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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 colonising and invading human tissues *C. albicans* is exposed to reactive oxygen (ROS) and reactive nitrogen intermediates (RNI). ROS and RNI 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 nitric oxide (NO) to nitrate by the flavohaemoglobin enzyme, CaYhb1. Members of the haemoglobin superfamily are highly conserved and are found in archaea, eukaryotes, and bacteria. Flavohemoglobins have a dioxygenase activity (NOD) and contain three domains: a globin domain, an FAD-binding domain, and an NAD(P)-binding domain. Here 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 summarise fungal responses to nitrosative stress.

Keywords

fungal stress response; flavohaemoglobin; nitric oxide; reactive nitrogen intermediates

Why study nitrosative stress responses in yeasts?

The evolutionarily divergent yeasts *Candida albicans, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* provide ideal model systems in which to compare nitrosative stress responses. These three yeasts, which diverged about 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, osmolartity and nutrient availability, as well as exposure to ROS and RNI [2] (the latter challenges being of particular interest in our laboratory). These unicellular yeasts have short life cycles, they can be grown on defined experimental conditions, their genomes have been sequenced [3]. Furthermore extensive molecular toolboxes that have facilitated the dissection of 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].

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Nitric oxide, RNI and their impact within the cell

Nitric oxide is an 'ancient' molecule and nitric oxide and its derivates were oxidizing substrates in the archaeal 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]. Nitric oxide is a gaseous radical that can have beneficial or unfavourable effects within cells depending on the 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 likely that the ability of pathogenic fungi to combat host-pathogen defences evolved through ancient interactions between fungi and phagocytic amoeba [13].

Nitrosative stress is mainly caused by three forms of NO: the nitric oxide radical, the nitrosonium cation and the nitroxyl 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 to the nitrite anion and further to the nitrate anion. Intermediates of this oxidation include dinitrogen trioxide and the nitrite anion which contributes to the nitric oxide toxicity by oxidising thiols and amines within the cell. Due 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^{3+} 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 aryl nitrite and S-nitrosothiols [20]. It has been proposed that NO is stored and carried as S-nitrosoglutathione (GSNO), and that GNSO is used as an NO pool within cells [21]. The nitroxyl anion is generated when one electron is added to NO. This reduction is supported by the Fe^{2+} ion and by Fe^{2+} containing metalloproteins which act as electron donors. The nitroxyl anion is believed to mediate sulfhydryl oxidation of target proteins. This process leads to the formation of nitrous oxide which is also the result of nitroxyl anion dimerisation [20].

In mammalian cells NO biosynthesis is catalysed by three isoforms of NO synthase (NOS): the inducible (iNOS), the constitutive neuronal (nNOS) and endothelial isoforms (eNOS). All nitric oxide synthases use L-arginine and NADPH to generate NO and citrulline [22]. As mentioned above, macrophages that have taken up microbial cells release RNS and RNI into the phagolysosome. Macrophages can produce up to 57 μM nitric oxide and up to 14 mM of hydrogen peroxide [23]. ROS such as superoxide anions ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are generated with the help of NADPH oxidase as by-products of the respiratory chain [24]. Furthermore the superoxide anion can also be converted with the help of the myeloperoxidase to hypochlorous acid (HClO). Parallel to the production of ROS, macrophages generate nitric oxide and nitrite with the help of iNOS. Furthermore NO reacts with the superoxide anion to create the strong oxidant peroxynitrite ($ONOO^{-}$) which has fungicidal activity and is more stable and a stronger oxidant than NO [12]. Due 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, the presence of bivalent metals and thiol groups [12].

