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Ferrous, But Not Ferric, Iron Maintains Homeostasis in *Histoplasma capsulatum* Triacylglycerides

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Abstract

Iron is an indispensable micronutrient for virtually all microorganisms, where it acts as a cofactor of many enzymes involved in regulation of multiple cellular and physiological functions. This metal is also considered an important determinant contributing to the pathogenesis of fungal infectious diseases, and therefore the identification of iron-regulated metabolic processes occurring within the invading fungal cell can help the development of new anti-fungal therapeutic strategies. In this study, we examined relationships between iron availability and neutral storage lipids in *Histoplasma capsulatum*, a dimorphic fungus responsible for the most common respiratory and systemic mycosis in humans. Yeast cells were grown in a defined minimal medium supplemented with or without iron. Lipids were extracted from cells at the log and late stationary growth phases, then separated by thin-layer chromatography, and fatty acids were analyzed by gas chromatography. A culture age-related decrease in the unsaturated fatty acid content was observed in all four neutral lipid classes examined. Iron-related alterations could be seen in relation to triacylglycerol and free fatty acid pools, whereas no iron-dependent effects were detected in diacylglycerol and steryl ester fractions. Regarding triacylglycerols, the presence of iron positively affected the content of unsaturated fatty acids, and this stabilizing action of iron was notably increased when ferrous ions were added. Subsequent iron uptake studies showed a definite preference of *H. capsulatum* to acquire iron in its reduced, more soluble, ferrous form, and therefore, the availability of iron may be the underlying reason for the observed iron-maintained homeostasis in *H. capsulatum* triacylglycerols.

Introduction

Iron is essential for virtually all living organisms, where it acts as a cofactor of many enzymes and is involved in multiple vital cellular and physiological functions. This metal exists in nature predominantly in the insoluble oxidized Fe^{3+} form and its accessibility to living organisms is,

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in general, drastically limited. In *Saccharomyces cerevisiae*, iron scarcity results in a substantial transcriptional remodeling of expression patterns of multiple genes leading to tremendous alterations in numerous metabolic traits, including biotin uptake and biosynthesis, nitrogen assimilation, and purine biosynthesis [13]. Iron also plays a crucial role in fungal infectious diseases and its deficit induces remarkable changes in a vast range of metabolic cascades within the fungal cell so that an invading fungus can multiply and establish a progressive infection, while tightly regulating iron acquisition to prevent accumulation of excessive toxic amounts of this metal [1,2,10].

Iron has been shown to play a critical role in both in vitro and in vivo growth of *Histoplasma capsulatum* [5,8,9,11,12]. This dimorphic human pathogenic fungus is the etiological agent of histoplasmosis, which is a severe life-threatening respiratory and systemic infectious disease in both immunocompromised and healthy individuals [14]. Although the importance of iron during *H. capsulatum* infection is indisputable, no information about the influence of iron availability on general metabolic processes in this fungus has been reported. These as yet unidentified iron-dependent biochemical and physiological routes present important questions about the pathobiology of this medically important microorganism and their identification could help to develop more efficient therapeutics. Here, we demonstrate a link between iron and neutral storage lipid metabolism in *H. capsulatum*. We also provide evidence that triacylglycerol homeostasis in fungal cultures is strictly dependent on the availability of ferrous, but not ferric, iron.

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 broth *Histoplasma capsulatum* minimal medium (HcMM) amended with ferrous iron [15]. In this study HcMM was also prepared without iron, or ferrous iron was substituted with ferric ions, or, alternatively, both its forms were used together. Yeast cultures were grown as reported elsewhere [11]. Before culture inoculation with 3-day-old cells, the inocula were incubated overnight in phosphate-buffered saline (PBS). This step allowed elimination of intracellular iron from fungal cells [12]. For all experiments, glass was initially treated with 5 N HCl and then thoroughly washed with Chelex-100 (BioRad Laboratories) resin-treated, double-distilled water.

