Hypoxia and Fungal Pathogenesis: To Air or Not to Air?

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Abstract

Over the last 3 decades, the frequency of life-threatening human fungal infections has increased as advances in medical therapies, solid-organ and hematopoietic stem cell transplantations, an increasing geriatric population, and HIV infections have resulted in significant rises in susceptible patient populations. Although significant advances have been made in understanding how fungi cause disease, our understanding of the dynamic microenvironments encountered by fungi during infection, and the mechanisms by which they adapt to these microenvironments, are not fully understood. As inhibiting and preventing in vivo fungal growth is a main goal of antifungal therapies, understanding in vivo fungal metabolism in these host microenvironments is critical for improvement of existing therapies or design of new approaches. In this review we focus on the emerging appreciation that pathogenic fungi like Candida albicans, Cryptococcus neoformans, and Aspergillus fumigatus, are exposed to oxygen-limited or hypoxic microenvironments during fungal pathogenesis. The implications of these in vivo hypoxic microenvironments on fungal metabolism and pathogenesis are discussed with an aim toward understanding the potential impact of hypoxia on invasive fungal infection outcomes.
Introduction

Molecular oxygen was an important driving force in the evolution of single cell and complex eukaryotic organisms (36, 50). Molecular oxygen plays an essential role as an electron acceptor in the generation of chemical energy via mitochondrial respiration, but is also critical for the biosynthesis of sterols, mono- and polyunsaturated fatty acids, nicotinamide adenine dinucleotide, porphyrin, and in other metabolic and biosynthetic pathways (41, 94, 115). Thus, the amount of available oxygen to eukaryotic cells is a critical factor in determining overall cellular metabolism. As most eukaryotic human fungal pathogens are generally considered obligate aerobes, oxygen availability during fungal pathogenesis may play a critical role in the outcome of infection from the perspective of both the host and fungus. In this review, we focus on the research generated to date that largely supports a significant role for in vivo oxygen availability in fungal pathogenesis. We seek here to expand upon other recent reviews in this area that dealt either primarily with fungal oxygen sensing mechanisms or hypoxia responses in Candida albicans and Cryptococcus neoformans and also raise questions regarding how manipulation of fungal and host oxygen responses may be utilized in the future to improve invasive fungal infection treatment outcomes (34, 42).

What is hypoxia and does it occur during human fungal pathogenesis?

In a given environment, oxygen availability is usually described as anaerobic or anoxic (complete absence of oxygen), hypoxic (reduction in available oxygen compared to atmospheric levels), and normoxic (atmospheric levels of oxygen, generally 21% O₂ or a partial pressure (pO₂) of 159mm Hg at sea level). In the context of microbial
pathogenesis, it is generally accepted that hypoxia occurs at sites of infection, thus

generating a significant environmental stress on most host and microbial pathogen cells

(25, 80, 84, 88). An exact oxygen level that defines localized hypoxia *in vivo* is difficult
to pinpoint and will likely vary with anatomical location and distinct pathologies.

However, the mammalian hypoxic response starts (as monitored through the induction
of the mammalian hypoxia transcription factor hypoxia inducible factor (HIF)-1) in most
cells at an oxygen level of ~6% (pO₂ 40 mm Hg) (110). In healthy tissues in the human
body, oxygen levels of 2.5% to 9% are considered normal, while oxygen levels of ≤1%
that have been described in tumors and wounds are typically considered hypoxic (1, 28,
77, 80, 109, 120). In the healthy human lung, the initial deposition site of many human
fungal infections, alveolar oxygen partial pressures are around 100-110 mm Hg (~14%
O₂) (57).

When an invading microbe interacts with host cells, tissue damage by

inflammation, thrombosis, and necrosis is thought to decrease available oxygen

centers due to decreased tissue perfusion at the site of infection (32, 80). For

example, it has recently been observed that production of the non-ribosomal peptide
toxin, gliotoxin, and other potential secondary metabolites by the mold *Aspergillus
fumigatus* contributes to the inhibition of angiogenesis in the lung (7). Inhibition of neo-
vascularization at the site of infection by the fungus is likely to cause significant tissue
necrosis, prevent tissue repair, and thus contribute to the development of localized and
perhaps systemic hypoxia. Moreover, it is likely that oxygen dependent metabolism of
both pathogen and host cells also contributes to the rapid utilization of available oxygen,
though this remains to be definitively confirmed. Importantly, at least within the lung, the
co-occurrence of microbial infection and hypoxia is often associated with poor clinical outcomes (3, 12, 101).

Oxygen concentrations at sites of human fungal infection have not been measured directly in vivo, though hypoxemia is often described as part of the clinical picture associated with these infections, even in the lung, that may require invasive or non-invasive oxygen therapy (13, 14, 53, 64, 70, 98). In addition, CO$_2$ production is directly coupled to oxygen consumption of eukaryotic cells and sites of hypoxia in vivo often also contain increased levels of this gas whose sensing has been linked to fungal virulence (31, 67). The interconnections and relationship between oxygen and carbon dioxide sensing with regard to fungal pathogenesis is an exciting area for further investigation (113).

With regard to Candida albicans, one of the most frequently occurring human fungal pathogens, its normal anatomical location is the human gastrointestinal tract that contains significant regions of hypoxia (49, 63). For cryptococcal meningitis caused by Cryptococcus neoformans, oxygen concentrations in the human brain are also significantly lower than in the atmosphere, indicating that C. neoformans is also faced with reduced oxygen levels during infection, at least within the brain (33, 106). In further support of the idea that fungal pathogens face significant oxygen depletion during pathogenesis, hypoxia at the site of infection has recently been confirmed in murine models of invasive pulmonary aspergillosis (IPA). In two related studies of murine IPA models, a luciferase-producing A. fumigatus strain showed decreased luminescence in vivo after reaching a maximum at day one post infection (despite an increase in fungal burden). The authors hypothesized that this observation was due to the severe tissue
damage caused during infection that may decrease oxygen availability. Thus, the lack of
luminescence may be attributable to hypoxia at the site of infection as oxygen is
essential for the light-producing luciferase reaction (15, 55).

