to saturated fatty acids of phospholipids (table 1). In whole cells of *M. cookei,* palmitoleic acid supplementation in-

Table 1. Effect of environmental conditions on the limpid composition of *M. gypseum* spheroplasts.

Carbon source	Additive (10 μg/ml)	Growth temp. (°C)	TPL (μg/mg protein)	Sterol (μg/mg protein)	Ratio	
					PC/PE	U/S
Glucose	—	27	12·16 ± 1·70	88·41 ± 1·11	1·36	1·06
Glucose	Palmitoleic acid	27	16·43 ± 2·30[a]	72·66 ± 7·50[a]	2·23	1·01
Glucose	Oleic acid	27	12·37 ± 1·30[NS]	76·43 ± 11·00[NS]	1·36	1·35
Glucose	Linoleic acid	27	21·48 ± 1·05[c]	78·80 ± 11·20[NS]	0·45	1·41
Glycerol	—	27	15·42 ± 0·52[a]	154 ± 4·50[c]	2·02	1·15
Glycerol	—	20	21·10 ± 1·48[c]	171 ± 10·4[c]	2·74	1·49
Glucose	—	20	18·36 ± 0·72[b]	47·90 ± 9·58[b]	1·74	1·77

Mean ± SD of 3 batches in duplicate.
NS, Non-significant. [a]$P \leq 0{\cdot}05$; [b]$P \leq 0{\cdot}01$; [c]$P \leq 0{\cdot}001$.
U/S, Unsaturated/saturated.

creased the saturation in phospholipid fatty acids (Bansal and Khuller, 1981). However, no significant change was observed in spheroplasts of *M. gypseum*. The changes in fatty acid composition were mainly confined to fatty acids of chain length 16-18 carbon atoms and the composition was qualitatively similar to fatty acid profile of phospholipids of *M. gypseum* mycelia, where 16:0, 18:0, 18:1 and 18:2 have been identified as the major phospholipid fatty acids (Larroya and Khuller, 1985, 1986).

In microorganisms, growth temperature and carbon source are critical for the proper function of normal cell physiology (Chopra and Khuller, 1983). Sub-optimal growth temperature (20°C) alters the lipid composition of various microorganisms including dermatophytes. Alterations in phospholipid polar head groups and apolar fatty acyl chains have been reported (Prasad, 1985; Chopra and Khuller, 1983). Similar efects were seen in *M. gypseum* whole cells when glucose was replaced with glycerol (Larroya and Khuller, 1985, 1986). TPL of *M. gypseum* spheroplasts increased significantly (25-75%) under these conditions (table 1) with maximum increase in spheroplasts of cells grown with glycerol at 20°C. Sterol content increased on substitution of glycerol for glucose, both at 27 and 20°C (optimum and sub-optimal temperature) while a reduced level was seen in spheroplasts of cells grown with glucose at 20°C (table 1). The ratio of PC to PE was also found to be altered. Cells grown at 20°C showed a 27% increase in the PC/PE ratio compared to cells grown at 27°C. The ratio was higher in glycerol substituted cells grown at 20°C (2-fold) and 27°C (1·5-fold). Increased unsaturation of phospholipid acyl groups was seen under all these conditions (table 1), and was mainly due to increase in the levels of oleic and linoleic acid (data not shown). The alterations observed in the lipid composition of spheroplasts are comparable with those of the whole cells of *M. gypseum* reported earlier (Larroya and Khuller, 1985, 1986). The minor diferences in lipid composition between spheroplasts and mycelia may be due to the loss of a few membrane lipid components during preparation of spheroplasts.

Changes in membrane lipid composition have been correlated with various membrane properties in microorganisms (Prasad, 1985). In particular, membrane permeability, which is intricately linked with membrane lipid composition, has been studied (Trivedi *et al.,* 1982; Larroya and Khuller, 1985, 1986). Therefore, amino acid uptake was studied in spheroplasts prepared from *M. gypseum* grown under different

Table 2. Effect of environmental conditions on the amino acid uptake in *M. gypseum* spheroplasts.

