Nitric oxide and nitrosative stress tolerance in yeast

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Abstract
The opportunistic human fungal pathogen Candida albicans encounters diverse environmental stresses when it is in contact with its host. When colonizing and invading human tissues, C. albicans is exposed to ROS (reactive oxygen species) and RNIs (reactive nitrogen intermediates). ROS and RNIs are generated in the first line of host defence by phagocytic cells such as macrophages and neutrophils. In order to escape these host-induced oxidative and nitrosative stresses, C. albicans has developed various detoxification mechanisms. One such mechanism is the detoxification of NO (nitric oxide) to nitrate by the flavohaemoglobin enzyme CaYhb1. Members of the haemoglobin superfamily are highly conserved and are found in archaea, eukaryotes and bacteria. Flavohaemoglobins have a dioxygenase activity [NOD (NO dioxygenase domain)] and contain three domains: a globin domain, an FAD-binding domain and an NAD(P)-binding domain. In the present paper, we examine the nitrosative stress response in three fungal models: the pathogenic yeast C. albicans, the benign budding yeast Saccharomyces cerevisiae and the benign fission yeast Schizosaccharomyces pombe. We compare their enzymatic and non-enzymatic NO and RNI detoxification mechanisms and summarize fungal responses to nitrosative stress.

Why study nitrosative stress responses in yeasts?
The evolutionarily divergent yeasts Candida albicans, Saccharomyces cerevisiae and Schizosaccharomyces pombe provide ideal model systems with which to compare nitrosative stress responses. These three yeasts, which diverged approx. 500 million years ago [1], exist in different environmental niches and therefore have been exposed to different evolutionary pressures. As a major fungal pathogen of humans, C. albicans has evolved robust stress responses that facilitate adaptation to environmental challenges such as changes in ambient pH, osmolarity and nutrient availability, as well as exposure to ROS (reactive oxygen species) and RNIs (reactive nitrogen intermediates) [2] (the latter challenges being of particular interest in our laboratory). These unicellular yeasts have short life cycles, they can be grown under defined experimental conditions and their genomes have been sequenced [3]. Furthermore, extensive molecular toolboxes have facilitated the dissection of their fundamental cellular processes, such as the cell cycle, signal transduction and stress responses [4–6]. The ability to survive these stresses contributes to the pathogenicity of C. albicans, as well as virulence factors such as adhesins, extracellular hydrolytic enzymes and phenotypic switching [7–9]. In contrast, the benign yeasts, S. cerevisiae and S. pombe, which are associated with environmental niches, tend to be more sensitive to stresses than C. albicans [10].

NO (nitric oxide), RNIs and their effect within the cell
NO is an ‘ancient’ molecule and it and its derivatives were oxidizing substrates in the archaenal world, driving the evolution of a pathway related to modern dissimilatory denitrification [1]. It has been suggested that aerobic respiration has emerged from this pathway by adaptation of the enzyme NO reductase to its new substrate, oxygen [11]. NO is a gaseous radical that can have beneficial or unfavourable effects within cells depending on its concentration. At low concentrations, NO can act as a second messenger controlling numerous physiological processes in animal cells [12]. At high concentrations, NO is cytotoxic and is exploited as a weapon in host–pathogen defences [12]. As mentioned above, fungal pathogens are relatively resistant to such stresses and it is probable that the ability of pathogenic fungi to combat host–pathogen defences evolved through ancient interactions between fungi and phagocytic amoebae [13].

Nitrosative stress is mainly caused by three forms of NO: the NO* radical, the nitrosonium cation and the nitroxy anion. The NO* radical is a signalling molecule that plays a regulatory role in cell proliferation, antimicrobial defence and inflammatory responses [14–17]. Within the cell, NO reacts with oxygen species, with thiol-containing proteins and with metalloproteins [18]. The NO* radical also reacts with oxygen to generate nitrogen dioxide which is converted...
into the nitrite anion and then further into the nitrate anion. Intermediates of this oxidation include dinitrogen trioxide and the nitrite anion which contributes to NO toxicity by oxidizing thiols and amines within the cell. Owing to its stability, the nitrate anion is thought to be the end metabolite of this NO pathway [19]. The nitrosonium cation is generated when one electron of NO is released. In this reaction, the iron atom of Fe$^{2+}$-containing metalloproteins acts as the electron acceptor. The Fe$^{2+}$–NO$^{+}$ complex serves as a NO carrier which releases NO at specific target sites. Additionally, the nitrosonium cation reacts with nucleophilic centres and is responsible for nitrosation, generating nitroso compounds, including nitrosamines, alkyl or amin nitrite and S-nitrosothiols [20]. It has been proposed that NO is stored and carried as GSNO (S-nitrosoglutathione), and that GSNOS is used as an NO pool within cells [21]. The nitroxy anion is generated when one electron is added to NO. This reduction is supported by the Fe$^{2+}$ ion and by Fe$^{3+}$-containing metalloproteins which act as electron donors. The nitroxy anion is believed to mediate thiol oxidation of target proteins. This process leads to the formation of nitrous oxide, which is also the result of nitroxyl anion dimerization [20].