Additional indirect evidence of hypoxia being an important component of the *in vivo* microenvironment during a pulmonary fungal infection comes from recent detection
of ethanol production in bronchoalveolar lavage fluid from a chemotherapeutic murine
model of IPA. It was further found that under normoxic conditions, *A. fumigatus* did not
produce detectable ethanol levels in culture supernatants, but that upon exposure to
hypoxia, *in vitro* culture supernatants from shake flask cultures contained significant
amounts of ethanol (44). These data suggest that in response to hypoxia, *A. fumigatus*
can ferment glucose or other fermentable carbon sources into ethanol. In addition to the
*in vivo* ethanol detection, hypoxia was directly monitored in the lung of three
immunologically distinct murine models of IPA utilizing the hypoxia detection agent
pimonidazole hydrochloride (hypoxyprobe-1) (Figure 1) (44). The results of this study
suggest that the influx and activity of host cells are strong contributors to the
development of hypoxia during an invasive pulmonary fungal infection as more
extensive hypoxia was detected in murine models characterized by strong inflammatory
responses (steroid treatment and chronic granulomatous disease) than those
characterized by fungal proliferation and tissue invasion (chemotherapeutic model).
However, the persistence and occurrence of hypoxia in the chemotherapeutic model
also suggests that direct actions of the invading fungus are important contributors to *in vivo* hypoxia. Studies on the occurrence of hypoxia in other models of fungal infections
await to be undertaken, but are essential to understanding when and where different
fungal pathogens are exposed to oxygen limitation. In summary, taken together, these observations suggest that human fungal pathogens are faced with rapidly changing oxygen availability during fungal pathogenesis, which may suggest that the ability to adapt to low oxygen environments is critical for fungal pathogenesis. In the following sections, we review our understanding of how normally aerobic fungal organisms adapt to hypoxia and whether these mechanisms are linked to the ability of these organisms to cause lethal disease.

**Fungal Adaptation to Hypoxia**

As the majority of human fungal pathogens do not normally inhabit the human body, at least as far as we currently understand, and are often only associated with infection in immunocompromised patients, a key question is how these fungi evolved and maintained their ability to adapt to hypoxia. The mold *A. fumigatus* is typically found in soil and decaying organic material such as compost heaps. These environments are relatively oxygen poor, and indeed, oxygen concentrations in compost piles rapidly change with the metabolic activity of the microflora and range from atmospheric (21%) to hypoxic (1.5% and lower) (122). This indicates that organisms that thrive in such environments have evolved hypoxia adaptation mechanisms. Moreover, the soil itself can become hypoxic after heavy rains or due to increased CO2 levels and thus soil borne organisms have evolved mechanisms to tolerate low and rapidly changing oxygen levels (26, 74). Although most molds have been traditionally considered obligate aerobes, *A. fumigatus* has been observed to tolerate oxygen levels as low as 0.1%, and several older studies even suggest that under the right nutrient conditions that *A.*
fumigatus can survive and grow anaerobically (47, 86, 117). In addition, Fusarium species seem particularly adept at tolerating hypoxic and even anoxic conditions, which is consistent with their resident ecological niche, the soil (45, 51). Thus, these studies strongly suggest that molds like A. fumigatus and F. oxysporum that cause human disease may not be typical obligate aerobes, but rather, are likely to be facultative anaerobes. More research on the metabolic physiology of these important pathogens is needed to further understand their ability to deal with low oxygen levels.

With regard to the human pathogenic yeasts, C. albicans is capable of low levels of growth under anaerobic conditions and can also ferment glucose to ethanol predominantly under low oxygen conditions suggesting a Pasteur effect typically associated with other facultative fermentative yeasts (99). In contrast, C. neoformans seems to be a true obligate aerobe, though the precise effects of low oxygen conditions on C. neoformans or Cryptococcus gattii growth have not been elucidated to the degree that they have in C. albicans. In RPMI 1640, a medium commonly used for Clinical and Laboratory Standards Institute (CLSI) antifungal drug susceptibility screening, oxygen is a limiting factor for C. neoformans growth, and cell cycle progression also seems dependent on oxygen availability (82, 83, 118). Taken together, these data suggest that human pathogenic fungi have evolved mechanisms to adapt to low oxygen environments that occur in their natural environments as well as during fungal pathogenesis. It is currently unclear whether these hypoxia adaptation mechanisms directly contribute to the distinction between pathogenic and non-pathogenic fungi, but data suggest that the responses are similar in non-pathogens such as Schizosaccharomyces pombe (119). A further detailed analysis of pathogenic and non-
pathogenic fungal hypoxia adaptation mechanisms is needed to determine whether
human pathogenic fungi contain unique mechanisms of hypoxia adaptation that allows
them to thrive in mammalian hosts.

