The effect of L-DOPA on Cryptococcus neoformans growth and gene expression

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Cryptococcus neoformans is unusual among melanotic fungi in that it requires an exogenous supply of precursor to synthesize melanin. C. neoformans melanizes during mammalian infection in a process that presumably uses host-supplied compounds such as catecholamines. L-3,4-dihydroxyphenylalanine (L-DOPA) is a natural catecholamine that is frequently used to induce melanization in C. neoformans and L-DOPA-melanized cryptococci manifest resistance to radiation, phagocytosis, detergents and heavy metals. Given that C. neoformans needs exogenous substrate for melanization one question in the field is the extent to which melanin-associated phenotypes reflect the presence of melanin or metabolic changes in response to substrates. In this study we analyze the response of C. neoformans to L-DOPA with respect to melanization, gene expression and metabolic incorporation. Increasing the concentration of L-DOPA promotes melanin formation up to concentrations >1 mM, after which toxicity is apparent as manifested by reduced growth. The timing of C. neoformans cells to melanization is affected by growth phase and cell density. Remarkably, growth of C. neoformans in the presence of L-DOPA results in the induction of relatively few genes, most of which could be related to stress metabolism. We interpret these results to suggest that the biological effects associated with melanization after growth in L-DOPA are largely due to the presence of the pigment. This in turn provides strong support for the view that melanin contributes to virulence directly through its presence in the cell wall.

Introduction

Many pathogenic fungi produce melanin in the cell wall. Melanin enhances the virulence of fungi in various ways. In the rice blast fungus, Magnaporthe grisea, melanin strengthens the cell wall, allowing for the buildup of high turgor pressure that aids in invasion of plant tissues.1 In Paracoccidioides brasiliensis, melanin interferes with normal macrophage function.2,3 In Exophiala dermatitidis, melanin contributes to fungal resistance to oxidative killing by neutrophils.4 In addition melanin increases the resistance of fungi to environmental damage, as in the melanized fungi isolated from the radioactively contaminated Chernobyl reactors.5 Melanin is one of the major virulence factors in Cryptococcus neoformans, a fungus that is a relatively frequent cause of life-threatening meningoencephalitis in immunocompromised individuals.6 Cryptococcosis is one of the more common secondary infections in AIDS patients. There are estimated to be nearly 1 million cases of the disease each year, the majority of which are in sub-Saharan Africa.7

In contrast to most other melanotic fungi that are able to make melanin from endogenous precursors, the synthesis of melanin by C. neoformans requires the addition of exogenous substrate in the medium, such as L-DOPA.8 The initial chemical step in the synthesis of melanin is the oxidation of L-DOPA to dopaquinone and is catalyzed by laccase.9 Subsequent steps are believed to occur spontaneously, producing dihydroxyindole or dihydroxyindole-2-carboxylic acid intermediates that finally polymerize into melanin.10,11 Melanins are amorphous polymers composed of crosslinked phenolic and/or indolic subunits.12 Since the exact composition of subunits in the polymer has not been determined, the definition of a compound as a melanin is based on physical characteristics, including dark color, insolubility in most solvents, resistance to acid, oxidation by hydrogen peroxide and degradation by alkalii.13 In C. neoformans, melanin has a complex architecture with multiple layers and a granular surface.14

Despite its importance to pathogenesis, many details of the cell biology of C. neoformans melanization remain unknown. In fact, the inability of C. neoformans to produce its own melanin without exogenous substrate makes this organism an ideal system to study melanization since it is possible to vary conditions to better understand this process. In this study we have explored the conditions that lead to optimum pigment expression and the response of the organism to the presence of substrate. The results provide new insights into the process of cryptococcal melanization that are relevant to understanding the role of melanin in pathogenesis.

Results

Dose-dependent responses of melanization and growth to L-DOPA. A dose-response assay was carried out with L-DOPA...
tested. At a concentration of 10 mM, growth of *C. neoformans* was completely inhibited.

