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Oxidative stress response to menadione and cumene hydroperoxide in the opportunistic fungal pathogen *Candida glabrata*

Mayra Cuéllar-Cruz¹,², Irene Castaño¹, Omar Arroyo-Helguera¹, Alejandro De Las Peñas¹/⁺

¹División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa San José 2055, 78216 San Luis Potosí, SLP, México ²Universidad Autónoma del Estado de Hidalgo, Instituto de Ciencias Básicas e Ingeniería, Área Académica de Química, 42060 Pachuca, HGO, México

*Candida glabrata* is an opportunistic fungal pathogen that can cause severe invasive infections and can evade phagocytic cell clearance. We are interested in understanding the virulence of this fungal pathogen, in particular its oxidative stress response. Here we investigated *C. glabrata*, Saccharomyces cerevisiae and *Candida albicans* responses to two different oxidants: menadione and cumene hydroperoxide (CHP). In log-phase, in the presence of menadione, *C. glabrata* requires Cta1p (catalase), while in a stationary phase (SP), Cta1p is dispensable. In addition, *C. glabrata* is less resistant to menadione than *C. albicans* in SP. The *S. cerevisiae* laboratory reference strain is less resistant to menadione than *C. glabrata* and *C. albicans*; however *S. cerevisiae* clinical isolates (CIs) are more resistant than the lab reference strain. Furthermore, *S. cerevisiae* CIs showed an increased catalase activity. Interestingly, in SP *C. glabrata* and *S. cerevisiae* are more resistant to CHP than *C. albicans* and Cta1p plays no apparent role in detoxifying this oxidant.

Key words: *Candida glabrata* - menadione - cumene - CTA1 - *Candida albicans* - Saccharomyces cerevisiae

Reactive oxygen species (ROS), including superoxide ion, hydrogen peroxide and hydroxyl radicals, are normal by-products of aerobic respiration. ROS can damage all biomolecules, but cells have developed enzymatic (catalases, superoxide dismutases and peroxidases) and non-enzymatic (glutathione and thioredoxin) mechanisms for keeping ROS levels low. Phagocytic cells, which are the first line of defence against fungal infections, generate ROS in order to eliminate invading pathogens (Mansour & Levitz 2002). Interestingly, pathogens have co-opted these well-conserved antioxidant mechanisms to evade phagocyte defences, thus survival and persistence are ensured (Thorpe et al. 2004, Temple et al. 2005).

*Candida glabrata* is an opportunistic fungal pathogen that can cause severe invasive infections and recent surveys show that *C. glabrata* is one of the most frequently isolated species in hospital acquired disseminated infections (Trick et al. 2002). *C. glabrata* virulence factors have only recently begun to be identified (Kaur et al. 2005). *C. glabrata* has a well-defined oxidative stress response and we have previously found that *C. glabrata* can withstand very high concentrations of H₂O₂ relative to that of Saccharomyces cerevisiae and even *Candida albicans* (Cuéllar-Cruz et al. 2008). Resistance of cells to H₂O₂ in stationary phase (SP) is dependent on the concerted role of Yap1, Skn7 and Msn4, well-conserved stress-related transcription factors. In log-phase cells (LP) *C. glabrata* adapts to high levels of H₂O₂ and this adaptive response is dependent on Yap1 and Skn7 and partially on Msn2 and Msn4. The single catalase gene *Cta1* is absolutely required for resistance to H₂O₂ in vitro, however in a mouse model of systemic infection the catalase is dispensable. A strain lacking *Cta1* showed no effect on virulence (Cuéllar-Cruz et al. 2008).

In this report, to better understand the oxidative stress response in *C. glabrata*, we screened the resistance of *C. glabrata* to two different oxidants: menadione and cumene hydroperoxide (CHP). We asked whether *C. glabrata* is more resistant to CHP than *C. albicans* and Cta1p is important for this response. We compared the resistance to these oxidants with the distantly related fungal opportunistic pathogen *C. albicans* and the non-pathogenic and closely related yeast *S. cerevisiae*. For *S. cerevisiae* we used a laboratory reference strain and two clinical isolates (CIs). In addition, we determined catalase activity in these strains.

MATERIALS AND METHODS

**Strains** - All strains used in this study are summarised in Table.

**Media** - Yeast media were prepared as described (Ausubel et al. 2001) and 2% agar was added for plates. YPD media contained yeast extract (10g/L) and peptone (20g/L), supplemented with 2% glucose.