Fungal Adaptation to Hypoxia – A Genomics Approach

In order to understand how human pathogenic fungi adapt to oxygen limitation
and whether this is important for fungal pathogenesis, the molecular mechanisms
associated with the ability to adapt to hypoxia have been investigated by global fungal
transcriptome and proteome profiling studies. Perhaps not surprisingly, hypoxia causes
significant changes in the levels of both mRNA and protein abundance in human
pathogenic fungi. While certain conserved themes have emerged from these studies, an
important consideration for their interpretation is the methodology utilized to generate
the RNA or protein samples examined for differential changes in response to hypoxia.
Oxygen concentrations between studies are often variable (typically between 0.2% and
1% or not defined when flasks were flushed with nitrogen), fungal material was
harvested after different periods of hypoxic exposure ranging from 2 hours to around 8
days, and carbon, nitrogen, and micronutrient sources in the growth media are also
variable (21, 71, 105, 116, 121). Despite these variables, there are general themes that
have emerged as to how human pathogenic fungi adapt to hypoxia that are discussed
below organized via the respective human fungal pathogens.
Three major transcriptome analyses of the *C. albicans* response to hypoxia have been published and generally have concordant results despite differences in experimental design particularly with how oxygen levels were manipulated and strain of the fungus utilized (2, 105, 116). Major themes of the *C. albicans* hypoxia response emerging from these studies include: transcript level increases of genes involved in iron metabolism, heme biosynthesis, fatty acid metabolism, ergosterol biosynthesis, glycolysis and fermentation, cell wall and membrane structure, and hyphae specific transcripts (2, 105, 116). In contrast, transcripts involved in oxidative respiration such as those from the TCA cycle and mitochondrial respiration chain along with general ATP synthesis were decreased in response to hypoxia (2, 105, 116). The increase in transcripts associated with ergosterol biosynthesis, heme, and unsaturated fatty acids is perhaps not surprising given the importance of oxygen in the biosynthesis of these critical molecules. Transcript increases in ergosterol biosynthesis due to hypoxia are highly dependent on the transcription factor *UPC2* (75, 108, 116), while the transcriptional regulator *EFG1* is critical for positively regulating fatty acid biosynthesis in hypoxia (105). Recently, the hypoxic induction of glycolytic genes in *C. albicans* was shown to be highly dependent on the transcription factors *TYE7* and *GAL4* (2). Hypoxia is associated with inducing hyphal formation in *C. albicans*, particularly under conditions in embedded agar, and the transcript profile of hypoxia grown yeast in liquid culture is partially similar to a hyphal gene pattern of expression (6, 16, 105). This may have particular relevance to fungal pathogenesis as the yeast to hyphal transition is thought to be important for *C. albicans* virulence (reviewed in: (114)). *EFG1* regulates about half
of all hypoxic regulated genes in *C. albicans* and plays a unique role in hypoxia signaling in that it also prevents expression of genes not required for the hypoxia response. *C. albicans* contains an *EFG1* homolog *EFH1* that is also involved in morphogenetic and hypoxic signaling (30). Deletion of both *EFG1* and *EFH1* derepresses an alternative pathway of hyphal formation that is dependent upon oxygen. In contrast, null mutants of the transcriptional regulator *ACE2* required for transcription of genes involved in cell separation are unable to filament under hypoxic conditions (79). Though the exact mechanism of the filamentation defect in hypoxia of the *ACE2* null mutant is unknown, it may be due to differences in activity of the respiration chain and/or defects in oxygen dependent steps in the ergosterol biosynthesis pathway.

*Cryptococcus neoformans*

Compared to *C. albicans*, less is known about the effects of hypoxia on *C. neoformans*. A transcriptome analysis of the *C. neoformans* response to hypoxia revealed that similar to the *C. albicans* hypoxia response, heme biosynthesis, fatty acid metabolism, ergosterol biosynthesis, and stress response transcripts were increased (21). However, in stark contrast to *C. albicans*, respiration related transcripts were also increased in response to hypoxia, while cell wall and capsule biosynthesis genes were reduced (21). Given the potentially different life-styles of *C. neoformans* and *C. albicans*, it may not be surprising that these two organisms have different transcriptional responses to hypoxia. In support of the key role of respiration in *C. neoformans* hypoxia adaptation, an *Agrobacterium tumefaciens* mediated forward genetics screen identified
several mutants defective in hypoxic growth that were associated with mitochondrial
function (56).

To date hypoxia responses in *C. neoformans* have been shown to be dependent
on the transcriptional regulator *SRE1*, a member of the sterol regulatory element
binding protein (SREBP) family, and *TCO1*, a member of a highly conserved family of
fungal specific histidine kinases (19, 21). *SRE1* was shown to be a key regulator of
genes involved in ergosterol biosynthesis and metal uptake in response to hypoxia, and
the *SRE1* null mutant is significantly impaired in hypoxic growth (19, 21). Surprisingly,
transcripts induced in hypoxia appear to not be affected in terms of quantity in the
absence of *TCO1* and the authors hypothesize that *TCO1* acts post-transcriptionally to
mediate its impact on hypoxic growth of *C. neoformans* (21). Thus, many exciting areas
for investigation of the *C. neoformans* hypoxic response remain to be elucidated, and it
will be important to determine whether these mechanisms are conserved in the related
human pathogen *C. gattii*.