In mammalian cells, NO biosynthesis is catalysed by three isoforms of NOS (nitric oxide synthase): iNOS (inducible NOS), nNOS (neuronal NOS) and eNOS (endothelial NOS). All NOSs use L-arginine and NADPH to generate NO and citrulline [22]. As mentioned above, macrophages that have taken up microbial cells release RNS (reactive nitrogen species) and RNIs into the phagolysosome. Macrophages can produce up to 57 μM NO and up to 14 mM H$_2$O$_2$ [23]. ROS, such as superoxide radicals (O$_2^{•−}$) and H$_2$O$_2$, are generated with the help of NADPH oxidase as by-products of the respiratory chain [24]. Furthermore, the superoxide anion (O$_2^{−}$) can also be converted with the help of the myeloperoxidase into hypochlorous acid (HClO). Parallel to the production of ROS, macrophages generate NO and nitrite with the help of iNOS. Furthermore, NO reacts with the O$_2^{−}$ anion to create the strong oxidant peroxynitrite (ONOO$^{−}$), which has fungicidal activity and is more stable and a stronger oxidant than NO [12]. Owing to the physical and chemical properties of NO, it is more accurate to imagine dynamic, temporary and local NO gradients within the cells. Hence, NO has a short half-life which varies depending on the intracellular and extracellular redox state [25], the NO concentration, the partial oxygen pressure, and the presence of bivalent metals and thiol groups [12].

**Nitrosative stress responses in the model yeasts**

*C. albicans* is exposed to NO and RNIs, which are generated during host defence by phagocytic cells, and to non-enzymatically generated NO from nitrates and nitrites of dietary products in the digestive system [26]. Alternatively, NO can be generated by bacteria in the oral cavity or gut [27,28]. On the other hand, *S. cerevisiae* is exposed to endogenous NO under hypoxic conditions and, given that the mitochondrial respiratory chain of *S. cerevisiae* can use endogenous nitrite instead of oxygen as an electron acceptor, it can generate NO within the cell itself [29]. Several mechanisms exist to counteract these nitrosative stresses: (i) the active detoxification of NO via flavohaemoglobin(s); (ii) the antioxidant system for scavenging NO via GSH or trehalose; and (iii) the up-regulation of repair systems to counteract the damage caused (Figure 1). The systems that repair RNI damage are poorly understood in yeasts [30].

A number of antioxidant systems contribute to nitrosative stress resistance, one of which is GSNO reductase. Interestingly, compared with *S. cerevisiae*, *S. pombe* is particularly sensitive to low concentrations of GSNO [31]. This might be due to the inactivation of GSNO reductase in *S. pombe* by peroxynitrite given that GSNO reductase activity is essentially required for the growth of *S. pombe*, unlike in *S. cerevisiae* [32,33]. This observation emphasizes the importance of repair functions, such as GSNO reductase, that are capable of reducing GSNO to ammonia and GSGG [34]. However, other enzymes, such as Tsa1 (thioredoxin peroxidase 1), contribute to resistance to endogenous RNIs. Tsa1 has also been shown to contribute to fungal virulence [30]. In addition, several non-enzymatic antioxidants help to counteract the effects of RNIs in yeasts, such as GSH and metalloporphyrins. For *C. albicans*, the antioxidant trehalose is essentially linked to stress adaptation [35]. The non-enzymatic antioxidant systems and NO-scavenger mechanisms are thought to have evolved a long time ago when cells were first exposed to an aerobic environment. From that time, gene-duplication events and the redundancy of stress-resistance pathways and antioxidant systems have facilitated the environmental adaptation of different yeast species.

Flavohaemoglobins are characterized by an NOD (NO dioxygenase domain), which is highly conserved in bacteria

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**Figure 1 | Simplified RNI response network in yeasts**

Yeasts cope with RNI stresses in different ways: they can enzymatically detoxify NO via flavohaemoglobin(s), they can scavenge NO through antioxidant systems or they can repair the caused damage. TRX, thioredoxin.
and yeast, and converts NO into nitrate [36]. As the name suggests, flavohaemoglobins contain an N-terminal haem group followed by the C-terminal FAD domain and a NAD(P)-domain [37]. S. cerevisiae and S. pombe each have a single flavohaemoglobin gene, ScYHB1 and the predicted SPAC869.02c respectively (Figure 2) [38]. In contrast, the C. albicans genome contains three flavohaemoglobin-like genes, namely CaYHB1, CaYHB4 and CaYHB5 [39]. The sequence identity between ScYHB1 and the three C. albicans flavohaemoglobins ranges from 31 to 25% [40].