**Menadione and CHP sensitivity assays** - All the starting overnight cultures of *C. glabrata*, *C. albicans* and *S. cerevisiae* were grown in YPD for 36 h at 30°C. Menadione and CHP (88%) were purchased from Sigma-Aldrich. All liquid cultures and plates were incubated at 30°C. For sensitivity assays for LP cells overnight cultures were diluted in fresh rich media, YPD, in such a
way that cells went through seven doublings (D) to reach OD_{600nm} 0.5. The cultures were divided, exposed to different menadione or CHP concentrations and incubated with shaking for 3 h. After the treatment the oxidant was removed and the cultures were adjusted when needed to O.D._{600nm} 0.5, serially diluted in 96-well plates and each dilution was spotted onto YPD plates and incubated at 30°C. Each dilution had the same amount of cells.

For SP cells, 36 h cultures were diluted to O.D._{600nm} 0.5 with spent media from the same culture. The cells were divided into aliquots and treated with menadione or CHP at different concentrations for 3 h at 30°C. After the treatment, the oxidant was removed and the cultures remained at the same O.D._{600nm} 0.5. The cultures were diluted in 96-well plates and spotted onto YPD plates.

All manipulations for these assays were performed in our 30°C temperature-controlled room to prevent abrupt changes in temperature because it has been reported that cold shock has an impact on oxidative stress resistance in S. cerevisiae. The experiments were repeated three times.

Preparation of cell-free extracts and catalase activity assay - The cells were grown for 48 h in YPD containing 2% glucose and were harvested by centrifugation at 800 g for 10 min. Cells were washed twice with distilled water and resuspended in lysis buffer [potassium phosphate (pH 7.0) supplemented with SIGMA FAST protease inhibitor from SIGMA] and 0.5 g of glass beads (SIGMA). Cells were frozen and thawed two times and the mixture was vortexed 4-6 times with 1 min cooling on ice. The lysate was centrifuged for 20 min at 4°C to remove cell debris and glass beads. The supernatant was used for enzymatic activity. Protein quantification was determined by Bradford assay (Bradford 1976). Bovine serum albumin from Sigma was used as standard. Catalase activity was determined in cell-free extracts by a spectrophotometric method that measures the breakdown of H_{2}O_{2} by catalase (Aebi 1984). Assays were performed four times. The catalase activity was normalised to total protein.

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**C. glabrata, C. albicans and S. cerevisiae catalase activity** - We have previously shown in plate assays that *C. glabrata* is more resistant to \( \text{H}_2\text{O}_2 \) than *C. albicans* and *S. cerevisiae* (Cuéllar-Cruz et al. 2008). Therefore, we decided to assay catalase activity in these strains. Surprisingly, *C.g.B14*, *C.a.CA5* and *S.c.W303* showed almost no difference in catalase activity (Fig. 4). As expected, the *C. glabrata* strain *C.g.cta1Δ* that lacks catalase showed no activity. However, *S. cerevisiae* CIs showed increased catalase activity, consistent with the fact that these *S. cerevisiae* CIs are more resistant to \( \text{H}_2\text{O}_2 \) than *C.a.CA5* and *S.c.W303* (Cuéllar-Cruz et al. 2008) (Fig. 4).

**DISCUSSION**

Pathogens are able to evade oxidative killing by phagocytic cells by using the well conserved enzymatic and non-enzymatic mechanisms that keep ROS, generated naturally by aerobic respiration, at low levels (Gonzalez-Parraga et al. 2003). In this way, pathogens can survive and persist in their host. The oxidative stress response for *C. glabrata* has only recently been described (Chen et al. 2007, Cuéllar-Cruz et al. 2008, Roetzer et al. 2008). *C. glabrata* is extremely resistant to very high levels of \( \text{H}_2\text{O}_2 \) and can evade phagocytic cell clearance (Kaur et al. 2007, Cuéllar-Cruz et al. 2008). *C. glabrata* has one catalase (*Cta1p*) that is required to respond to oxidative stress generated by \( \text{H}_2\text{O}_2 \) (Cuéllar-Cruz et al. 2008). Furthermore, the well-conserved transcription factors *Yap1*, *Skn7*, *Msn2* and *Msn4* coordinate, in part, the oxidative stress response in *C. glabrata* (Chen et al. 2007, Cuéllar-Cruz et al. 2008, Roetzer et al. 2008). In this report, we investigated the natural resistance of *C. glabrata, C. albicans* and *S. cerevisiae* to menadione and CHP. In addition, we measured their catalase activity in SP. Fig. 1A shows that LP cells of *C.g.BG14* (wt) can resist up to 0.2 mM menadione and that this resistance is dependent on Cta1. Menadione is a cytotoxic quinone that generates superoxide and it has been shown that exposure to menadione induces the expression of *S.c.* and *S.p.Cta1* (Nakagawa et al. 1995, Osorio et al. 2003). This result suggests that the superoxide generated by menadione is dismutated to \( \text{H}_2\text{O}_2 \), which is then reduced by the...
Fig. 2: *Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae* stationary phase resistance to menadione. Saturated cultures of *C. glabrata* strain BG14, *C. albicans* strain CA5, *S. cerevisiae* strains W303, CIs YJM128 and YJM336 were treated as described in Fig. 1B.