Aspergillus fumigatus

In addition to *C. albicans* and *C. neoformans*, recent studies have been
conducted assessing transcriptome and proteome responses to hypoxia in the mold *A.
fumigatus* (5, 121). These studies differ in design, as one study focuses on early time
points of hypoxia adaptation (2 to 24 hours) in batch culture (5), while the other study
focuses on long-term hypoxic exposure with an incubation time of around 8 days in
chemostat cultures with glucose limitation (121). In the *A. fumigatus* early hypoxia
adaptation study, the authors observe that transcripts involved in glycolysis,
fermentation, ergosterol biosynthesis, the \( \gamma \)-aminobutyrate (GABA) shunt, and iron uptake were induced in hypoxia (5). The induction of the GABA shunt has not been previously observed to be induced in response to hypoxia in either \textit{C. albicans} or \textit{C. neoformans}, but has in the related filamentous fungus, \textit{A. nidulans} (76, 107). The GABA shunt bypasses the TCA cycle, contributes to glutamate formation, and is hypothesized to be involved in the prevention of NADH accumulation in the absence of a terminal electron acceptor such as oxygen (35). This indicates that \textit{Aspergillus} species not only utilize fermentation to replenish sources of NAD\(^+\) for continued glycolytic flux, they also appear to use the GABA shunt to prevent NADH accumulation. In the culture conditions examined, TCA cycle and mitochondrial respiration chain associated transcripts were found to largely be decreased in \textit{A. fumigatus} during the early hypoxia response similar to \textit{C. albicans} (5, 105, 116). However, in the \textit{A. nidulans} hypoxia transcriptome analysis several TCA-cycle associated transcripts were increased in response to hypoxia though respiration associated transcripts were largely decreased (76). An \textit{A. nidulans} hypoxia proteomics study from the same laboratory found that respiration and TCA cycle protein abundance was not altered in hypoxia (107). Thus, it is unclear whether the observed TCA hypoxia responses are due to differences in culture conditions used between the studies. However, this question warrants further investigation as the reductive TCA cycle has been observed to be important for \textit{Mycobacterium tuberculosis} adaptation to oxygen limiting environments through increased fumarate reductase activity (124). \textit{A. fumigatus} contains a putative fumarate reductase ortholog, OsmA, which to date has been uncharacterized.
In contrast to the transcriptomic and proteomic studies conducted at early hypoxia adaptation time points, Vödisch et al. found that after long-term exposure to hypoxia protein levels of glycolysis, TCA cycle, and respiration, but not fermentation associated enzymes are increased in *A. fumigatus* (121). This suggests that oxidative respiration might be important for long-term growth under hypoxic conditions, while fermentation seems to be utilized for early hypoxia adaptation. However, the lack of fermentation in this study is likely a result of the extreme glucose-restricted conditions and constant turnover of the media utilized in the chemostat (121).

As in *C. neoformans*, *A. fumigatus* has a member of the SREBP family of transcriptional regulators named SrbA. Loss of SrbA in *A. fumigatus* as in *C. neoformans* significantly impairs growth in hypoxia and many of the SREBP dependent transcripts in *C. neoformans* were also found to be SREBP dependent in *A. fumigatus* including transcripts involved in ergosterol biosynthesis and metal uptake (10, 128).

Taken together, transcriptome and proteome profiling experiments have revealed common themes among the human pathogenic fungi with regard to mechanisms of hypoxia adaptation that have facilitated mechanistic analyses through molecular genetic based gene replacement approaches. The impact of these studies on our understanding of human fungal pathogenesis is addressed in the following section.

**Fungal Adaptation to Hypoxia – Links to fungal pathogenesis**

Taken together, null mutants of key hypoxia regulated genes or regulators of fungal hypoxia adaptation generally display attenuated virulence in murine models of fungal infection (Table 1). A common theme of many of the genes listed in Table 1 is

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that they are key regulatory proteins that mediate a significant number of biological responses in fungi not exclusive to hypoxia adaptation. Thus, defects in regulation of basic fungal metabolism often result in significant growth attenuation under low oxygen environments when metabolic control becomes essential for survival in the absence of oxygen (particularly with regard to the biosynthesis of sterols, fatty acids, heme, and iron homeostasis mechanisms). In theory, many of the molecules whose biosynthesis is reduced or inhibited in hypoxia may be available to the invading fungus from host cells. However, whether fungi have appropriate uptake systems to utilize these molecules in the absence of oxygen is unclear. What does seem clear is that these molecules that require oxygen for their biosynthesis are likely indirect sensors of oxygen levels that stimulate changes in overall fungal metabolism to allow the organisms to adapt in hypoxia. How pathogenic fungi actually sense and respond to changing oxygen levels is still an area of ongoing investigation (reviewed in: (42)).

A common theme in fungal hypoxia responses that appears directly related to fungal pathogenesis is the ability of fungi to sense changes in sterol levels. In the human fungal pathogen C. albicans, an UPC2 ortholog has been identified and shown to be activated in hypoxic conditions and in response to lowered sterol levels (52, 108, 116, 127). UPC2 null mutants have significant growth reductions in hypoxia and no longer filament, but UPC2’s link with pathogenesis is currently unknown (Table 1). However, a main line of evidence for a direct link between fungal hypoxia adaptation and the ability to cause lethal disease comes from studies on the SREBPs in the yeast C. neoformans and the mold A. fumigatus (8, 19-21, 128). SREBPs are conserved in a wide range of eukaryotes and are membrane bound transcription factors that are...
activated by proteolytic cleavage. The SREBP pathway in fungi was first identified and studied in the non-pathogenic yeast *S. pombe* where it was found to be a major regulator of the hypoxic response and essential for growth under these conditions (9, 54). In both *S. pombe* and *C. neoformans*, *SRE1* has been shown to be activated by proteolytic cleavage in response to hypoxia (which stimulates a depletion in total cellular ergosterol levels) and direct ergosterol depletion (as mediated by various antifungal agents that target ergosterol biosynthesis such as the triazoles) (9, 19, 54, 90, 119). In both *C. neoformans* and *A. fumigatus*, the respective SREBP ortholog (*Sre1* for *C. neoformans* and *SrbA* for *A. fumigatus*) is required for fungal growth in hypoxia and responses to triazole antifungal drugs. Subsequently, null mutants of *sre1* and *srbA* are strongly attenuated in virulence in murine models of cryptococcosis and IPA (19, 21, 128). Deletion of other regulatory components of the SREBP pathway in *C. neoformans* also results in hypoxia growth defects and attenuated virulence (8, 20, 21). A more in depth review of the mechanisms of SREBP regulation in yeast was recently published (9).