**Timing of melanization.** When cells were cultured in liquid medium, we noted that it often took several days for the cells to turn black, depending on the strain. We reasoned that this delay in melanization could have several explanations. For example, the melanization lag could have indicated that L-DOPA was incorporated very slowly into melanin. Alternatively, it could have indicated that the melanization process took several days to commence, but occurred rapidly once begun. To distinguish between these possibilities, cells were grown for seven days in minimal medium without L-DOPA, and then L-DOPA was added to a concentration of 1 mM. After one or two days, the “old” cultures melanized (Fig. 2). In contrast, when cells were cultured for two days in “new” media containing L-DOPA, they did not melanize. This result suggested that the rate of melanization in culture was limited by the initiation of melanization, not the production of melanin.

**Density of cells influenced melanization.** The results with old cultures suggested that cell density and/or age impacted the rate of melanization, since older, denser cultures melanized rapidly and new cultures did not. Cell density effects were tested by concentrating an overnight culture 10- and 100-fold. L-DOPA was added and the cultures were incubated overnight. Only the densest culture turned black; no color change was observed for the less dense cultures (Fig. 3A).

Similar to growth in liquid, the timing at which melanization of *C. neoformans* cells grown in agar became visually apparent was variable. To test if the timing of melanization in agar was related to density, L-DOPA agar was inoculated with variable amounts
of *C. neoformans*. When grown in agar, melanization occurred faster when more CFUs were plated per culture dish (Fig. 3B).

**Genes induced by L-DOPA.** Gene expression in melanizing cells was analyzed. L-DOPA was added to a culture of *C. neoformans* grown to a cell density of 4 x 10^7 CFU/ml. After four hours of incubation with L-DOPA, RNA was extracted from the cells and purified. Microarray analysis was carried out comparing cells grown in the presence of L-DOPA to those without L-DOPA. Of the approximately 6,500 genes in *C. neoformans*, eight were identified with increased expression in the presence of L-DOPA (Table 2). Real-time PCR was used to confirm the induction of the genes. Induction of five of the eight genes was confirmed by real-time PCR analysis (CNB04110, CNK02300, CND03820, CNG04630 and CNB04240). To determine the putative functions of the induced genes, homology searches were carried out by entering the predicted protein sequence into the NCBI BLAST search webpage (www.ncbi.nlm.nih.gov/BLAST). Among these genes, several were putative enzymes with redox functions (Table 2).

To test the relationship of these genes to melanization, we determined their expression in the presence of L-DOPA under non-melanizing conditions. To do so, gene induction was measured by real-time PCR in a *C. neoformans* strain without laccase (*LAC1* deletion strain 2E-TU and the complemented strain, 2E-TUC, of a related genetic background). The same five genes with confirmed increase in gene expression had little or no induction in the laccase deletion strain. The complemented strain had variable expression, perhaps due to differences in laccase expression between this strain and the wild-type (Table 2).

Next, cultures were incubated with L-DOPA for several days and changes in gene expression were evaluated over time (Fig. 4). The maximum levels of induction were considerably higher at these longer times in comparison to the previous experiments in which cultures were incubated with L-DOPA for 4 h (Table 2).

For example, gene CNB04110 had a maximum induction of 350-fold after three days of incubation with L-DOPA. A similar pattern of induction was observed for most of the genes, with low expression on day 1, a peak of gene expression on day 3, and gradual decrease over time. This peak of gene expression coincided with the cultures reaching a density at which melanization occurs. Gene induction was generally not observed in the laccase mutant strain.

**Uptake of L-DOPA by *C. neoformans* cells.** The gene expression studies using the *LAC1* deletion strain suggested that
melanization was required for induction of the genes in the presence of L-DOPA. To further address the issue of whether the gene expression changes were related to melanization, the ability of cells to incorporate L-DOPA was tested. Cells were incubated with ¹⁴C-labeled L-DOPA and the level of incorporation into the cells was determined by liquid scintillation counting. Initial experiments showed a low level of incorporation for all cells (data not shown). To increase the level of L-DOPA incorporation, cells were incubated in starvation media prior to adding ¹⁴C-L-DOPA, a condition known to induce melanization. Incorporation was measured at 5 min, 1 h, 4 h and 23 h of incubation. After overnight incubation, the wild-type and laccase complemented strains accumulated much of the ¹⁴C-L-DOPA (77% and 70%, respectively), while the laccase deletion strain and heat-killed
Indeed, these results add to considerable work that has been done in our lab16,17 suggesting that \textit{C. neoformans} melanization is controlled by quorum sensing. In this regard, a quorum sensing-like phenomenon has been described for \textit{C. neoformans} mediated by a peptide.18 Although this peptide has not been studied for its effect on the rate of melanization, it has protean changes in \textit{C. neoformans} metabolism that include effects on melanization 19 and it is conceivable that a related mechanism is responsible for our results reported in this manuscript.