Fig. 3: *Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae* stationary phase resistance to cumene hydroperoxide (CHP). Saturated cultures of *C. glabrata* strains BG14 and CGM295 (cta1Δ), *C. albicans* strain CA5, *S. cerevisiae* strains W303, CIs YJM128 and YJM336 were grown and treated as in Fig. 2, but cultures were exposed to CHP at 0, 0.4 and 0.8 mM.

Fig. 4: *Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae* catalase activity. *C. glabrata* strains BG14 and CGM295 (cta1Δ), *C. albicans* strain CA5, *S. cerevisiae* strains W303, CIs YJM128 and YJM336 were grown for 48 h (stationary phase) in YPD containing 2% glucose and cell extracts were prepared. The catalase activity was normalized to total protein from the lysate and expressed as units per mg of protein. One unit is defined as the amount of catalase required for degradation of 1.0 µmol H₂O₂ per min. Data is presented as the mean ± S.D. of four independent experiments.

In SP the resistance to menadione is increased and there is no difference in resistance between BG14 and the strain lacking the catalase (cta1Δ) (Fig. 1B). However, the increased resistance in SP is not surprising. It has previously been shown that SP cells of different pathogens are more resistant to different stresses including oxidative stress (Cyrne et al. 2003). Interestingly, Cta1 is dispensable in SP and this suggests that other genes are compensating for the lack of Cta1. However, this phenomenon appears to occur only in SP. In LP these genes may be silent. Other elements in SP that could be playing important roles in detoxifying oxidative stress are superoxide dismutases, glutathione, glutathione peroxidases and thioredoxins (Jamieson 1992, Jamieson et al. 1994, Stephen & Jamieson 1996, Zadzinski et al. 1998). In any case, the regulatory network of the oxidative stress response in SP in *C. glabrata* is an important aspect that requires further studies.
C. albicans superoxide dismutases showed that C.g. SODs are more closely related to S.c. SODs than to C.a. SODs [C.g. Sod1p (CAGL0C04741g) 83% identical/7% similar to S.c. Sod1p, C.g. Sod2p (CAGL0E04356g) 70% identical/11% similar to S.c. Sod2p, 58% identical/14% similar to C.a. Sod2p; 54% identical/13% similar to C.a. Sod3p]. Interestingly, both S.c. CIs YJM128 and YJM336 are more resistant to menadione than W303 (Fig. 2) and it has been shown that these CIs are also more resistant to H₂O₂ than W303 reference strain (Cuéllar-Cruz et al. 2008). These results support the idea that pathogens need to have a proper response to oxidative damage in order to survive within the host (Clemons et al. 1994, McCullough et al. 1998, Cassone et al. 2003, Munoz et al. 2005). These CIs could have acquired an increased expression/activity of SOD and multiple drug resistant genes and/or reduced permeability.

We investigated the natural resistance of C. glabrata to CHP, an organic hydroperoxide. As shown in Fig. 3, C. glabrata is more resistant than C. albicans. S. cerevisiae (W303 and CIs) are almost as resistant as C. glabrata. The oxidative stress generated by CHP is removed in part by glutathione peroxidases and glutaredoxins. One likely explanation of the difference in resistance between C. glabrata and S. cerevisiae and C. albicans could be gene dosage. S. cerevisiae possesses two phospholipid hydroperoxide glutathione peroxidase genes, GPX1 and GPX2, eight glutaredoxin genes, GRX1-8. C. glabrata possesses five glutaredoxin genes, GRX1-5 and four glutathione peroxidase genes, GPX1-4. Whereas C. albicans possesses only three glutaredoxin genes, GRX1-3.

Analysis of the catalase activity (Fig. 4) showed almost no difference between C. glabrata, C. albicans and S. cerevisiae, whereas C. glabrata has been shown to be more resistant to H₂O₂ than C. albicans and S. cerevisiae (Cuéllar-Cruz et al. 2008). This result indicates that there must be additional elements in C. glabrata that determine the increased resistance. Interestingly, S. cerevisiae CIs showed increased catalase activity. This result suggests that S. cerevisiae CIs could have acquired a more robust response to oxidative stress in order to evade the attack and elimination by phagocytic cells. It would be interesting to determine if Cta1 expression is upregulated and/or the activity of Cta1 is increased in S. cerevisiae CIs.

Currently, we are making knockout mutants of C. glabrata SOD, GPX and GRX genes in order to evaluate their role in the response to menadione and CHP. Furthermore, we are determining whether expression of S. cerevisiae Cta1 is modified in CIs.

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