The flavohaemoglobins in S. cerevisiae and S. pombe are fully functional, and the deletion of ScYHB1 leads to growth-inhibition and the loss of the NOD function in S. cerevisiae [40]. In S. cerevisiae, the ScYhb1 protein is translocated to the mitochondria under hypoxic conditions where it detoxifies NO [41]. This suggests that flavohaemoglobins are able to both protect yeasts against external, as well as internal, sources of NO and RNIs. In C. albicans, only CaYHB1 deletion attenuates virulence slightly [39,42]. Inactivation of CaYHB4 or CaYHB5 does not inhibit NO consumption under the experimental conditions used, or attenuate virulence in the mouse model of systemic candidiasis [39], but this does not exclude the possibility that these gene products are important under other conditions or at specific stages of infection.

These differences in YHB gene-copy number and flavohaemoglobin functionality might relate to the different environmental niches of these yeasts and thus their individual adaptation requirements. In vivo, CaYHB1 is expressed in C. albicans cells on epithelial surfaces during oral infection [43] and in cells infecting the mouse gastrointestinal tract [44]. However, CaYHB1 is not up-regulated in deep-tissue infections of liver, for example [45].

It is not clear how yeasts detect NO and which signalling pathways mediate NO and RNI responses. In contrast with mammalian cells, yeasts do not express an obvious NO receptor. However, Chiranand et al. [46] found that, in C. albicans, CaYHB1 expression is activated by the regulator CaCta4. By mutating the regulatory region of CaYHB1, they identified a NORE (NO-responsive element) which is crucial for CaYHB1 gene regulation in response to NO. Once this NORE promoter element was identified, CaCta4 [a Zn(II)2-Cys6 transcription factor] was then shown to bind directly to NORE. Furthermore, Chiranand et al. [46] demonstrated that inactivation of CaCTA4 inhibits CaYHB1 induction in response to NO. Moreover, the C. albicans Δcta4-null mutant displayed attenuated virulence in the mouse model of systemic candidiasis, reinforcing the idea that robust nitrosative stress responses contribute to the pathogenicity of C. albicans. CaCta4 also up-regulates a putative sulfite transporter gene (CaSSU1) in response to RNIs. Interestingly, C. albicans Δssu1 cells are not sensitive to NO, unlike in S. cerevisiae where SSU1 mediates NO resistance under certain environmental conditions [46].

Comparisons of NO-induced genes in S. cerevisiae and C. albicans are intriguing [42,47]. For instance, catalase and iron-acquisition genes are up-regulated in both species. However, as illustrated by the case of SSU1, even where apparent orthologues are highly expressed in both S. cerevisiae and C. albicans, the molecular activities and responses appear to be specific for each yeast species and might only be explainable by their evolutionary adaptation to their environmental requirements [46]. Furthermore, the transcription factors that regulate the nitrosative stress responses in these yeasts are even more divergent. The closest homologue of CaCTA4 in S. cerevisiae is ScOAF1 [48], an oleate receptor. The next closest homologue of CaCTA4 in S. cerevisiae is ScHAP1 [49], a haem-responsive transcription factor. Neither ScOAF1 nor ScHAP1 nor ScFzf1 are involved in nitrosative stress response in S. cerevisiae. Instead, Sarver and DeRisi [50] have shown that the C2H2 zinc-finger transcription factor ScFzf1 is involved in NO sensing in S. cerevisiae. In S. pombe, the AP-1 (activating protein 1)-like bZIP (basic leucine zipper) transcription factor SpPap1 regulates nitrosative, as well as oxidative and nutritional, stress responses [51]. The orthologue of SpPap1, ScTap1, regulates the oxidative stress response in S. cerevisiae. These observations illustrate the functional reassignment of transcription factors across these evolutionarily divergent yeasts, an observation that also holds true between S. cerevisiae and C. albicans [52–53].

Conclusions and future perspectives

Our understanding of nitrosative stress pathways in most organisms is rudimentary at best, and much work remains to be carried out to elucidate fungal nitrosative stress-response mechanisms. This cannot be simply carried out by genome sequence comparisons because fungi lack obvious homologues.
of many nitrosative stress functions that are present in other organisms. In addition, there has been a rebirring of nitrosative stress regulators across the ascomycetes [54]. Nevertheless, it is important to study NO and RNI defence mechanisms in yeasts because they contribute to fungal pathogenicity and presumably to the survival of yeasts in other environmental niches. Understanding the cross-talk between nitrosative and oxidative stress responses is likely to lead to a better understanding of host—pathogen interactions and fungal virulence, because pathogenic yeasts are exposed to both ROS and RNIs during contact with host immune defences. Finally, new antifungal drug targets may be revealed by a more complete understanding of the biochemical detoxification pathways of pathogenic fungi.

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