Review Article

Cryptococcus neoformans and oxygen

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Summary

Oxygen is essential to life of all organisms except for obligate anaerobic species, because it is necessary for energy generation and also for some biosynthetic pathways. However, sensitivity to low oxygen levels can vary widely in different organisms and cell types. The pathogenic yeast species *Cryptococcus neoformans* is known to love oxygen. In response to the lack of oxygen (hypoxia), this yeast delays budding without resigning DNA replication, which eventually results in unique cell cycle arrest in the unbudded G_2-phase. Potential mechanisms of oxygen sensing and hypoxia signaling will be discussed, as well as the possible role of gradual adaptation to decreased oxygen supply during systemic cryptococcal infection in mammals.

Cryptococcus loves oxygen

Oxygen is essential to life of all organisms except for obligate anaerobic species, because it is necessary for energy generation and also for some biosynthetic pathways. Thus, all aerobic species have to develop a strategy how to cope with fluctuations in oxygen availability and how to possibly survive severe oxygen limitation. However, sensitivity lo low oxygen levels can vary widely in different organisms and cell types. The pathogenic yeast species *Cryptococcus neoformans* is known to love oxygen. The first indices about the essential need of adequate oxygen supply for its proliferation came from Roberts *et al* [1], who have shown that rates of isolation of yeasts from blood cultures were significantly enhanced by venting vacuum blood culture bottles in studies of both simulated and patients' blood cultures. Although they do not comment on this matter, their data clearly show that the isolation rate of *C. neoformans* decreased more markedly in unvented blood culture bottles compared to other yeast species (66% recovery rate in simulated *C. neoformans* samples compared to 84-100% in *C. albicans*, *tropicalis* and *parapsilosis*). Later, Huahua *et al* [2] pointed at the key role of oxygen when they demonstrated that addition of hydrogen peroxide to blood culture bottles resulted both in increased pO_2 levels and increased CFU/ml yields of *Cryptococcus neoformans*. At last, Odds *et al* [3] demonstrated that oxygen should be the limiting nutrient for growth of *C. neoformans* in microtiter plates, because agitation or 100% oxygen environment both resulted in highest OD readings regardless of the glucose concentration. It is obvious that such sensitivity to oxygen levels must rely on a delicate oxygen sensing system which controls cell proliferation. However, the above-mentioned studies were targeted on resolving technical issues in microbiological diagnostics, and nobody has followed this line of research initially.

Clue comes from studies on ploidy

New incentive to the study of cryptococcal hypoxia response surprisingly came from the other side of the coin. Until the year 1995, our knowledge of cell cycle regulation in budding yeasts was based almost exclusively on the observations made in the model yeasts *Saccharomyces cerevisiae*. In this organism, initiation of budding and DNA-replication are coupled tightly, thus unbudded yeast cells are always G_1-cells harboring 1n DNA content in haploid strains. This paradigm was challenged and overcome by Takeo *et al* [4], who recognized that *Cryptococcus neoformans* is able to undergo DNA-replication without starting to bud, resulting in a

fraction of unbudded G_2-cells harboring 2n DNA content. This unique ability of *C. neoformans* to arrest in unbudded G_2-phase of the cell cycle has not been described in any other yeast species so far. Subsequently, the same group has shown that this arrest develops gradually during transition to stationary phase, when budding is delayed from S to G_2[5]. At the end, Ohkusu *et al*[6] identified deficit in oxygen as the key cause for delayed budding resulting eventually in the unbudded G_2-arrest.

