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Neutral Storage Lipids of *Histoplasma capsulatum*: Effect of Culture Age

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Abstract

Lipids contribute significantly to the pathogenesis of fungal infectious diseases and an understanding of lipid metabolism occurring in fungal pathogens can help the development of more efficient antifungal therapeutic strategies. In this study, the effect of culture age on the distribution of fatty acids among different neutral lipid (NL) classes in the dimorphic fungus *Histoplasma capsulatum* was investigated. Yeast cells of the G217B strain grown in two different media were collected after 4 and 7 days of growth, which roughly correspond to log and stationary culture growth phases, respectively. Neither culture age nor medium type had any influence on qualitative fatty acid (FA) profiles; however, the FA percentage composition varied with culture growth. A culture age-related decrease in the content of unsaturated FAs could be observed in all four of the NL classes examined, but the most intensive changes were detected in diacylglycerol and free FA fractions. Conversely, an increase in saturated FAs was observed. The transcriptional analysis of two major Δ^9- and Δ^{12}-FA desaturase genes, *ode1* and *sde1*, showed no differences in their expression levels under experimental conditions. These results showing the dynamics of changes in FA composition in the NL fraction were concomitant with nutrient exhaustion in aging *H. capsulatum* cultures. Overall, the results presented in this work not only have implications for our knowledge of basic lipid biochemistry of *H. capsulatum*, but also will contribute to better understanding of biology and pathogenesis of this fungus and, consequently, can help in the discovery of more effective antifungal drugs.

Introduction

In all types of living cells, neutral lipids (NLs) serve as reduced reservoirs of oxidizable energy and depots of simple acyl-CoA units needed for membrane formation [3]. Triacylglycerols (TAGs) and steryl esters (STEs) are the most prominent storage NLs, also in many fungal species [16,24]. Unfortunately, our present knowledge of lipid biochemistry in medically important dimorphic fungal pathogens remains rather scarce. Dimorphic fungi display considerably slower metabolism and growth than *Saccharomyces cerevisiae* and many other yeasts. One of the dimorphic fungi, *Histoplasma capsulatum*, is responsible for the most common systemic mycosis in humans, histoplasmosis. This is a severe respiratory disease, mostly of the reticuloendothelial system, manifesting itself in the lungs, bone marrow, liver, and spleen [22]. To date, there are very few reports focused on *H. capsulatum* lipids [1,2,8,9,

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18,20], but only our recent study provided a thorough analysis of cellular fatty acid (FA) patterns observed in this primary human mycopathogen [26].

As a result of physiological and metabolic adaptations, all lipids (TAG in particular) in eukaryotic cells are influenced by various developmental and environmental factors [15]. In this work, we have directed our attention to NLs of *H. capsulatum* and variability in their composition as a function of culture aging. To provide deeper insight into the dynamics of those changes, we examined NL isolated from *H. capsulatum* cells grown on two different microbiological media.

Materials and Methods

Fungal Strains and Cultivation

The G217B strain (ATCC 26032) of *H. capsulatum* var. *capsulatum* Darling was used in this study. The fungus was cultivated in either liquid HMM [23] or HcMM (Liesener and Woods, unpublished). The latter consisted of 50 mM glucose, 10 mM KH_2PO_4, 10 μM $FeSO_4$, 15 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.35 mM cystine, 50 mM NaCl, and 10 mM HEPES, pH 7.5. Yeast cultures were grown in 0.5-L batch cultures at 37°C in a 5% CO_2/95% air atmosphere for 4 and 7 days, respectively. These time points, corresponding to log and stationary growth phases of the G217B strain, were determined experimentally as reported elsewhere [19].

Lipid Extraction and Analysis

Cells were harvested by centrifugation (3000g, 5 min) and washed twice with sterile distilled water. The pellet was resuspended in 0.5 ml of 0.97% potassium chloride. Lipid extraction and separation and subsequent FA analysis were performed as described elsewhere [25,26].

