



Original Article

β -Endorphin enhances the phospholipase activity of the dandruff causing fungi *Malassezia globosa* and *Malassezia restricta*

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Abstract

β -Endorphin is known to stimulate phospholipase production by *Malassezia pachydermatis* during canine dermatoses. The role of β -endorphin in *Malassezia* infection in humans is not well studied. The present study compares the influence of β -endorphin on *Malassezia globosa* and *Malassezia restricta* isolated from patients with seborrhoeic dermatitis/dandruff (SD/D) and healthy controls. *Malassezia* isolates (five each of the two species from patients and healthy controls) were grown on modified Dixon's agar with or without 100 nmol/L β -endorphin. Phospholipase activity was quantified based on its ability to hydrolyze L- α -phosphatidylcholine dimyristoyl (phospholipid substrate). Free fatty acid was measured by a colorimetry method. In isolates from patients, the phospholipase activity significantly increased after exposure to β -endorphin (*M. globosa*, $P = .04$; *M. restricta*, $P = .001$), which did not occur in isolates from healthy controls. Moreover, after β -endorphin exposure the patient isolates had significantly higher ($P = .0004$) phospholipase activity compared to the healthy control isolates. The results suggest that isolates of *M. globosa* and *M. restricta* from patients may differ from those of healthy humans.

Key words: *Malassezia*, dandruff, phospholipase activity, β -endorphin.

Introduction

Malassezia species are known to cause seborrhoeic dermatitis/dandruff (SD/D) in humans. The fungi are unable

to synthesize myristic acid, an essential fatty acid required for its growth.¹ Various lipolytic enzymes, including lipase, esterase, phospholipase, and lysophospholipase, produced

by *Malassezia* species help in the utilization of essential fatty acids from exogenous lipid sources.² Among these enzymes, phospholipases are considered as a virulent factor. *Malassezia pachydermatis* isolated from lesional areas of dogs produces higher amount of phospholipase compared to those isolated from non-lesional area.³ The β -endorphin present in skin of dog with dermatoses stimulates phospholipase production from *M. pachydermatis*.⁴ The nerve endings, keratinocytes, sebocytes, and melanocytes contain β -endorphin μ -opioid receptors. The β -endorphin activation modulates immunological and chemical activity of the skin.⁵ β -Endorphin also affects lipogenesis of long-chain fatty acids and pigmentation.⁶

Malassezia globosa and *Malassezia restricta* are commonly isolated from patients with SD/D.⁷ The role of β -endorphin in modulating phospholipase production by these species during human infection is not well understood or explored. Hence, the present study was conducted to analyze and compare phospholipase activity of *M. globosa* and *M. restricta* isolated from patients with SD/D and healthy controls before and after stimulation with β -endorphin.

Materials and methods

Specimen collection and strains cultivation

Specimens were collected using a blunt scalpel from severely affected scalp lesions of SD/D patients and from vertex of healthy control scalp. The sampling was done at convenience of patients and controls by random screening. All isolates were cultured on Leeming and Notman agar (LNA) plates and incubated at 34°C for 2–4 weeks. The isolates were identified based on phenotypic and molecular technique as described earlier.⁸ Ten isolates of *Malassezia* (five-*M. globosa* and five-*M. restricta*) isolated from the lesional area of SD/D patients and the same number from healthy controls were included in the study. The study protocol was approved by the Institute Ethics Committee of Postgraduate Institute of Medical Education and Research, Chandigarh. The samples were collected after obtaining the informed consent of each subject.

Phospholipase activity of *Malassezia* before and after β -endorphin exposure

Malassezia isolates were inoculated on modified Dixon's agar plate with or without 100 nmol/L β -endorphin (Sigma Aldrich, St. Louis, Missouri, USA) and incubated for seven days.⁹ Extraction of extracellular protein was performed by the method described earlier with few modifications.¹⁰ Each isolate was cultured on five sets of LNA plates at 34°C for seven days. The cells were collected by scraping

the agar surface using a sterile scalpel. After scraping the cells from the surface of the agar medium, the surface was rinsed twice with distilled water. LNA medium was crushed in a pestle-mortar and the resultant slurry was collected in 100 ml protein extraction buffer kept at 4°C for 24 h.¹⁰ The mixture was filtrated through Whatmann filter paper followed by 0.2 mm membrane filter (Millipore corporation, MA, USA). The resultant solution was concentrated with Amicon ultra centrifugal filter, ultracel-30 K (Millipore corporation, MA, USA). Phospholipase activity was quantified by the assay based on the hydrolysis of phospholipid substrate, L- α -phosphatidylcholine dimyristoyl. The assay was performed at 30°C for 1 h and terminated by adding 1.25 ml methanol.¹¹ The released fatty acids were extracted by the rapid single step method.¹² The concentration of free fatty acid was measured by a colorimetry method using the half micro test kit (Roche, Basel, Switzerland).¹¹ The phospholipase activity on the basal media (LNA) was deducted from the value of the tests and controls to remove the background activity of the basal media. Each strain was tested in triplicate. One unit of phospholipase activity was defined as the amount of enzyme that released 1 μ M of free fatty acid per minute.