This discovery opened a completely new field for research, posing the questions on the oxygen sensor and on the signaling pathway from this sensor towards control of cell cycle progression. These questions appear to be important from the general perspective, because commitments to budding, replication and SPB duplication are all controlled by the Cdc28 downstream signaling and are inseparable in the model ascomycetous yeast *Saccharomyces cerevisiae*. This paradigm appears not to fit the cryptococcal cell cycle control at all points, although the cryptococcal Cdc28 homologue, CnCdk1, cannot be questioned as its master cell cycle regulator[7]. However, as already noted by Ohkusu *et al*[5], a specific checkpoint control may exists in *C. neoformans* to control cell cycle progression. Its cells either have to pass an oxygen sensitive cell cycle checkpoint to start budding, regardless of how far DNA synthesis had proceeded, or, in other words, an unknown oxygen sensitive mechanism should be able to delay budding. Consequently, low oxygen supply could cause shorter or longer or even long-lasting delay in the onset of budding, resulting in G2/M arrest through the well conserved morphogenetic checkpoint[8]. The rapid onset of budding upon reoxygenation of G_2-arrested culture demonstrates that budding initiation and/or progress should be the only limiting steps when all other conditions to cell cycle progression are completed[9].

Established hypoxia signaling in mammals and *Saccharomyces* yeast provide little guidance

The easiest way to look for a clue to the control of cryptococcal cell cycle progression by oxygen availability should be to go through the knowledge gathered on the response to low oxygen levels in other eukaryotes. In mammals, HIF-1 (hypoxia-inducible factor) is the master player in hypoxia response[10]. HIF-1 is a heterodimeric transcription factor, one component of which is polyubiquitylated and subsequently degraded by the proteasome under normoxic conditions. Prolyl hydroxylases, which require dioxygen for their activity, serve as the oxygen sensors, which postranslationally modify the HIF-1 α and thus mark it for degradation. Once the oxygen level is reduced, HIF is stabilized and transcription of genes needed to cope with hypoxia is upregulated. However, this type of response has developed during evolution of multicellular organisms only and there is no circumstantial evidence for its presence in unicellular eukaryotes. Also, when it comes to cell cycle arrest in response to hypoxia in mammalian cells, this is mediated through the PP1 phosphatase action on pRb resulting in G1 cell cycle arrest prior to DNA replication[11].

When unicellular eukaryotes are considered as the source of knowledge about analogous signaling, the model yeast *Saccharomyces cerevisiae* should be the first choice. Two types of transcription factors are involved in activation and repression of genes under normoxic and hypoxic conditions in *Saccharomyces* yeast (for review see Kwast *et al*[12]). However, in contrast to the strictly aerobic basidiomycetous yeast *Cryptococcus neoformans*, this ascomycetous yeast is facultative anaerobic and switches to a completely different metabolic behavior in response to oxygen limitation, which enables it to continue cell cycle progression. Thus, scarcely any useful hints can be learned from its Hap/Rox hypoxia response system for cryptococcal signaling.

In contrast, several interesting observations have been done in the aerobic yeast *Pichia stipitis*. Activation of its pyruvate decarboxylase was observed during aerobic cultivation in a cell number-correlated manner[13]. Activation of this enzyme is also strongly triggered under hypoxic conditions, indicating that transient oxygen limitation due to accumulation of rapidly growing oxygen-demanding cells can by the reason for its cell number-correlated manner of activation in aerobic culture. Transient delay of budding was also observed in late log-phase of cryptococcal growth by us (unpublished data). Later it was shown that *Pichia stipitis* obviously responds to a decrease in dissolved oxygen tension while the culture is still aerobic, most probably to reduce oxygen consumption, to prevent a real limitation[14]. This

type of response is very similar to the response of *Cryptococcus neoformans*, which also reduces its growth rate under limited aeration and eventually arrests without exploiting the glucose available in medium completely[6]. Based on yet unpublished observations made in *Pichia stipitis*, Klinner *et al*[14] were the first to suggest possible role of nitric oxide synthase in hypoxia sensing of this yeast.

Can nitric oxide be the master?