An apparent SREBP independent pathway of sterol homeostasis has also been found to be critical for *A. fumigatus* hypoxia adaptation and virulence, the fungal unfolded protein response (UPR), which in mammals is known to be critical for hypoxia responses (96). A null mutant of the *A. fumigatus* ER stress sensor IreA was found to be moderately attenuated in hypoxic growth compared to normoxia and was also essentially avirulent in a murine model of IPA (37). Like SREBP null mutants, the IreA null mutant displays sensitivity to triazole antifungal drugs, alterations in transcript levels of key ergosterol biosynthesis genes, and a reduction in total cellular ergosterol content.
Thus, perturbations in ergosterol biosynthesis are a common theme among some of the isolated fungal null mutants with defects in hypoxic growth. Another group of molecules that may be critical for hypoxia responses in fungi are reactive oxygen and nitrogen species (ROS and RNS). In the model yeast *Saccharomyces cerevisiae*, it has been proposed that the fungal respiratory chain is involved in oxygen sensing, growth in hypoxia, and hypoxic gene regulation through production of ROS and RNS (17, 27, 46, 56, 68, 91, 92). Furthermore, several studies have suggested that increased oxidative stress observed in hypoxia may act as a putative second messenger that activates redox-sensitive transcription factors to enable hypoxia adaptation (18, 29, 46). Recently, loss of cytochrome C (CycA) in *A. fumigatus* was shown to decrease hypoxic growth and concomitantly increase ROS resistance with a significant attenuation in virulence (43). While the mechanism is speculative at this point, it may be that alteration of ROS homeostasis and RNS production from complex IV of the mitochondrial electron transport chain interferes with the ability of *A. fumigatus* to adapt to hypoxia and pulmonary microenvironments. Thus, the exact role of the respiratory chain in hypoxic signaling in human fungal pathogens is still undefined but promises to be an exciting area of further investigation.

An intriguing finding with the *A. fumigatus* cytochrome C mutant is an inability of this mutant to produce ethanol in response to hypoxia (43). While ethanol production per se was found to not be essential for *A. fumigatus* growth in hypoxia and *in vivo*, ethanol fermentation mutants stimulated increases in IL-8 homolog levels and neutrophil levels in the lung in a corticosteroid model of IPA that correlated with decreased fungal burden (44). These results suggest that fungal responses to hypoxia *in vivo* may not...
only affect fungal growth directly, but also affect the production of fungal metabolites that can subsequently alter host immune responses.

Further evidence for this hypothesis, that remains to be further examined, are the significant changes in cell wall biosynthesis transcripts in response to hypoxia in *C. albicans* and *A. fumigatus* that could alter pathogen associated molecular pattern (PAMP) expression and/or exposure (Figure 2). It has been shown that the cell wall protein expression is altered in response to hypoxia in *C. albicans* (111). Thus, the effects of hypoxia on fungal pathogenesis are likely to be more complex than simply altering rates of *in vivo* fungal growth. Consequently, in future studies it will be intriguing to elucidate how different fungal metabolites are produced in response to changing oxygen levels and whether this affects fungal pathogenesis.

In addition to fungal transcription factors that respond to changes in sterol levels, other transcriptional regulators associated with hypoxia responses have been investigated for their role in fungal pathogenesis. In *C. albicans* these include *EFG1*, *EFH1*, *ACE2*, and *TYE7* (2, 30, 79, 105). *EFG1* null mutants are hyperfilamentous under low oxygen conditions possibly due to downregulation of the filamentous repressor *NRG1* under these conditions, and thus *EFG1* negatively regulates hypoxia-induced filamentation in *C. albicans* (105). Consequently, in an *in vivo* infection model an *efg1* null mutant grew more than a wild type strain (66). Thus, this *in vivo* result partially supports a role for hypoxia in *C. albicans* pathogenesis, as the yeast-to-hyphae switch is generally thought to be a major virulence attribute of this organism. In general the *in vivo* phenotype of the *EFG1* null mutant is surprising given the importance of this transcription factor in regulating gene expression in *C. albicans*. Furthermore, a recent
study observed that during hypoxic conditions EFG1 induces all major classes of genes known to be associated with biofilm formation, a major cause of persistence and antifungal resistance in C. albicans infections, and that EFG1 is required for biofilm formation (112). As biofilms contain significant layers of hypoxia, it is tempting to speculate that regulators of biofilm formation may also have roles in fungal hypoxia adaptation that have been largely unexamined (39, 81, 97). Another important role for the Efg1/Efh1 gene expression network and thus hypoxia is in Candida commensalism. EFH1/EFH1 null mutants of C. albicans are able to persist in the GI tract at higher levels than wild type strains while cells that over-express EFH1 are significantly reduced in GI tract colonization (126). Thus, given the hypoxic environment in the GI tract, and Candida’s propensity to colonize this location, it is tempting to speculate that Candida responses to hypoxia are critical for host immune evasion under these conditions to allow commensalism.

With regard to ACE2 null mutants that display a significant attenuation in their ability to filament under hypoxia, they are attenuated in virulence in a murine model of systemic candidiasis (Table 1) (65, 79). The exact molecules that stimulate ACE2 activity are unknown. In contrast, a TYE7 null mutant displays a slight reduction in hypoxia growth on solid agar and a corresponding moderate virulence attenuation in a Galleria model of infection. Additional deletion of GAL4 leading to a gal4/gal4/tye7/tye7 double mutant significantly attenuates hypoxic growth and virulence in a murine model of systemic candidiasis (2). Both transcription factors are important for controlling glycolysis, a pathway important in fungal responses to hypoxia. Thus, it is intriguing to speculate that glycolytic intermediates may be involved in regulating hypoxia responses
in C. albicans. In addition to the transcriptional regulators discussed above, hypoxia
induced filamentation in C. albicans has been observed to be dependent on both Ras1
and Cdc35 (Cyr1) that are important in virulence, but the exact mechanisms remain
unclear (116). In the future, it will be intriguing to monitor the development of hypoxia at
sites of Candida infection in available animal models and observe whether the
occurrence of hypoxia correlates with filamentation and virulence in vivo.