Lipid Extraction and Analysis

Cells were harvested by centrifugation (3000 g, 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 fatty acid (FA) analysis were performed as described elsewhere [15,16].

Statistics

Lipid data were evaluated by analysis of variance (ANOVA; Statistica for Windows v. 5.1, StatSoft Ltd.). Mean values labeled with different letters in figures are significantly different at $p < 0.005$. FA profiles specific for each of the fungal cultures were also used to construct a proper matrix that was processed using principal component analysis (Statistica). Proper dendrograms were drawn using calculated Varimax-rotated principal component loadings as described elsewhere [16].

⁵⁹Fe Uptake Assay

Late-exponential-phase cells were collected by centrifugation. Cells were washed twice in Chelex-treated PBS, resuspended in iron-free HcMM, and incubated for 3 additional days at 37°C in 5% CO₂/95% air atmosphere. Cultures were subsequently spun down at 3500 g for 10 min, and cells were then resuspended in PBS and transferred into fresh Eppendorf tubes. Prior to the assay, samples were allowed to equilibrate by gentle stirring in Eppendorf tubes at 37°C for 5 min. The experiment was initiated by adding the required concentration of ⁵⁹FeCl₃ (Perkin Elmer). The radiolabeled iron was added either in its reduced Fe²⁺ form (prepared by 10-fold dilution of the stock of ⁵⁹FeCl₃ in 1 M sodium ascorbate) or as Fe³⁺-citrate (the stock was diluted 10 times in 1 M sodium citrate). Unlabeled Fe²⁺ and Fe³⁺ were prepared in the same way and the final iron concentration in all experiments was 1 μM. After 1-h incubation at 37°C, Fe uptake was stopped by layering 175-μl samples onto 0.3 ml of dimethicone oil (Ace Glass Inc.) and centrifugation at 13,000 g for 4 min through viscous siloxane oil. The aqueous layer was aspirated, and the oily surface was washed twice with 0.3 ml Chelex-treated PBS. The oily layer was removed, and the cell pellet was resuspended in 175 μl of OptiPhase Super-Mix liquid scintillation cocktail (Perkin-Elmer) and transferred to a polyethylene terephthalate 96-well sample plate (Wallac). Cell-associated ⁵⁹Fe was measured by liquid scintillation counting using a Perkin-Elmer 1450 LSC and Luminescence Counter MicroBeta TriLux device. Assay mixtures containing no cells were used to calculate blank values for correction.

Results and Discussion

Neutral lipids serve in eukaryotes as reduced reservoirs of highly oxidizable energy and provide pools of accessible acyl-CoA units needed for cellular metabolism. The importance of neutral lipid metabolism and its implication in maintenance of cellular homeostasis as well as in development of certain infectious diseases have been well documented [3,4]. In many fungi including *H. capsulatum*, triacylglycerols (TAGs) and steryl esters (STEs) constitute the majority of neutral lipids, whereas diacylglycerol (DAG) and free FA fractions are less abundant [7,15,17]. Neutral lipids in eukaryotic cells are altered by numerous factors that often set the pace of metabolism leading to desirable cellular and physiological adaptations [6]. Interestingly, neutral lipids are utilized extensively by aging *H. capsulatum* cultures and this process manifests itself in a significant decrease in unsaturated FA content [15]. In this study, we examined alterations in FA patterns within neutral lipids of *H. capsulatum* cultures as a function of iron availability. Yeast cells of the *H. capsulatum* G217B strain were grown in a defined minimal medium differentially supplemented with iron, which is deemed essential for growth of this fungus [14]. The medium was therefore amended with ferric iron, ferrous iron, or both iron forms or contained no iron at all. The presence of contaminating trace amounts of iron in the iron-deprived medium is highly unlikely, and therefore, these cultures should be considered to be literally free of iron.