Carbon source	Additive (10 μg/ml)	Growth temp. (°C)	Uptake of amino acid*		
			L-lysine	L-aspartic acid	L-glycine
Glucose	—	27	98·63 ± 9·80	75·72 ± 15·60	64·25 ± 14·80
Glucose	Palmitoleic acid	27	80·32 ± 13·10^a	60·24 ± 9·02^a	87·02 ± 12·80^a
Glucose	Oleic acid	27	86·50 ± 2·80^a	43·80 ± 3·09^b	70·09 ± 7·9^{NS}
Glucose	Linoleic acid	27	76·70 ± 6·12^b	61·42 ± 4·12^a	64·74 ± 4·50^{NS}
Glycerol	—	27	89·90 ± 1·70^{NS}	47·78 ± 6·67^b	64·25 ± 14·80^{NS}
Glycerol	—	20	230 ± 22·00^c	120 ± 3·9^b	163 ± 9·95^c
Glucose	—	20	300 ± 16·00^c	133 ± 17·0^b	109 ± 9·4^b

*n mol/mg protein/7 min.
Same notations as in table 1.

conditions (table 2). The uptake of each of the 3 amino acids used (lysine, aspartic acid and glycine) was different for different conditions of growth and maximum increase in accumulation (2-3 fold) was seen in spheroplasts of cells grown at 20°C. It appears from these results that the uptake of these amino acids follows independent mechanisms and that membrane permeability is altered as a result of altered polar head group and fatty acyl chain composition of phospholipids, as has been reported earlier for *M. gypseum* mycelia (Larroya and Khuller, 1985, 1986).

The role of lipids in the structural organization of plasma membranes in dermato-

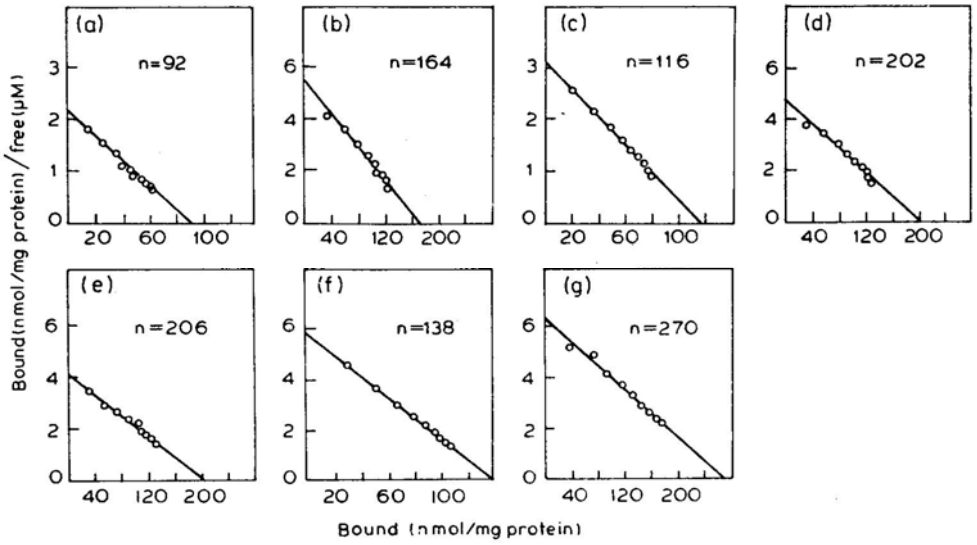

Figure 1. Scatchard plot of ANS binding (based on one batch run in duplicate) of M. *gypseum* spheroplasts. **a.** Spheroplasts of control, **b.** Spheroplast of palmitoleic acid supplemented cells, **c.** Spheroplast of oleic acid supplemented cells, **d.** Spheroplast of linoleic acid supplemented cells, **e.** Spheroplast of glycerol grown cells al 27°C. **f.** Spheroplast of glycerol grown cells at 20°C. **g.** Spheroplast of glucose grown cells at 20°C.
n. Number of binding sites for ANS, expressed in mol/mg protein.