An additional transcriptional regulator involved in hypoxia responses has been
examined in C. neoformans, Tco1p. Tco1p is a fungal-specific hybrid histidine kinase
family member involved in two-component signal transduction (4, 21). Null mutants of
Tco1p are attenuated in their ability to grow in hypoxia and in murine model of
cryptococcosis. To date, the mechanisms behind Tco1p mediated hypoxic adaptation
and fungal virulence are still largely undefined and remain to be determined.

Importantly, TCO1 null mutant data strongly suggest that other SREBP independent
pathways of hypoxia adaptation exist in pathogenic fungi and remain to be elucidated.

Not all fungal mutants with attenuated hypoxic growth display a reduction in
virulence. An intriguing C. neoformans mutant that is defective in hypoxic growth is the
nuclear transport protein Kap123. While this mutant is highly attenuated in hypoxic
growth, it remains fully virulent in a murine model of cryptococcosis suggesting that not
all fungal mutants defective in low oxygen growth are attenuated in virulence (20).

However, the in vivo proliferation of the Kap123 null mutant was not measured, and
thus it is unclear if the observed mortality was due to fungal growth or an enhanced host
inflammatory response due to other unstudied defects in the mutant. Regardless, the
Kap123 mutant remains an interesting tool for further understanding the link between fungal hypoxia adaptation and virulence.

Pleiotropic phenotypes of many fungal hypoxia mutants with attenuated virulence needs to be taken into consideration when assessing the link between fungal hypoxia adaptation and virulence. A good example of this important observation are studies with the fungal SREBPs that have demonstrated that in both C. neoformans and A. fumigatus regulation of iron acquisition is altered in SREBP null mutants (10, 19). As both A. fumigatus (e.g. ΔhapX) and C. neoformans (Δcir1 and ΔhapX) iron acquisition/homeostasis mutants are also attenuated in virulence (59-62, 102, 103), these studies suggest a potential role for SrbA mediated iron acquisition in fungal virulence and complicates the direct link between the hypoxia growth defect of the SREBP null mutants and virulence (10, 19). Given the importance of iron in some of the ergosterol biosynthesis enzymes, co-regulating gene expression of these two important metabolic pathways is logical and this is a promising area of investigation.

However, taken together, our current knowledge suggests that the ability of human pathogenic fungi to cause lethal disease is in part mediated by their ability to adapt to hypoxic microenvironments that occur during fungal pathogenesis. To definitively confirm that in vivo hypoxia, fungal hypoxia adaptation, and fungal virulence are really “cause and effect”, studies on spatial and temporal aspects of hypoxia development during invasive fungal infections and additional knowledge of fungal hypoxia adaptation mechanisms are needed. Thus, the identification and discovery of a gene solely required for fungal hypoxia adaptation and growth remains a “holy grail” that may or may not exist.
One clear area for further investigation is the expansion of fungal hypoxia adaptation mechanism research into other clinically relevant fungi. Mechanisms of hypoxia adaptation have not been extensively explored in many important human fungal pathogens, particularly the dimorphic systemic fungi such as *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*. It will also be intriguing to determine whether hypoxia adaptation is important for Mucorales (some require low oxygen for dimorphism (85)) pathogenesis and other pathogens such as *Penicillium marneffei* and *Fusarium* species (which grow well anaerobically through novel fermentation systems). Along these lines of investigation, whether plant fungal pathogens also face *in vivo* oxygen limitation and whether this is critical for virulence in fungal-plant pathosystems should be further studied. Some support for an affirmative answer in these plant based pathosystems was recently observed with alcohol dehydrogenase (Adh1) mutants in the root pathogen *Fusarium oxysporum* that were attenuated in virulence (22). Thus, many areas of investigation remain to be explored with regard to fungal pathogenesis and its relationship to hypoxia adaptation mechanisms.

**Hypoxia and Host Immune Responses to Fungi**

Outcomes of invasive fungal infections are two-way streets depending on both fungal and host factors that are dynamic in response to the interaction. Consequently, we would be remiss not to discuss the implications of *in vivo* hypoxia on host antifungal responses. It should be noted that this is an almost completely unexplored area of fungal pathogenesis, but data from other pathosystems strongly suggest that it could be
a significant area for investigation with regard to human fungal infections. It has been shown that hypoxia inducible factor 1 (HIF1), a key host transcription factor mediating mammalian hypoxia responses, plays an important role in the immune response and host defense to microbial pathogens (reviewed in: (80)). More recently, with the establishment of conditional knockouts, the ability to study the role of HIF1α during infection has been made possible. For example, the deletion of HIF1α, the oxygen tension-regulated α subunit of HIF, from the myeloid lineage has demonstrated the importance HIF1α in bacterial pathogenesis. Using macrophages from HIF1α conditional knockout mice, it was demonstrated that survival of Group B Streptococcus was increased compared to the functional bactericidal activity of wild type macrophages (25). Additionally, HIF1α is critical for macrophage effector functions against Staphylococcus aureus and Group A Streptococcus in normoxia and hypoxia (24, 25, 89, 129). The importance of HIF1α in innate responses is also supported by pathogens, such as Chlamydia pneumoniae, that have been shown to block the actions of HIF1α in order to survive within the host (100). HIF1α is also required for controlling Yersinia enterocolitica in the intestine, suggesting the importance of HIF1α in mucosal defense (48). Recently, it was found that C. albicans along with other microbial pathogens could stabilize HIF1α in the presence of oxygen supporting the idea that HIF1α may be important for phagocytic cell responses to fungi (125). A mechanistic understanding of how hypoxia affects the killing and clearance of fungal infections by cells of the innate immune system is a fruitful area for further investigation (Figure 2). This holds potential for an increased understanding of fungal pathogenesis, improvement of existing treatment strategies, and development of new therapeutic options.
In summary, the occurrence of hypoxia during human fungal infections and the apparent need for fungal adaptation to oxygen limitation for host adaptation and virulence suggest that further exploration of these mechanisms may prove to be clinically beneficial. While “to air or not to air” may be an abuse of a famous literary quote, it does reflect the idea that manipulation of oxygen (and/or CO$_2$) levels at sites of human fungal infection may have promise as a therapeutic approach. The effects of oxygen on fungal-host interactions are likely to be multifaceted and manipulation of oxygen levels and/or oxygen mediated signaling pathways in vivo may have both positive and negative effects on the outcome of these infections. For example, while controversial and undefined, the potential use of hyperbaric oxygen to perfuse tissue to increase host cell antifungal activities, and possibly thwart pathogen growth should also not be overlooked (23, 38, 40, 58, 78, 93, 104). It is currently unclear whether increased tissue oxygen perfusion would inhibit fungal growth, promote fungal growth, or how it would affect the antifungal immune response in an immunocompromised patient. In addition to the potential for manipulating oxygen levels at the site of infection, direct fungal centric approaches to turn off key fungal hypoxia adaptation pathways that are critical for virulence is another area for therapeutic exploration. As discussed in this review, hypoxia has significant impacts on ergosterol biosynthesis, a target for two classes of antifungal drugs (triazoles and polyenes). In general, hypoxia induces an increase in mRNA levels of the triazole target Erg11A, which is coincidentally regulated by the A. fumigatus SREBP, SrbA (11). In vitro these antifungal agents are often highly efficacious but in vivo fungal eradication is difficult often necessitating very long
therapeutic regimens of antifungal treatment. It is thus tempting to speculate that in vivo hypoxic microenvironments adversely affect antifungal drug delivery to sites of infection and their efficacy due to changes in target gene expression. In vitro analyses of oxygen effects on antifungal drug activity provide some support for this idea, but further experimentation is needed (87, 123).