Nitric oxide (NO) is known to be widely involved in signaling in multicellular eukaryotes including plants. Dioxygen and L-arginine serve as substrates for nitric oxide synthases (NOS), being converted into NO and citruline. This reaction is dependent on the availability of several co-factors and co-substrates, including NADPH, FAD, and tetrahydrobiopterin (BH4). Activity of NOS was shown to be proportional to a wide range of dioxygen levels. Therefore, it is argued that the enzyme can serve as an oxygen sensor[15]. Recently, there is a growing body of evidence suggesting that the mechanisms by which cells adapt to hypoxia involve NOS activity[16]. In addition, presumable nitric oxide synthase (NOS) homologues have been detected in the model yeast *Sachharomyces cerevisiae*, although the evidence relies on the study of proteins, whereas the coding genes have not been described yet[17, 18]. Furthermore, Klinner *et al*[14] reported that they have recently detected NOS activity in *P. stipitis* (submitted for publication). If the model on the role of NO in hypoxia signaling proposed in mammals would be applied to yeasts, then, increased dioxygen levels should result in increased NO production followed by downstream signaling.

However, another piece of puzzling evidence was added by Castello *et al*[19] recently, who showed that yeast mitochondria produce NO at dissolved oxygen concentrations below 20μM. This NO production is nitrite (NO_2-) dependent, requires an electron donor, and is carried out by cytochrome *c* oxidase (COX) in a pH-dependent fashion. In such case, not increased, but decreased dioxygen levels should result in increased NO production followed by downstream signaling. Nonetheless, the above cited remark on NOS activity in *Pichia stipitis*[14] and the observed mitochondrial NO production in *Saccharomyces cerevisiae*[19] strongly suggest that NO production should play its part in hypoxia signaling in *Cryptococcus*. Certainly, the accurate picture and significance of the role of NO in cryptococcal hypoxia response has to be established only. Due to the complexity of cellular systems, it is even conceivable, that both NOS and COX systems work in NO generation in yeast cells, being possibly activated differently at different dioxygen levels. Because the state of regulatory networks can also be different under different conditions, same levels of NO can even have converse effects. Also, the role of the two proposed systems may vary among aerobic a facultative anaerobic yeasts. Anyway, the possible role of NO in hypoxia signaling in yeasts represents an attractive hypothesis, because this molecule can also transmit signal to closely adjacent cells in culture, offering an intriguing explanation for the observed quorum sensing response in *Pichia stipitis*[13].

Concluding remarks

It is noteworthy that hypoxia signaling and cell cycle regulation in *Cryptococcus neoformans* deserves to be studied not only because its general significance for understanding the biology of lower eukaryotes other than *Saccharomyces cerevisiae*. In addition, the role of cryptococcal adaptation to varying dioxygen levels during human infection highly deserves investigation and evaluation. As widely known, lung tissue is the typical port of entry of cryptococcal infectious propagules into the human body. From this primary site, it typically spreads into the CNS, causing meningoencephalitis. It is beyond reasonable doubt that inhalation of aerosol plays key role in its pathogenesis. There is also supportive evidence explaining cryptococcal predilection for the CNS because of its ability to utilize catecholamines for melanogenesis[20], although this has been questioned recently[21].

However, lungs and brain tissue also share one character of possibly major importance for cryptococcal survival in human tissue, i.e. they show high levels of oxygenation. Although oxygen levels are lower in brain compared to lungs, they are kept still quite high and also within a rather narrow range because of the sensitivity of the brain tissue to damage by hypoxia. Thus, easier adaptation to gradually decreasing, but yet stable oxygen supply during invasion from environment to brain tissue through lungs may provide an intriguing explanation for the typical course of cryptococcal

infection, without questioning the other well known virulence factors further supporting its survival ability. In such case, adaptation to hypoxia may play significant role in pathogenesis of cryptococcal infection. Notably, in a murine model, cryptococcal cells which crossed the blood-brain barrier were always closely associated with the brain capillaries and were rarely found within the brain parenchyma as early as 3 hours postinjection. By 22h postinjection, they were found in the brain parenchyma, but still mainly near the capillaries. At 10 days postinjection, brains showed large cystic lesions throughout the brain parenchyma, where the largest numbers of yeast cells were again localized in perivascular cysts, i.e. close to the vessels providing them with oxygen (all Chang et al[22]).

Thus, it can be concluded that the study of hypoxia signaling and cell cycle regulation in *Cryptococcus neoformans* represents a challenging and hot topic.

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