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The MAPK Hog1 mediates the response to amphotericin B in *Candida albicans*

José Pedro Guirao-Abad[a,b,1], Ruth Sánchez-Fresneda[b], Elvira Román[a], Jesús Pla[a], Juan Carlos Argüelles[b], Rebeca Alonso-Monge[a,*]

[a] Departamento de Microbiología y Parasitología, Facultad de Farmacia, Instituto Ramón y Cajal de Investigaciones Sanitarias (IRYCIS), Universidad Complutense de Madrid, Plaza de Ramón y Cajal s/n, E-28040 Madrid, Spain
[b] Área de Microbiología, Facultad de Biología, Universidad de Murcia, E-30071 Murcia, Spain

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ABSTRACT

The HOG MAP kinase pathway plays a crucial role in the response to different stresses in the opportunistic pathogen *Candida albicans*. The polyene amphotericin B (AMB) has been reported to trigger oxidative stress in several pathogenic fungi, including *C. albicans*. In the present work, we have analyzed the role of the MAPK Hog1 in sensing and survival to AMB treatment. Mutants lacking Hog1 are more susceptible to AMB than their parental strains and Hog1 became phosphorylated in the presence of this polyene. A set of mutated versions of Hog1 revealed that both the kinase activity and phosphorylation of Hog1 are required to cope with AMB treatment. Flow cytometry analysis showed that AMB induced intracellular ROS accumulation in both parental and *hog1* null mutant strains. In addition, AMB triggered a Hog1-independent synthesis of trehalose. The addition of rotenone to AMB-treated cells improved cell viability, decreased intracellular ROS and prevented intracellular trehalose accumulation, suggesting that AMB-induced ROS is associated to a functional electron transport chain but the presence of rotenone did not impair Hog1 phosphorylation in AMB-treated cells. Our results indicate that Hog1 is necessary during AMB treatment to increase its survival.

1. Introduction

Despite the increasing isolation of new fungal species as responsible of community and nosocomial outbreaks, *Candida albicans* remains the most prevalent fungal pathogen in humans, being considered an important etiological cause of life-threatening mycotic infections in immunocompromised individuals (Perlroth et al., 2007; Pfaller and Castanheira, 2016; Pfaller and Diekema, 2010). *C. albicans* also causes superficial infections (involving skin and mucosal surfaces) which have higher clinical incidence than invasive infections. The polyene amphotericin B (AMB) introduced in the 1950s (Oura et al., 1955) was the first extensively used antifungal, and, in spite of its toxicity, it is still widely prescribed to treat superficial and systemic candidiasis (Pfaller and Diekema, 2007). The classical mechanism of action of the AMB consists in the specific binding to the ergosterol present in fungal membranes which leads to alterations in the selective permeability eventually causing pore formation and cell killing (Akins, 2005; Brajtburg et al., 1990). However, the requirement of channel formation as an essential requisite for the fungicidal activity is open up to dispute (Gray et al., 2012). In recent years, it has convincingly been proven that the generation of an oxidative stress through the production of endogenous ROS is a universal action mechanism of AMB against relevant pathogenic yeasts (Mesa-Arango et al., 2014). AMB also induces the accumulation of the protective disaccharide trehalose and the activation of several antioxidant enzymatic activities in *C. albicans* (Gonzalez-Parraga et al., 2011). Proteomics analyses have allowed the identification of a specific set of AMB-induced proteins involved in both oxidative stress protection and osmotic tolerance (Hoehamer et al., 2010). These results reinforce the theory that generation of an internal oxidative damage is an important contributory factor to the fungicidal action of AMB.

Eukaryotic cells sense and respond to sudden environmental challenges through specific signal transduction pathways. The MAPK (Mitogen Activated Protein Kinase) pathways are well-established

Abbreviations: AMB, amphotericin B; ROS, reactive oxygen species

* Corresponding author.
E-mail address: realonso@ucm.es (R. Alonso-Monge).
[1] Current address: Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati, Medical Sciences Building, 231 Albert Sabin Way, Cincinnati, OH 45267, United States.

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signaling mechanisms, which have rigorously been conserved throughout evolution and play critical functions in cell physiology and response to stress (Kultz, 1998; Kultz and Burg, 1998). These signaling pathways are integrated, among other elements, by a core of three MAP kinases that become sequentially activated by phosphorylation. In *C. albicans*, diverse elements of the MAPK pathways have been identified and characterized in detail (Alonso-Monge et al., 2009b). The HOG pathway is directly involved in counteracting several environmental injuries and *hog1* mutants are highly sensitive to osmotic stress, a variety of oxidative and nitrosative agents, as well as certain metals or metalloids (Alonso-Monge et al., 2003; Alonso-Monge et al., 2009b; Herrero-de-Dios et al., 2018; San José et al., 1996; Urrialde et al., 2015). Notably, the HOG pathway is strictly required for *C. albicans* cells to colonize gastrointestinal tract in a murine commensalism model (Prieto et al., 2014). This pathway also plays a relevant role in virulence and survival to phagocytes (Alonso-Monge et al., 1999; Arana et al., 2007). In the present work the role of the Hog1 MAPK in the response of *C. albicans* to AMB has been carefully studied. We show that Hog1 became phosphorylated in the presence of AMB and that this MAPK is important to trigger an adequate response. Nevertheless, the AMB-induced oxidative stress is independent of the presence of Hog1.