Fungal cells were collected after 4 and 7 days of growth, which roughly correspond to the log and stationary culture growth phases, respectively [15]. Although iron supplementation accelerated fungal growth, there was no growth variation when ferric or ferrous iron was used (data not shown), and this observation corroborates previous studies [11]. Minimal growth rates of the fungus scarcely observed in iron-deprived cultures might possibly be driven by hypothetical intracellular pools of iron, which could not be readily immobilized by initial iron starvation, or by intra-cellular iron pools released from degraded iron-containing proteins in fungal cells grown under iron-depleted conditions. Regardless of the presence or absence of iron in cultures, neutral lipids isolated from yeast cells of *H. capsulatum* contained a wide range of FAs, with predominantly palmitic (16:0), oleic (18:1), and linoleic (18:2) acids, whereas myristic (14:0), palmitoleic (16:1), stearic (18:0), linolenic (18:3), eicosanoic (20:0),

eicosaenoic (20:1), eicosatetraenoic (20:4), and docosanoic (22:0) acids were present in considerably lower amounts (data not shown). In general, qualitative FA patterns remained constant over the time course of this study and this finding is in good agreement with our previous report [15]; however, some modifications in quantitative FA patterns within particular neutral lipids were noted with reference to iron availability (Fig. 1). Among all four neutral lipid fractions studied, the most essential iron-dependent changes were observed in TAGs (Fig. 1A). In this case, the lack of iron induced a previously described, culture age-related 67.2% increase in saturated FAs, while its presence exerted a strong protective effect on TAGs, especially when cultures contained ferrous iron. Intriguingly, the content of mono-unsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) in cells growing in ferrous iron-amended HcMM was reduced by 13.8 and 3.2%, respectively. The presence of ferric iron induced more noticeable changes in FAs in the TAG fraction (21.1% and 22.0% decrease, respectively), while supplementation with both iron ions yielded intermediate, but still statistically significant changes in FA unsaturation levels (as indicated by different-letter labels in Fig. 1A). A slightly different effect was observed with reference to free FAs, where the addition of ferrous iron resulted in an increase in FA saturation and about a twofold decrease in PUFAs, but the presence of ferric ions had no effect (Fig. 1B). There was no significant iron-related variability observed in FA patterns of DAG and STE pools, two other neutral lipid fractions found in *H. capsulatum* (Fig. 1C and D).

To gain more detailed insight into the observed iron availability effect on *H. capsulatum* neutral lipids, the data obtained were then compared statistically using principal component analysis. This approach yielded as many as six principal component loadings, which were subsequently used to construct corresponding dendrograms delineating correlation between FA patterns in *H. capsulatum* neutral lipids and differential culture supplementation with iron. Those changes in FA profiles were clearly less significant in 4-day-old log-growth-phase cells but were escalated after 3 additional days of growth in stationary fungal cultures, and the effect of ferrous iron supplementation could be readily distinguished (Fig. 2A). Further analysis also allowed definition of the variability in FA profiles obtained from differently aged cultures grown in the same form of iron. In general, the lowest change ratio was observed in a cluster combining the cultures containing no iron, whereas the other types of iron supplementation generated distinguishable alterations in neutral lipids represented by spatial shifts of the proper objects within particular clusters (Fig. 2B).