Statistical analysis

The impact of β -endorphin exposure on phospholipase activity of *M. globosa* and *M. restricta* isolated from SD/D patients and healthy controls were compared by employing the unpaired t-test using the software GraphPad Prism, version 6.01. $P < .05$ was considered statistically significant.

Results

The source of the isolates and its phospholipase activity before and after stimulation with β -endorphin is provided in Table 1 and in Supplementary Table S1. For both *M. globosa* and *M. restricta* isolates from SD/D patients, phospholipase activity significantly increased after stimulation with β -endorphin (*M. globosa*, $P = .04$; *M. restricta*, $P = .001$). In addition, after β -endorphin stimulation the overall phospholipase activity of the isolates from SD/D patients was significantly higher compared to isolates from healthy controls (Fig. 1b, $P = .0004$), indicating the presence of possible pathogenic strains within these two *Malassezia* species. The phospholipase activity after β -endorphin stimulation of *M. globosa* isolated from SD/D patients was significantly higher when compared to the isolates from healthy controls (Fig. 1d, $P = .022$). Similarly, phospholipase activity after β -endorphin stimulation of *M. restricta* isolated from SD/D patients was significantly higher compared to the isolates from healthy controls (Fig. 1f, $P = .017$). No significant difference of phospholipase activity between isolates from

Table 1. Phospholipase activity of *M. globosa* and *M. restricta* isolated from SD/D patients and healthy controls, before and after stimulation of β -endorphin.

	Seborrheic dermatitis/dandruff patients		Healthy controls		P value			
	Before β -endorphin stimulation (A)	After β -endorphin (100 nmol/L) stimulation (B)	Before β -endorphin stimulation (C)	After β -endorphin (100 nmol/L) stimulation (D)	A v/s C	B v/s D	A v/s B	C v/s D
<i>M. globosa</i>	1.232 $\pm$ 0.072	1.535 $\pm$ 0.1	1.277 $\pm$ 0.031	1.230 $\pm$ 0.038	.586	.022*	.041*	.367
<i>M. restricta</i>	1.277 $\pm$ 0.031	1.561 $\pm$ 0.049	1.233 $\pm$ 0.072	1.171 $\pm$ 0.119	.586	.017*	.001*	.673
All <i>Malassezia</i>	1.255 $\pm$ 0.038	1.548 $\pm$ 0.053	1.255 $\pm$ 0.038	1.201 $\pm$ 0.059	.999	.0004*	.0003*	.454

One unit of phospholipase activity is defined as the amount of enzyme that released 1 μ M of free fatty acid per minute. Phospholipase activity expressed in mean $\pm$ SEM, * $P < 0.05$ was considered statistically significant

patients and control was noted without β -endorphin stimulation

Discussion

In the present study, a significant increase in phospholipase activity after β -endorphin exposure was noted in patient isolates compared to those from healthy controls, whereas similar difference was not observed without β -endorphin stimulation. The finding was similar for both *M. globosa* and *M. restricta* isolates from SD/D patients. The whole genome sequence of *M. globosa* has revealed the presence of six phospholipase-C encoding genes, which are predicted to be transcribed and secreted enzymes,¹ and two phospholipase-D genes and one phospholipase-B gene, which are not known to be secreted.¹ Besides, many genes encoding lipases have been identified and characterized namely, *MfLIP1* in *M. furfur*,¹³ phospholipase D in *M. pachydermatis*,¹⁴ *MgLIP1* and *MgLIP2* in *M. globosa*,^{15,16} *MrLIP1*, *MrLIP2*, and *MrLIP3* in *M. restricta*.¹⁷ The comparative genomics delineated the importance of lipases, phospholipases, peptidases and aspartyl proteases and their potential role in virulence and *Malassezia* niche-specificity.¹⁸ Phospholipase of *M. pachydermatis* has been reported as a potential virulence factor in canine dermatoses.³ *M. sympodialis* strains isolated from pityriasis versicolor patients showed higher phospholipase activity than strains derived from healthy controls.¹⁹ These data suggest that phospholipase plays a major role in the pathogenesis of *Malassezia*-associated diseases. The occurrence of pathogenic subtypes has been described within *M. furfur*, *M. globosa* and *M. restricta* on the basis of molecular typing.^{20,21} Depending on the β -endorphin action (switch-on and switch-off phenomena) in a susceptible individual, the potential pathogenic subtypes may get activated to release higher amounts of phospholipase leading to dysfunction of the stratum corneum in SD/D. In concordance with our

observation, Vlachos et al. showed recently that phospholipase activity of *Malassezia* isolated from SD lesions was stimulated by β -endorphin.⁹