Nitrosative stress responses in the model yeasts

C. albicans is exposed to NO and RNI, 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, since the mitochondrial respiratory chain of *S. cerevisiae* can use endogenous nitrite instead of oxygen as an electron acceptor, thereby generating NO within the cell [29]. Several mechanisms exist to counteract these nitrosative stresses: (1) the active detoxification of NO via flavohemoglobins; (2) the antioxidant system for scavenging NO via GSH or trehalose; and (3) the up-regulation of repair systems to counteract the caused damage. 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 S-nitrosoglutathione reductase (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 *S. pombe* GSNO reductase by peroxynitrite since GSNO reductase activity is essentially required for the growth of *S. pombe*, unlike in *S. cerevisiae* [32-33]. This observation emphasises the importance of repair functions such as GSNO reductase that are capable of reducing GSNO to ammonia and glutathione disulfide (GSSG) [34]. However, other enzymes such as thioredoxin peroxidase (Tsa1) contribute to resistance to endogenous RNI, and Tsa1 has also been shown to contribute to fungal virulence [30]. In addition, several non-enzymatic antioxidants help to counteract the effects of RNI 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 first were exposed to an aerobic environment. Since then gene duplication events and the redundancy of stress resistance pathways and antioxidant systems have facilitated the environmental adaptation of different yeast species.

Flavohemoglobins are characterised by an NO dioxygenase domain (NOD) which is highly conserved in bacteria and yeast and converts nitric oxide to nitrate [36]. As the name suggests, flavohemoglobins contain a N-terminal heme group followed by the C-terminal FAD (flavin adenine dinucleotide) domain and a NAD(P) domain [37]. *S. cerevisiae* and *S. pombe* each have a single flavohemoglobin gene: *ScYHB1* and the predicted *SPAC869.02c* respectively (see Figure 1) [38]. In contrast, the *C. albicans* genome contains three flavohemoglobin-like genes, namely *CaYHB1, CaYHB4* and *CaYHB5* [39]. The sequence identity between ScYhb1 and the three *C. albicans* flavohemoglobins ranges from 31% to 25% [40].

The flavohemoglobins 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 flavohemoglobins are able to both protect yeasts against external as well as internal sources of NO and RNI. In *C. albicans* only the deletion of *CaYHB1* deletion attenuates virulence slightly [39, 42]. Inactivation of *CaYHB4* or *CaYHB5* did not inhibit NO consumption under the experimental conditions tested 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 flavohemoglobin 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 to mammalian cells, yeasts do not express an obvious NO receptor. However Chiranand et al. [46] recently found that in *C. albicans, CaYHB1* expression is activated by the regulator CaCta4. By mutating the regulatory region of *CaYHB1* they identified a nitric oxide-responsive element (NORE) 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 and coworkers demonstrated that inactivation of *CaCTA4* inhibits *CaYHB1* induction in response to NO [47]. Moreover *C. albicans Δcta4* null mutant display attenuated virulence in the mouse model of systemic candidiasis, reinforcing the idea that robust nitrosative stress responses contribute to the pathogenicity of *C. albicans. CaCTA* also up-regulates a putative sulphite transporter gene (*CaSSU1*) in response to RNI. Interestingly *C. albicans Δssu1* cells are not sensitive to NO, unlike the situation in *S. cerevisiae* where *SSU1* mediates NO resistance under certain environmental conditions [47].

Comparisons of NO-induced genes in *S. cerevisiae* and *C. albicans* are intriguing [42, 48]. 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 to *CaCTA4* in *S. cerevisiae* is *ScOAF1* [49], an oleate receptor. The next closest homologue of *CaCTA4* in *S. cerevisiae* is *ScHAP1* [50], a heme-responsive transcription factor. Neither *ScOAF1* nor *ScHAP1* appears to be involved in nitrosative stress response in *S. cerevisiae*. Instead, Sarver and DeRisi have shown that the C_2H_2 zinc finger transcription factor *ScFZF1* is involved in NO sensing in *S. cerevisiae* [46]. In *S. pombe,* the AP-1-like bZIP transcription factor, SpPap1, regulates nitrosative as well as oxidative and nutritional stress responses [51]. The orthologue of SpPap1, ScYap1, 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 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 done to elucidate fungal nitrosative stress response mechanisms. This cannot be simply done by genome sequence comparisons because fungi lack obvious homologues of many nitrosative stress functions that are present in other organisms. Also there has been rewiring 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 crosstalk 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 RNI during contact with host immune defences. Finally new antifungal drug targets might be revealed by a more complete understanding of the biochemical detoxification pathways of pathogenic fungi.