Both melanization and growth are inhibited in the presence of a high concentration of L-DOPA or norepinephrine (10 mM). The inhibition of melanization may be due to autopolymerization of the substrate in the medium making it unavailable for use in melanin formation. Examination of the plates shows that the agar surrounding the colonies is dark at this concentration. The inhibition of growth suggests that L-DOPA, or a metabolic product of L-DOPA, presents some toxicity to \textit{C. neoformans}. This hypothesis is supported by the gene expression data, as discussed below.

These data elucidate the optimal conditions for in vitro melanization of \textit{C. neoformans}. The relationship between in vitro and in vivo melanization is an important topic about which very little is known. Melanized cryptococcal cells have been observed in the brains of patients as well as in experimental animals.20,21 The in vivo substrate has yet to be identified, but it is hypothesized to be one or a combination of catecholamines such as dopamine, norepinephrine, homovanillic acid and L-DOPA. Several of these compounds are normally present at concentrations of μg/g in
which growth of media containing substrate. The levels of these catecholamines in cryptococcosis patients are not known. Likewise, cell density effects on in vivo melanization are hard to determine. In vivo, cryptococcal cells may not be evenly distributed as in a liquid culture, but may be inside host macrophages or a cryptococcoma. The effects of immune attack and hypoxia on further complicate the scenario.

One of the unanswered questions in the field is the extent to which growth of C. neoformans in media containing substrate affects fungal metabolism independently of the capacity of substrate to induce melanization. Published studies in melanization have identified genes required for C. neoformans to produce pigment, resulting in a white phenotype when mutated. These include genes in cell wall synthesis, metal ion transport and virulence regulators. In contrast, our study identifies novel genes that are upregulated in the presence of melanization substrate. Gene induction is only observed in cells capable of melanization, suggesting the genes either have a role in this process or are induced as a consequence of melanization. In addition, the density at which maximal gene expression occurs is similar to the density at which melanization becomes visible in liquid culture. Furthermore, analysis of L-DOPA incorporation by C. neoformans cells shows that only wild-type cells capable of melanization incorporate the substrate. This suggests that L-DOPA does not accumulate inside cells and is unlikely to have an effect on gene expression that is unrelated to melanization. Together, these data support the hypothesis that the identified genes play a role in melanization.

We hypothesize that the genes identified function in regulating melanization and/or protecting cells from toxic by-products produced during melanization. In general, melanin and its synthetic intermediates are highly reactive molecules. For example, the intermediate dopaquinone can react with amine and thiol groups, such as in proteins, resulting in crosslinking of melanin and other molecules. Thus, it seems logical that melanization is regulated in some way. The toxicity hypothesis is supported by published data showing that two of the genes, CNB04110 and CNB04240, are also induced by nitric oxide stress. Furthermore, homology searches also provided information consistent with the toxicity hypothesis. CNG04630 has homology to glutathione transferases, proteins involved in removal of toxic substances from the cell, whereas CNK02300 has homology to glutathione transferases, proteins involved in metabolizing toxins inside cells.