phytes has not been well investigated. Phospholipids, being polar, amphipathic, charged molecules, participate in electrostatic interactions with other molecules in the membrane. Hence, changes in the membrane surface charge density can be interpreted as structural and compositional changes in the membrane. ANS was used as a membrane probe to monitor these aspects (Slavik, 1982). This compound binds with the membrane phospholipids in such a way that its hydrophobic ring intercalates in the membrane interior. ANS also binds to proteins though to a smaller extent but only when a hydrophobic domain is available (Slavik, 1982). The number of ANS binding sites in *M. gypseum* spheroplast membranes was calculated from a Scatchard plot (figure 1). There was an increase in the number of binding sites in spheroplasts when cells were grown under varied growth conditions. This increase could be correlated with changes in the lipid composition under these conditions (table 1); however, it is not possible to specify a particular lipid component responsible for these changes. Similar observations have been made earlier with spheroplasts of *M. gypseum* prepared from cells grown in medium supplemented with different nitrogenous bases (Pandey *et al.,* 1987). The changes in PC/PE ratio result in alteration in membrane charge, which regulates ANS binding to membranes (Trivedi *et al.,* 1982). Apolar fatty acyl chains also affect the steric conformation of surrounding molecules. These factors have a net combined effect, signifying the structural changes in the membrane as a result of altered membrane charge and microviscosity (Slavik, 1982). However, it is not possible to draw a definite conclusion about which particular lipid component is important for proper membrane function till a system to induce controlled alterations in a specific membrane component is developed.

Acknowledgement

This work was financed in part by a grant from the Indian Council of Medical Research, New Delhi.

References

Azzi, A. (1974) *Methods EnzymoL,* **B32,** 234.
Bansal, V. S. and Khuller, G. K. (1980) *FEMS Microbiol Lett,,* **9,** 167.
Bansal, V. S. and Khuller, G. K. (1981) *Sabouraudia,* **19,** 307.
Basu, J., Kundu, M. and Chakrabarti, P. (1986) *Arch. Biochem. Biophys.,* **250,** 382.
Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol,* **37,** 911.
Bloch, K. E. (1983) *CRC Crit. Rev. Microbiol,* **14,** 47.
Chopra, A. and Khuller, G. K. (1983) *Prog. Lipid Res.,* **22,** 189.
Khuller, G. K. and Brennan, P. J. (1972) *Biochem. J.,* **127,** 369.
Khuller, G. K., Chopra, A., Bansal, V. S. and Masih, R. (1981) *Lipids* **16,** 20.
Koh, T. Y., Marriott, M. S,, Taylor, J. and Gale, E. F. (1977) *J. Gen. Microbiol,* **102,** 105.
Larroya, S. and Khuller, G. K. (1985) *Lipids,* **20,** 11.·
Larroya, S. and Khuller, G. K. (1986) *Indian J. Biochem. Biophys.,* **23,** 9.
Larroya, S., Johl, P. P., Pancholi, V. and Khuller, G. K. (1984) *IRCS Med. Sci.,* **12,** 1064.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol Chem.,* **193,** 265.
Marrinetti, G. V. (1962) *J. Lipid Res.,* **3,** 1.
Pandey, R., Verma, R. S. and Khuller, G. K. (1987) *Lipids,* **22,** 530.
Prasad, R. (1985) *Adv. Lipid Res.,* **21,** 186.
Singh, M., Jayakumar, A. and Prasad, R. (1978) *Arch. Biochem. Biophys.,* **191,** 680.
Slavik, J. (1982) *Biochim. Biophys. Acta.,* **694,** 1.
Trivedi, A., Khare, S., Singhal, G. S. and Prasad, R. (1982) *Biochim. Biophys. Acta.,* **692,** 202.
Zlatkis, A., Zak, B. and Boyle, A. J. (1953) *J. Lab. Clin. Med.,* **41,** 486.