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Abbreviations used

GSNO	*S*-nitrosoglutathione
ROS	reactive oxygen species
RNI	reactive nitrogen intermediates
NO	nitric oxide
FAD	flavin adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate

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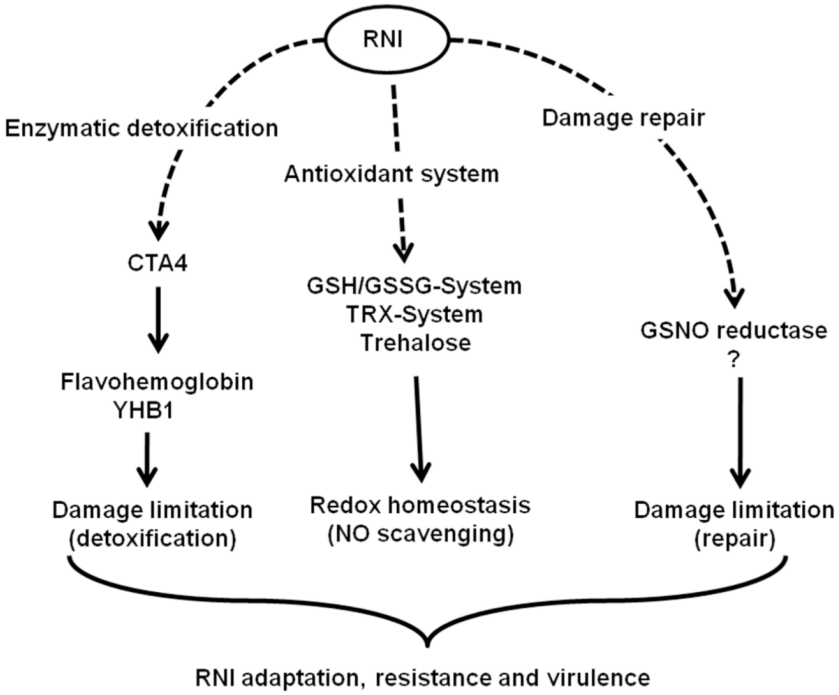

Figure 1.
Simplified RNI response network in yeasts.
Yeasts cope with RNI stresses in different ways: they can enzymatically detoxify NO via flavohemoglobins, they can scavenge NO through antioxidant systems or they can repair the caused damage.

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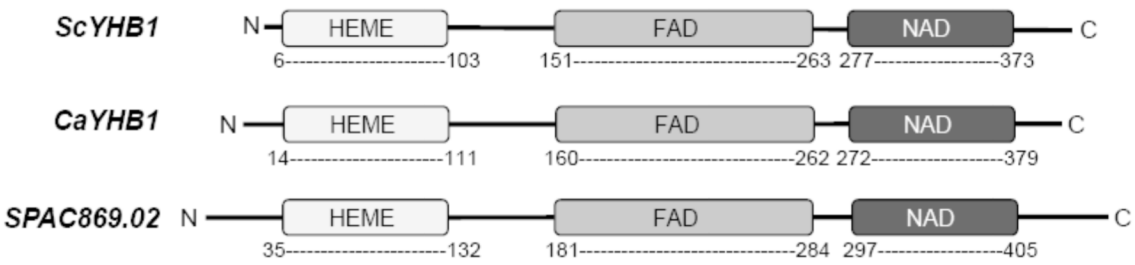

Figure 2.

Flavohemoglobins of *S. cerevisiae* (*ScYHB1*), *C. albicans* (*CaYHB1*) and *S. pombe* (*SPAC869.02c*).

Flavohemoglobins consist of three highly conserved domains an N-terminal globin domain (HEME), followed by a FAD-binding domain (FAD) and an C-terminal NAD(P)-binding domain (NAD).