Metabolic pathways are frequently regulated at the transcriptional level both by substrates that are consumed in the pathway and by the products of the pathway. More complex mechanisms of regulation of metabolic pathways have been reported in yeasts [13]. Here, we have described a mode of regulation in which the availability of iron can influence the TAG metabolism in *H. capsulatum*. It is unclear, however, whether iron acts directly as a cofactor required for TAG-related enzyme activities or whether this metal affects the expression of TAG-related genes. The rationale for the existence of the observed iron-maintained homeostasis in *H. capsulatum* TAG might also result from unique physicochemical properties of this metal. Thermodynamically, the equilibrium between both reduced ferrous and oxidized ferric ions at physiological pH is normally heavily biased in favor of the latter type, which forms extremely stable and insoluble ferric (hydr)oxide complexes. Under physiological conditions ferrous iron is definitely much more soluble than its oxidized equivalents, and thereby this form should be more acquirable by *H. capsulatum*. To verify this hypothesis, we tested whether *H. capsulatum* G217B preferentially acquires ferrous or ferric iron. We measured iron uptake of Fe^{2+} and Fe^{3+} ions in late-exponential-phase cells grown in iron-depleted HcMM. Approximately 10^6 cells of *H. capsulatum* were able to take up about 79.98 ± 9.22 fmol of $^{59}\text{Fe}^{2+}$ min^{-1} (Fig. 3). Fe^{2+} uptake was over fivefold more efficient than that of Fe^{3+} , which was accumulated in fungal cells at a maximum concentration of 13.95 ± 3.97 fmol $^{59}\text{Fe}^{3+}$ min^{-1} . Interestingly, the uptake of iron when both forms were present yielded an intermediate

value of $48.63 \pm 7.86 \text{ fmol Fe } (10^6 \text{ CFU})^{-1} \text{ min}^{-1}$. These results demonstrated a definite preference of *H. capsulatum* G217B for iron in its reduced, more soluble form, which is acquired in a dose-dependent way. The availability of iron may, therefore, be the underlying cause for the observed iron-maintained homeostasis in *H. capsulatum* TAGs; however, the functional consequences of this phenomenon and its contribution to the pathobiology of *H. capsulatum* remain unclear. It is rather unlikely that neutral lipids, which are mostly localized in the intracellular milieu, are directly involved in avoidance or survival of the host-generated oxidative stress during infection. In fact, no significant changes in FA profiles in neutral lipids isolated from *H. capsulatum* cells challenged with sublethal doses of hydrogen peroxide were seen (data not shown). We are, therefore, interested in further characterizing iron-regulated TAG metabolism in *H. capsulatum*, identifying the relevant genes and their corresponding enzymes, and understanding their regulation by iron. Molecular biology-based alterations in the expression of these genes and the corresponding enzyme activities involved in intracellular neutral lipid metabolism in *H. capsulatum* will elucidate the biological and pathogenic roles of this mechanism.

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Fig. 1.

Variations in saturated, monounsaturated, and polyunsaturated fatty acids of neutral storage lipids isolated from *H. capsulatum* grown in cultures differentially supplemented with iron. The fungus was grown as reported under Materials and Methods. Lipids were extracted at the appropriate time points, then separated on TLC plates, and isolated fatty acids were converted into corresponding methyl esters. Each data bar is the mean of four independent replicates and represents percentage changes in saturated, monounsaturated, and polyunsaturated fatty acids measured over the time course of this study. Data were evaluated by ANOVA, and mean values labeled with different letters are significantly different (at $p < 0.005$). (A) Triacylglycerols; (B) free fatty acids; (C) diacylglycerols; (D) sterol esters

Fig. 2.

Correlation between fatty acid patterns in *H. capsulatum* neutral lipids and differential culture supplementation with iron. Data were compared statistically using principal component (PC) analysis, and three-dimensional score plots were created based on calculated Varimax-rotated PC loadings. Values in brackets refer to percentage variations observed in the original data. (A) PC1 (48.82 %); PC2 (44.64 %); PC3 (5.17 %). (B) PC4 (0.76 %); PC5 (0.22 %); PC6 (0.21 %)

Fig. 3.

Iron uptake by *H. capsulatum* yeast cells. The experiment was initiated by the addition of ⁵⁹Fe²⁺, ⁵⁹Fe³⁺, or both iron forms to late-exponential-phase yeast cells grown in Chelex-treated, iron-deprived HcMM. The final concentration of iron in all experiments was 1 μM. Iron uptake was determined after 1-h incubation and data represent mean values ± SD from one representative of two independent experiments carried out in triplicate. Iron uptake values were normalized per 10⁶ fungal cells per minute