However, in the majority of the earlier studies phospholipase activity was measured by the semi-quantitative method using egg yolk plate. This technique is not specific as the presence of nonspecific substrates in the egg yolk along with triglycerides and phospholipids interfere with the assay. In addition, the formation of a zone of white precipitate renders its interpretation difficult. Hence in the present study, the phospholipase activity was quantitatively measured using a specific substrate following the technique described by Juntachai et al. with few modifications.¹⁰ In that study, they compared the extracellular phospholipase activity of standard reference strains of seven *Malassezia* species. *M. pachydermatis* (CBS 1891) had the highest phospholipase activity compared to six other *Malassezia* spp. (*M. furfur*, *M. globosa*, *M. slooffiae*, *M. sympodialis*, *M. obtusa*, and *M. restricta*). Both the standard strains of *M. globosa* (CBS 7966^T, CBS 7986, CBS 8745) and *M. restricta* (IFM 15950, IFM 15951) had very low phospholipase activity.¹⁰

Serum and skin concentrations of β -endorphin have been shown to increase in psoriasis, atopic dermatitis, and other inflammatory dermatoses.⁵ A pathogenic link has also been established between the increasing level of β -endorphin mediated stress and acne.⁶ β -endorphin activates the μ -opiate receptor present on the cell wall of *M. pachydermatis* to overproduce the phospholipase enzyme.²² Similar mechanisms may exist in pathogenic strains of *M. restricta* and *M. globosa* causing SD/D. We propose the following hypotheses; in a susceptible individual, alteration in the microenvironment (physical/chemical/immunological factors) of the skin, such as stress, pH, salts, bacterial microbiota, keratinocytes, sebocytes, and melanocytes, may induce β -endorphin resulting in the stimulation of receptors present on the cell wall of pathogenic strains of *M. restricta* and *M. globosa*. This stimulus may cause the abundant release