Perhaps the most surprising aspect of the gene expression analysis is the paucity of genes that are differentially regulated in the melanized state. Given that melanization is associated with diverse new attributes for melanized cells such as resistance to radiation, heavy metals, oxidants, enzymatic degradation, antifungal drugs and defenses, one might have expected melanin synthesis to be associated with more global changes in gene expression. In fact, the relative paucity of gene expression changes implies that melanization in C. neoformans is a relatively simple system from a genetic point of view and this, in turn, supports the association of new cellular properties in melanized cells with the formation of pigment rather than substrate-induced metabolic changes. The paucity of gene responses is also consistent with our recent proposal that melanin is synthesized in vesicles that are subsequently exported to the cell wall since this model involves pigment synthesis from highly reactive and toxic intermediates of L-DOPA oxidation in a confined membrane enclosed compartment that shields cellular processes. A similar strategy for avoiding melanin precursor toxicity is used by mammalian cells, which make melamins within melanosomes. In this regard, defective melanosomes have been associated with necrosis in melanoma cells. Nevertheless, the observation that most differentially regulated genes are associated with redox functions suggests that C. neoformans growth in the presence of L-DOPA is a stressful condition for this microbe. In summary, our results show that melanization in C. neoformans is a cell density dependent process that is associated with the induction of very few genes relative to the protean effects conferred by pigmentation that reduce fungal cell susceptibility to numerous insults. This in turn provides strong evidence for the view that the pigment itself is responsible for many protective effects associated with melanization and further strengthens the connection between melanin synthesis and virulence.

Materials and Methods

Fungal strains. JEC21, is a serotype D, MATα strain that was produced by crossing an environmental isolate with a clinical isolate. The serotype A, MATα strain H99 is a clinical isolate. The serotype D LAC1 deletion strain (2E-TU) contains a partial deletion of LAC1 near the 5’ end of the gene, and the complemented strain (2E-TUC) contains an integrated copy of LAC1. The laccase deletion and complemented strains are related to JEC21.

Growth and melanization assays. C. neoformans cells were grown in chemically defined minimal medium (15 mM dextrose, 10 mM MgSO4, 29.4 mM KH2PO4, 13 mM glycine and 3 μM thiamine, pH 5.5) with or without 1 mM L-DOPA (Sigma-Aldrich) and incubated at 30°C, 150 rotations per minute (RPM) in the dark. Cultures were examined daily to monitor growth and pigment production.

Cell density assays. Cell densities were determined by hemocytometer count and/or plating on agar. For the plate assay, the indicated numbers of CFUs were plated on chemically defined minimal medium agar containing 1 mM L-DOPA. For the liquid assay, an overnight culture grown in chemically defined minimal medium at a density of 3 x 10^5 CFU/ml was concentrated 10- and 100-fold. L-DOPA (1 mM) was added to the concentrated cultures and they were incubated for 18 h before photographing. All incubations were done at 30°C, 150 RPM in the dark.

Microarray analysis of genes induced by L-DOPA. C. neoformans cells were grown in minimal medium without substrate at 30°C, 150 RPM to a density of 4 x 10^7 CFU/ml. L-DOPA was then added at a concentration of 1 mM and the cells were allowed to grow for an additional four hours.
After the incubation, cells grown in the presence of L-DOPA were slightly grayish in color, indicating that melanization was occurring in the cells. Two independent biological replicates were performed. Total RNA was isolated from the cultures using the RNeasy kit (Qiagen) and incubated with DNase (GenHunter) to remove contaminating DNA. The purified RNA was sent to the Washington University Genome Sequencing Center for microarray analysis with the JEC21 genomic microarray as follows. Comparisons were done with both RNA pools and a Cy3-Cy5 dye swap was done between the sample with and without L-DOPA. Immediately after hybridization, the microarray slides were scanned on a ScanArray Express HT Scanner (Perkin Elmer) to detect fluorescence. Gridding and analysis of images were performed with ScanArray Software Express V2.0 (Perkin Elmer). Gene expression data were averaged across the two RNA pools and analyzed using the GeneSpring 7.2 software (Agilent) to identify genes in which the mean of the replicates had >2-fold change and p < 0.05. The Benjamini & Hochberg false discovery rate was performed with ScanArray Software Express V2.0 (Perkin Elmer) to detect fluorescence. Gridding and analysis of images were performed with ScanArray Software Express V2.0 (Perkin Elmer).