Expression Analysis of Genes Encoding for Fatty Acid Desaturases

Total cellular RNA was isolated from yeast cells using a RiboPure-Yeast kit (Ambion). Equivalent amounts of total cellular RNA (1 μg) were used in the synthesis of first-strand cDNA using an oligo$(dT)_{20}$ primer and Superscript III reverse transcriptase (Invitrogen) according to the supplier's protocol. For RT-PCR expression analysis of Δ^9-and Δ^{12}-FA desaturase genes, primers were designed based on sequences found in the GenBank database. The primer pairs were designed to amplify short regions (~400–600 bp) within the respective open reading frames. The nucleotide sequences of the sense and antisense primers were 5′-GGACTCCTATGTTGGTGATGAAGC-3′ and 5′-CAGCAGTGGTGGAGAAAGAATAAC-3′ (for actin, *act1*) [10], 5′-TCATTTGGTCGGGTCGTATTG-3′ and 5′-TCATCGTCTTTGGGCACACTGC-3′ (for Δ^9-desaturase, *sde1*) [12], and 5′-AAGCCGTTTCTCCAAATGAATG-3′ and 5′-TCCGAGCGTAAGCATACTACACCG-3′ (for Δ^{12}-desaturase, *ode1*) [4]. PCR was performed using Taq polymerase with buffer components provided by the supplier (Promega). The amplification parameters were as follows: an initial denaturation step at 95°C for 3 min, followed by 24 cycles which consisted of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min. PCR reaction products were analyzed by electrophoresis on a 1% agarose gel.

Results and Discussion

TAG and STE are the main NLs in most fungi, where they serve as reduced stores of oxidizable energy in eukaryotic cells [3,17]. TAG and STE, and their biosynthesis, have also been implicated in several important biological processes, such as regulation of biological membrane composition, as a source of FAs for biosynthesis of polar lipids, and as agents for

remobilization of membrane lipids during cell aging. The importance of TAG metabolism and its involvement in lipid homeostasis in eukaryotic organisms and in the development of some human diseases has also been well described, for example, in toxoplasmosis [6] and tuberculosis [13].

There are only a few reports focused on lipids from *H. capsulatum* [1,2,8,9,18,20,26], but only studies from the Maresca lab [5,12,21] cast some light on the role of lipids in adaptation to sudden changes in temperature shifts and expression of heat shock proteins in this fungus. In this study, we analyzed FA compositions of NL fractions from *H. capsulatum* G217B strain cultures grown continuously as yeast in two different microbiological media. Depending on the lipid class, the sets of 6 to 13 different FAs were identified (Tables 1 and 2). The major FAs of *H. capsulatum* grown for 4 days were oleic (18:1), linoleic (18:2), palmitic (16:0), and palmitoleic (16:1) acids. In addition, myristic (14:0) and stearic (18:0) acids were detected in abundance in the STE fraction. After an additional 3 successive days of growth, FA patterns of late stationary-phase *H. capsulatum* cultures were considerably changed and a trend toward accumulation of saturated palmitic, stearic (18:0), and myristic (14:0) acids was noted.

Regardless of the medium used, levels of saturated FAs in TAG, diacylglycerol (DAG), free FA (FFA), and STE fractions tended to increase during culture growth, whereas the content of unsaturated FAs was considerably reduced. Among the lipid classes of the NL fraction, the most variable were DAG and FFAs, while the lowest variability ratio was observed in the TAG fraction. Levels of unsaturation in DAG and FFAs were decreased by 46.0% and 53.6% in cultures grown in HcMM (Table 1), and the contents of saturated FAs were increased by 46.1 and 53.8%, respectively. Only slightly different variability ratios in FAs were observed in extracts from yeast cells grown in *Histoplasma*-macrophage medium (HMM) (Table 2). The content of saturated FAs was increased during *H. capsulatum* growth by 10.4%, 34.5%, and 42.0% in the TAG, DAG, and FFA fractions, respectively. The decrease in unsaturated FAs was more equitable when cells were grown in HMM (Table 2) and averaged 10.5%, 34.5%, and 42.2% in the TAG, DAG, and FFA fractions, respectively. Interestingly, variations in the STE pool were quite similar in both media used. The accumulation of saturated and unsaturated FAs over the culture growth was comparable in *Histoplasma capsulatum* minimal medium (HcMM) and HMM (24.8% and 27.8% for saturated FAs and 24.6% and 27.8% for unsaturated FAs).