With regard to fungal SREBP s, low oxygen conditions induce activation of this pathway likely in response to sterol depletion upon hypoxia exposure and are required for virulence. Thus it has been proposed that fungal SREBP s may be excellent drug targets due to their absolute requirement for virulence in C. neoformans and A. fumigatus (9). With regard to SREBP s, amino acid sequence similarity suggests substantial differences between human and fungal orthologs that indicate that it may be possible to design an effective therapeutic targeting this protein. However, whether those specific differences can result in a functionally attenuated protein or fungal SREBP signaling pathway is unclear. A similar strategy can certainly be pursued with regard to other fungal proteins essential for hypoxia adaptation and virulence, especially if they are conserved in function across the different fungal pathogens. Yet, using oxygen to “turn off” these pathways may stimulate other pathways that exacerbate disease caused by fungi. Only experimental validation of oxygen use and targeting of these pathways in models of mammalian fungal infection can begin to answer these questions.

Finally, besides direct fungal centric targeted approaches, the idea that hypoxia can alter key innate immune system antifungal responses is worthy of further investigation. Immunomodulation strategies targeting host hypoxia responses, either
agonistically or antagonistically, could have great therapeutic benefits in many fungal disease settings.

In conclusion, much remains to be learned about fungal hypoxia adaptation and how it relates to outcomes of fungal pathogenesis. Many areas for further investigation remain, as outlined in this review, but our knowledge has progressed to the point where hypotheses can be proposed and experiments designed to answer the question whether "to air or not to air" is the best approach for improving treatment outcomes of these often lethal and increasingly common infections.

ACKNOWLEDGEMENTS

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REFERENCES


Hollis JP. 1948. Oxygen and Carbon Dioxide Relations of Fusarium oxysporum Schlecht and Fusarium eumartii Carp. Phytopathology 38:761-775.


Hughes AL, Todd BL, and Espenshade PJ. 2005. SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast. Cell 120:831-842.