Table 1. Primers used for real-time PCR amplification

| Locus       | 5’ primer             | 3’ Primer             |
|------------|------------------------|------------------------|
| CNB04110   | GTC GTC ATT TCG GCC ATT | GAC CAG GGA TGC TGA TTT CT |
| CNK02300   | AAC ACT GGA TTG ATC CGA CA | CTT GAT GTG GTG GAA ATT GG |
| CND02380   | GCT GAC CGA GTC GCC GTC | GAC GAG AGT CAC CGG TAG TG |
| CND03820   | TGC GTG TGT TAC TGA AAC CC | GCA TCC TCC TCT TCT TCT TG |
| CNG04630   | GAG CGC TAC ATT CCT GAT GA | GAA GCG ATT GCT AGT GAG TAG G |
| CNB04240   | GTT GGC AAG TTT GTT TCC CT | ACC AAC AGA GGG CTC AGA GT |

Table 2. Fold changes in gene expression upon addition of L-DOPA to cultures

| Locus       | Putative function | Microarray | Real-Time PCR |
|------------|-------------------|------------|---------------|
|            |               | Wild-type | LAC deletion | Complemented |
| CNB04110   | Isoflavone reductase | 12.9 | 16.6 | 0.85 | 4.2 |
| CNK02300   | Glutathione transferase | 3.9 | 4.2 | 2.2 | 40 |
| CND02380   | NADH flavin oxidoreductase | 2.7 | 1.6 | 1.5 | 4 |
| CND03820   | Myosin I binding protein | 2.1 | 2.5 | 0.77 | 1 |
| CNG04630   | Permease | 2.1 | 4.8 | 0.86 | 1.4 |
| CNB04240   | Benzoquinone oxidoreductase | 2.0 | 2.1 | 0.87 | 1.6 |
| CND00360   | Unknown | 6.7 | Undetectable | |
| CNM01700   | Unknown | 2.0 | Not determined | |

Real-time PCR analysis of identified genes. L-DOPA was added to C. neoformans cells of strain JEC21, 2E-TU or 2E-TUC at densities of between 2 and 5 x 10⁷ CFU/ml and RNA was isolated after four hours as described above. cDNA was made by reverse transcription of two independent pools of RNA using the Quantitect Reverse Transcription kit (Qiagen). For the time course analysis, L-DOPA was added to cells at a density of 1 x 10⁶ CFU/ml (day 0) and RNA was isolated days 1, 3, 5 and 8. Primers were designed based on the JEC21 genome sequences to amplify approximately 100 base pairs (Table 1). PCR products were amplified with SYBR® Green PCR Master Mix (Applied Biosystems) in an ABL PRISM 7900HT Sequence Detection System (Applied Biosystems). Fold change in gene expression was determined relative to untreated cells of the same strain according to the method of Pfaffl.47 The gene encoding glyceraldehyde-3-phosphate dehydrogenase was used as the reference.

[14C]L-DOPA incorporation analysis. Cells of C. neoformans strains JEC21, 2E-TU and 2E-TUC were cultured for two days in chemically defined minimal medium at 30°C, 150 RPM. Cells were collected by centrifugation, washed and suspended in starvation medium (0.2 g/l, K₂HPO₄, 0.1 g/l KH₂PO₄). The cells were then incubated overnight at 30°C and 150 RPM. Before incubation with the labeled substrate, cells were collected by centrifugation again and suspended in starvation medium to a final density of 1–2 x 10⁸ CFU/ml. Heat-killed cells were incubated at 65°C for 1 h. Cell suspensions were incubated with L-3,4-dihydroxyphenyl[3-¹⁴C]alanine ([μCi of a 54 mM solution (GE Healthcare)] such that the concentration of L-DOPA was approximately 0.005 mM. At indicated time points, aliquots were removed and briefly centrifuged to pellet cells. The pellets were washed twice with PBS to remove unincorporated label. Liquid scintillation counting was used to determine the total counts per minute (CPM) in the supernatants and pellets (LKB Wallac 1217 Rackbeta, PerkinElmer). Percent incorporation was calculated using the formula: CPM pellet ÷ (CPM pellet + CPM supernatant) x 100. A multivariate analysis of variance (MANOVA) was used to test for incorporation differences between C. neoformans strain and time. Simple contrasts were then done to determine significant differences among strains at a particular time.


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