The changes observed in unsaturation levels within analyzed FAs prompted us to evaluate the expression of two major fatty acid desaturases, *sde1* and *ode1*, respectively. *sde1* (alternatively called *ole1*) is a Δ^9-desaturase responsible for the conversion of palmitic acid (16:0) and stearic acid (18:0) to palmitoleic acid (16:1) and oleic acid (18:1), whereas *ode1* is a Δ^{12}-desaturase that converts oleic acid into corresponding polyunsaturated linoleic (18:2) and linolenic (18:3) acids. Downregulation of Δ^9- and Δ^{12}-desaturase expression is one of the well-described mechanisms of aging in mammalian study models [14]. In this study, mRNAs encoding for *sde1* and *ode1* genes obtained from cultures grown in liquid HcMM were maintained at a relatively similar level independently of the culture age (Fig. 1). Therefore, we postulate that the decrease in lipid/fatty acid unsaturation levels was related to nutrient exhaustion in starving/aging cultures and relied on a selective withdrawal of unsaturated FAs from accumulated lipids and their further redistribution into cellular metabolism. This might help to replenish exhausted nutrients in aging cultures.

Our findings concerning NLs in *H. capsulatum* cells are in good agreement with previously published reports [8,9,18], although it is difficult to compare data obtained for different strains using different media, growth conditions, and subsequent procedures for lipid extraction and analysis. Our observation is also in good agreement with studies of other fungal species, where a decrease in unsaturated FA content accompanied by an increase in FA saturation has been

reported (e.g., Refs. 7 and 11). Overall, the results presented in this work not only have implications for our knowledge of the basic lipid biochemistry and physiology of *H. capsulatum*, but also will contribute to a better understanding of the biology and pathogenesis of this fungus and, consequently, can help in the discovery of more effective antifungal lipophilic drugs.

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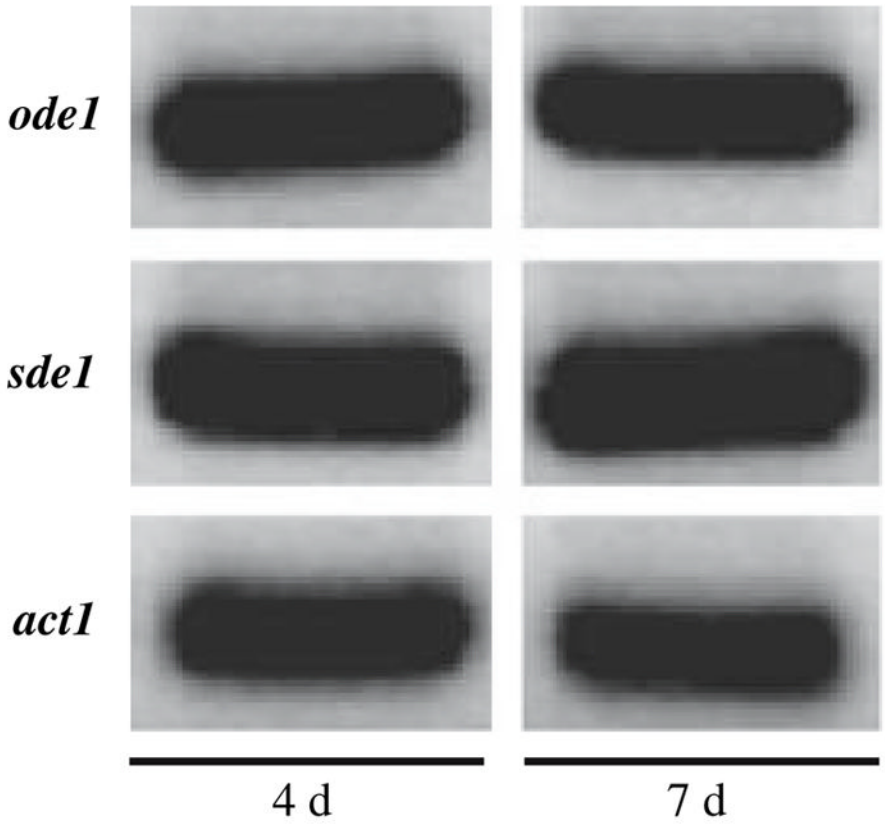

Fig. 1.