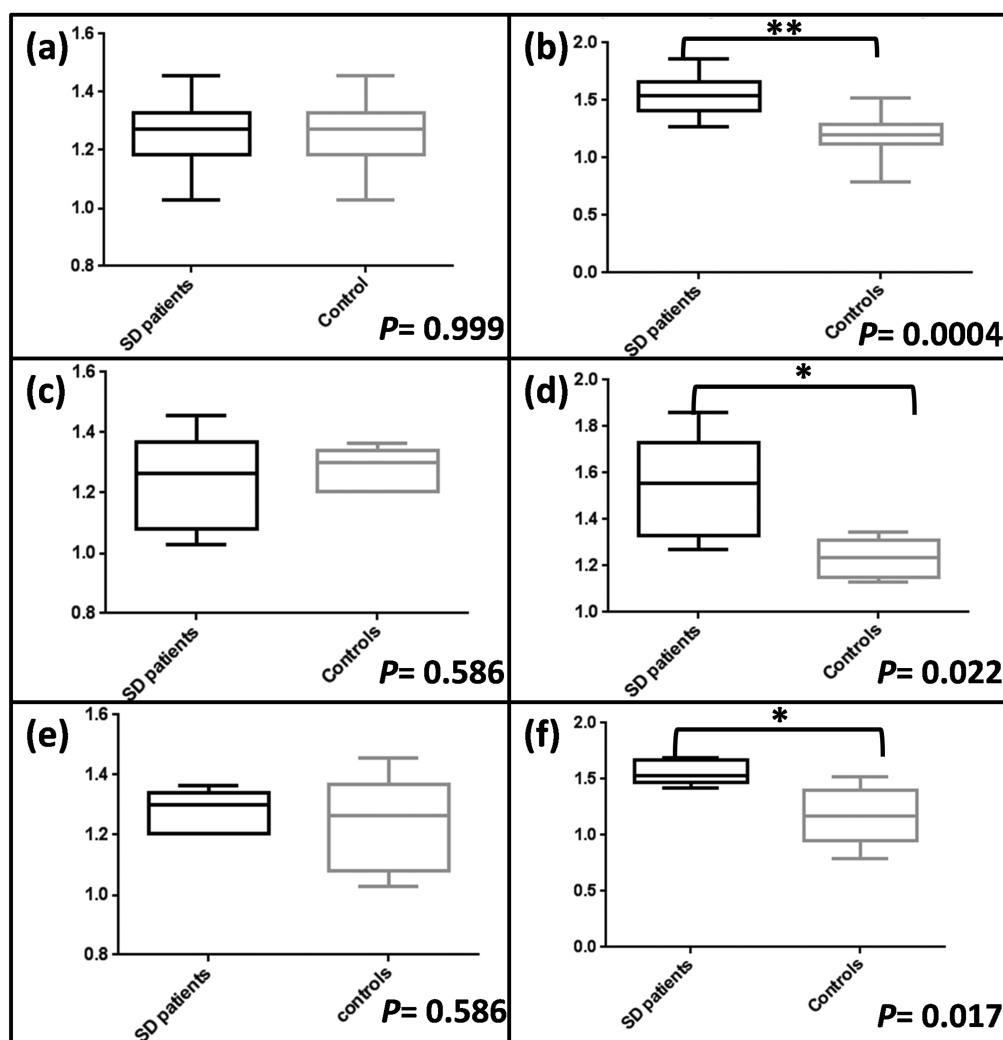


Figure 1. Comparison of phospholipase activity of strains isolated from seborrhoeic dermatitis/dandruff patients and healthy controls. (a) Background phospholipase activity; (b) Phospholipase activity after β -endorphin stimulation; (c) Background phospholipase activity of *M. globosa*; (d) Phospholipase activity of *M. globosa* after β -endorphin stimulation; (e) Background phospholipase activity of *M. restricta*; (f) Phospholipase activity of *M. restricta* after β -endorphin stimulation.

of phospholipase. The phospholipase may degrade the intercellular lipid of the stratum corneum resulting in dysfunction of the stratum corneum and exfoliations leading to SD/D. Future *in vivo* studies are warranted to prove this hypothesis.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary material is available at *Medical Mycology* online (<http://www.mmy.oxfordjournals.org/>).

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Supplementary table 1: Phospholipase activity (in mM) of *M. globosa* and *M. restricta* strains isolated from SD/D patients and healthy controls.

	Background activity				Phospholipase activity after 100 nM β -endorphin stimulation			
	Strain	OD*	OD-blank	Conc (mM)	Strain	OD*	OD-blank	Conc (mM)
<i>M. globosa</i> (SD/D patients)	u10	0.982	0.861	1.026312	B u10	1.185	1.064	1.268288
	u66	1.342	1.221	1.455432	B u66	1.683	1.56	1.861904
	u71	1.073	0.952	1.134784	B u71	1.288	1.167	1.391064
	u77	1.18	1.06	1.265904	B u77	1.462	1.341	1.598472
	u60	1.195	1.074	1.280208	B u60	1.424	1.303	1.553176
<i>M. restricta</i> (SD/D patients)	u54	1.133	1.012	1.206304	B u54	1.542	1.421	1.693832
	u64	1.264	1.143	1.362456	B u64	1.508	1.387	1.653304
	u65	1.21	1.089	1.298088	B u65	1.402	1.281	1.526952
	u73	1.13	1.009	1.202728	B u73	1.313	1.192	1.420864
	u74	1.226	1.105	1.31716	B u74	1.388	1.267	1.510264
<i>M. globosa</i> (healthy controls)	Nu11	1.21	1.089	1.298088	BNu11	1.156	1.035	1.23372
	Nu68	1.13	1.009	1.202728	BNu68	1.192	1.071	1.276632
	Nu70	1.226	1.105	1.31716	BNu70	1.248	1.127	1.343384
	Nu75	1.133	1.012	1.206304	BNu75	1.067	0.946	1.127632
	Nu60	1.264	1.143	1.362456	BNu60	1.101	0.98	1.16816
<i>M. restricta</i> (healthy controls)	Nu55	1.073	0.952	1.134784	BNu55	1.046	0.925	1.1026
	Nu63	1.183	1.062	1.265904	BNu63	1.188	1.067	1.271864
	Nu65	1.195	1.074	1.280208	BNu65	1.104	0.983	1.171736
	Nu78	0.982	0.861	1.026312	BNu78	0.783	0.662	0.789104
	Nu80	1.342	1.221	1.455432	BNu80	1.398	1.277	1.522184

* The values are the average of experiments performed in triplicate; OD- Optical Density