Table 1. List of genes involved in hypoxia adaptation in human pathogenic fungi and their role in virulence*.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Function</th>
<th>Organism</th>
<th>Hypoxia Null Mutant Phenotype</th>
<th>Role in Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPC2</td>
<td>Transcription Factor, zinc cluster family</td>
<td><em>Candida albicans</em></td>
<td>Significant Growth attenuation (75, 116)</td>
<td>Not reported</td>
</tr>
<tr>
<td>CZF1</td>
<td>Transcription factor, zinc finger domain</td>
<td><em>C. albicans</em></td>
<td>Required for filamentous response to hypoxia (16)</td>
<td>Not reported</td>
</tr>
<tr>
<td>EFG1</td>
<td>Transcription Factor, APSES Family</td>
<td><em>C. albicans</em></td>
<td>Hyperfilamentous response to hypoxia (105)</td>
<td>Increased in vivo proliferation (66) Virulent but kinetics of mortality altered (73)</td>
</tr>
<tr>
<td>EFH1</td>
<td>Transcription Factor, APSES Family</td>
<td><em>C. albicans</em></td>
<td>No phenotype</td>
<td>Increased intestinal tract persistence (126)</td>
</tr>
<tr>
<td>TYE7</td>
<td>Transcription Factor</td>
<td><em>C. albicans</em></td>
<td>Moderate Growth Defect (2)</td>
<td>Significant attenuation** (2)</td>
</tr>
<tr>
<td>ACE2</td>
<td>Transcription Factor, Swi5 family</td>
<td><em>C. albicans</em></td>
<td>Required for filamentous response to hypoxia (79)</td>
<td>Significant attenuation (65)</td>
</tr>
<tr>
<td>RAS1</td>
<td>Small GTPase</td>
<td><em>C. albicans</em></td>
<td>Required for filamentous response to</td>
<td>Significant attenuation (69)</td>
</tr>
<tr>
<td>Gene</td>
<td>Function</td>
<td>Organism</td>
<td>Hypoxia Response</td>
<td>Significant Attenuation</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------------------------------</td>
<td>---------------------------</td>
<td>------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>CDC35</td>
<td>Adenylate cyclase</td>
<td><em>C. albicans</em></td>
<td>Required for filamentous response to hypoxia (116)</td>
<td>Significant attenuation (95)</td>
</tr>
<tr>
<td>SCH9</td>
<td>AGC Protein kinase</td>
<td><em>C. albicans</em></td>
<td>Required for filamentous response to hypoxia (113)</td>
<td>Significant attenuation (72)</td>
</tr>
<tr>
<td>TCO1</td>
<td>Histidine Kinase part of two component regulatory system</td>
<td><em>Cryptococcus neoformans</em></td>
<td>Significant growth attenuation (21)</td>
<td>Significant attenuation (21)</td>
</tr>
<tr>
<td>SRE1</td>
<td>Transcription Factor, SREBP family, bHLH DNA binding</td>
<td><em>C. neoformans</em></td>
<td>Significant growth attenuation (19, 21)</td>
<td>Significant attenuation (19, 21)</td>
</tr>
<tr>
<td>SCP1</td>
<td>Sterol cleavage activating protein, senses sterols and involved in Sre1 signaling</td>
<td><em>C. neoformans</em></td>
<td>Significant growth attenuation (19, 21)</td>
<td>Significant attenuation (21)</td>
</tr>
<tr>
<td>STP1</td>
<td>Protease involved in proteolytic cleavage of Sre1</td>
<td><em>C. neoformans</em></td>
<td>Significant growth attenuation (8, 21)</td>
<td>Significant attenuation (8, 21)</td>
</tr>
<tr>
<td>KAP123</td>
<td>Nuclear transport, involved in SRE1 pathway</td>
<td><em>C. neoformans</em></td>
<td>Significant growth attenuation (20)</td>
<td>No attenuation (20)</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase, involved in Sre1 turnover/phosphorylation</td>
<td><em>C. neoformans</em></td>
<td>Significant growth attenuation (20)</td>
<td>Significant attenuation (20)</td>
</tr>
<tr>
<td>srbA</td>
<td>Transcription Factor, SREBP family, bHLH DNA binding</td>
<td><em>Aspergillus fumigatus</em></td>
<td>Significant Growth attenuation (128)</td>
<td>Significant attenuation (128)</td>
</tr>
<tr>
<td>ireA</td>
<td>Type 1 membrane protein, protein kinase domain and endoribonuclease domain</td>
<td><em>A. fumigatus</em></td>
<td>Moderate growth attenuation (37)</td>
<td>Significant attenuation (37)</td>
</tr>
<tr>
<td>cycA</td>
<td>Cytochrome C</td>
<td><em>A. fumigatus</em></td>
<td>Moderate growth attenuation</td>
<td>Significant attenuation</td>
</tr>
<tr>
<td>ADH1</td>
<td>Alcohol dehydrogenase</td>
<td><em>Fusarium oxysporum</em></td>
<td>Moderate growth attenuation (22)</td>
<td>Moderate attenuation (22)</td>
</tr>
</tbody>
</table>
*Does not include the Cobalt Chloride sensitive *Cryptococcus neoformans* mutants isolated from a T-DNA insertion mutagenesis library that are also sensitive to hypoxic growth. A list of these genes can be found in the original publication, Table 1 (56).

**Significant attenuation in *Galleria mellonella* model. Single *Tye7* mutant virulence not reported in mice, but a *gal4/tye7* strain is highly attenuated in A/J mice (4).
FIGURE LEGENDS

Figure 1. Hypoxia occurs in murine models of invasive pulmonary aspergillosis.

The hypoxia detection agent pimonidazole hydrochloride, hypoxyprobe-1, was utilized to monitor in vivo hypoxia in 3 immunologically distinct murine models of invasive aspergillosis. Green = A. fumigatus, Blue = DAPI stained host cells, Red = hypoxia as detected by hypoxyprobe-1. All images are from day +4 after inoculation and represent merged images as described in (44). (A) Chemotherapy model, outbred CD1 mice immunosuppressed with cyclophosphamide and triamcinolone and inoculated with A. fumigatus conidia. (B) Corticosteroid model, outbred CD1 mice immunosuppressed with a single dose of triamcinolone and inoculated with A. fumigatus conidia. (C) Chronic granulomatous disease (X-CGD) model, gp91phox−/− mice, deficient in NADPH oxidase activity, and inoculated with A. fumigatus conidia. Figure adapted from experiments in reference (44).

Figure 2. Confirmed and potential effects of hypoxia on fungal pathogenesis.

Molecular oxygen is critical for numerous cellular processes including ATP production and the biosynthesis of key molecules such as sterols, fatty acids and NAD. Thus, the loss of oxygen at sites of fungal infections influences the physiology of both the fungal pathogen and immune cells of the host that affect the final outcome of the fungal-host interaction. Images from left to right: Aspergillus fumigatus colony on nutrient agar, Cryptococcus neoformans yeast, and monocytes, neutrophils and macrophages from a lung bronchoalveolar lavage. PAMP = pathogen associated molecular pattern, PRR =
pattern recognition receptor. Suggested impacts on the host side are based on data largely from bacterial pathosystems and have not been confirmed to be active in fungal-host interactions to date.
Hypoxia
Fungal Pathogen

- Alterations in growth: ergosterol levels, heme levels, cell wall components, fermentation, respiration, ribosome biogenesis, secondary metabolism
- Virulence factor and PAMP expression
- Pathogen Specific Responses: i.e. glycolysis up in Candida albicans and Aspergillus fumigatus but no change in Cryptococcus neoformans

O₂ Sensing
Sterol, heme, respiration by products (ROS, RNS)

Host Cells

- Stabilization of hypoxia inducible factor-1 protein
- Stabilized/increased effector cell antifungal responses via unknown mechanisms possibly HIF1 dependent?
- Effects on PRR expression and recognition of fungal PAMPs?

Outcome of Infection