RT-PCR transcriptional analysis of Δ^9- and Δ^{12}-fatty acid desaturase genes of *H. capsulatum* G217B. Total cellular RNA was isolated from 4- and 7-day-old yeast cells grown in liquid *H. capsulatum* minimal medium. Equivalent amounts of total cellular RNA (1 μg) were used in the synthesis of first-strand cDNA, which was subsequently used as a template in RT-PCR gene expression analysis of Δ^9- and Δ^{12}-fatty acid desaturases, sde1 and ode1. Transcript levels of both desaturases were normalized with reference to act1

Table 1

Culture age-related changes in fatty acid composition in neutral lipids of *H. capsulatum* G217B grown in *Histoplasma capsulatum* minimal medium[a]

Culture growth stage (age)	Lipid class[b]	Fatty acid composition [%][c]												
		14:0	**16:0**	**16:1**	**17:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:1**	**20:4**	**20:5**	**22:0**	**22:6**
Log phase (4 days)	TGA	0.4	17.1	8.8	t	0.9	39.2	32.9	0.2	0.2	t	t	ND	t
	DAG	0.4	22.6	10.6	ND	0.8	25.6	39.3	t	t	ND	0.6	ND	ND
	FFA	0.7	28.9	5.9	0.3	1.7	26.2	29.7	0.6	0.5	ND	5.5	ND	ND
	STE	11.0	8.4	2.5	4.4	43.2	29.2	0.2	ND	t	1.0	ND	ND	ND
Stationary phase (7 days)	TGA	6.3	26.3	5.9	ND	2.8	28.2	28.7	ND	0.3	0.5	0.9	t	ND
	DAG	1.4	63.2	2.7	t	5.3	11.0	13.4	ND	ND	ND	3.0	ND	ND
	FFA	2.6	74.0	0.6	0.5	8.3	2.4	ND	3.6	ND	ND	8.2	ND	ND
	STE	43.7	32.0	5.9	15.3	0.8	ND	ND	ND	ND	2.4	ND	ND	ND

[a] Mean values of three different determinations from a representative experiment. The SE did not exceed 3%

[b] TAG, triacylglycerol; DAG, diacylglycerol; FFA, free fatty acid; STE, sterol ester

[c] *t*, trace (<0.1 %); ND, not detected

Table 2

Culture age-related changes in fatty acid composition in neutral lipids of *H. capsulatum* G217B grown in *Histoplasma*-macrophage medium[a]

Culture growth stage (age)	Lipid class[b]	Fatty acid composition [%][c]										
		14:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:1	20:4	20:5
Log phase (4 days)	TGA	0.5	8.8	8.1	0.2	0.6	55.1	25.7	0.2	0.5	0.2	ND
	DAG	0.9	16.3	9.5	ND	0.9	38.4	33.5	ND	ND	0.4	ND
	FFA	0.7	11.8	5.3	0.4	3.3	41.2	36.2	0.5	0.5	ND	ND
	STE	4.3	13.6	1.6	10.5	34.3	33.8	0.9	ND	ND	0.9	ND
Stationary phase (7 days)	TGA	1.0	18.0	5.2	0.2	1.3	51.4	21.5	t	0.6	0.3	0.3
	DAG	1.9	47.1	1.6	t	3.6	23.8	19.9	ND	ND	0.4	1.6
	FFA	1.2	43.1	t	0.6	13.3	20.7	16.5	2.5	ND	ND	1.8
	STE	37.0	37.2	1.8	16.3	T	4.6	0.7	ND	ND	2.3	ND

[a] Mean values of three different determinations from a representative experiment. The SE did not exceed 3%

[b] TAG, triacylglycerol; DAG, diacylglycerol; FFA, free fatty acid; STE, sterol ester

[c] *t*, trace (<0.1 %); ND, not detected