2. Materials and methods

2.1. Yeast strains and growth conditions

C. albicans strains are listed in Table 1. Unless otherwise stated, yeast cell cultures were grown at 37 ˚C with shaking in YPD (2% peptone, 1% yeast extract and 2% glucose) medium. Usually, preinoculated overnight cultures were harvested, resuspended in fresh YPD and incubated further until they reached exponential phase. The growth was monitored by measuring the OD_{600nm} of cultures.

2.2. ROS determination

Intracellular ROS were measured with dihydrofluorescein diacetate (DHF), following the procedure described elsewhere (Sangalli-Leite et al., 2011). Briefly, overnight cultures from the *C. albicans* strains were washed and resuspended in PBS at 1 × at 10^7 cells/mL. The strains were treated with 0.156 mM rotenone for 1 h, then 0.25 mg/L AMB was added and incubated for another 1 h at 37 ˚C. Control cells were treated similarly but without drug addition. Then, DHF was added to the samples to a final concentration of 40 μM and incubated at 37 ˚C for 30 min. This compound emits fluorescence when cleaved into fluorescein by free radicals. Fluorescence intensity was determined by flow cytometry using the EPICS XLMCL4 cytometer (Beckman Coulter, High Wycombe, UK) equipped with an argon ion laser with an excitation power of 15 mW at 488 nm. Forward scatter (FSC) and side scatter (SSC) were analyzed on linear scales, while the analyses of green (FL1) fluorescence intensity were made on logarithmic scale. Analysis gates were set around debris and intact cells, on an FSC *vs* SSC dot plot. The fluorescence histograms corresponding to 5000 cells were generated using the gated data. Data acquisition and analysis were performed using Flowing Software (http://flowingsoftware.btk.fi/).

2.3. Real time quantitative RT-PCR (qRT-PCR) analysis

Exponential yeast cultures growing in YPD at 37 ˚C were split on two subcultures, then antifungal treatment was added to one of them and samples taken at different time points. Total RNA was isolated using the “mechanical disruption” protocol and RNeasy mini kits with column DNase treatment (Qiagen). The amount of RNA was quantified spectrophotometrically and 2 μg of total RNA from yeasts were reverse transcribed by the Superscript First-Strand synthesis system for RT-PCR (Invitrogen). Q-PCR was carried out using the SYBR-green method with *ACT1* as the internal standard. The Applied Biosystems 7500 Real-Time PCR System was used to run samples and analyze data. All samples were analyzed in triplicate, normalized to *ACT1* gene expression level, and the results were expressed as fold induction compared to untreated controls. The primers used in the assay are listed in table 2.

2.4. Protein extracts and immunoblot analysis

Exponentially growing cultures (O.D. = 1) in YPD liquid medium at 37 ˚C were challenged with rotenone (0.15 mM) or not for 1 h. and then AMB was added to the concentration indicated in the legends to figures. Samples were taken at different time points and processed for western-blot as previously described (Martín et al., 1993). Proteins were quantified by 280 nm absorbance and equal amount were loaded onto gels. Blots were probed with the following antibodies: ScHog1 polyclonal antibody (Santa Cruz Biotechnology) that recognizes CaHog1 protein; Ab-p38-P (Thr180/Tyr182) 28B10 monoclonal antibody (Cell Signaling Technology, Inc.) which detects phosphorylated Hog1. Western blots were performed according to the manufacturer's instructions using the Hybond ECL kit (AmershamPharmacia Biotech).

3. Results

3.1. The hog1 mutant is hypersensitive to AMB

Since AMB causes oxidative stress in *C. albicans* (Mesa-Arango et al., 2014) and Hog1 mediates the response to osmotic and oxidative stress

Table 1
C. albicans strains used in this work.

Strain	Genotype	Nomenclature in Manuscript and Figures	Source
SC5314	*wt*		Gillum et al. (1984)
RM100	*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG-URA3-hisG*	*RM100*	Alonso-Monge et al. (2003)
RM1000	ura3::imm434/ura3::imm434 his1::hisG/his1::hisG		Alonso-Monge et al. (2003)
CNC13	*RM1000 hog1::hisG-URA3-hisG/hog1::hisG*	*hog1*	San José et al. (1996)
CNC15-10	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG hog1::hisG/hog1::hisG LEU2/ leu2::HOG1URA3*	$hog1^{reint}$	Alonso-Monge et al. (2003)
HI3-21	*ura3Δ::imm434/ura3Δ::imm434 hog1::hisG-URA3-hisG/hog1::hisG*	*hog1*	Prieto et al. (2014)
HHH	*ura3Δ::imm434/ura3Δ::imm434 hog1::hisG-URA3-hisG/hog1::hisG ADH1/adh1::tTA Ptet –HOG1-SAT1*	*hog1:HOG1myc*	Morales-Menchen et al. (2018)
HHA	*ura3Δ::imm434/ura3Δ::imm434 hog1::hisG-URA3-hisG/hog1::hisG ADH1/adh1::tTA Ptet –*$hog1^{F321L}$*-SAT1*	$hog1^{F321L}$	Morales-Menchen et al. (2018)
HKD	*ura3Δ::imm434/ura3Δ::imm434 hog1::hisG-URA3-hisG/hog1::hisG ADH1/adh1::tTA Ptet –*$hog1^{K52RL}$*-SAT1*	$hog1^{K52R}$	Morales-Menchen et al. (2018)
HDP	*ura3Δ::imm434/ura3Δ::imm434 hog1::hisG-URA3-hisG/hog1::hisG ADH1/adh1::tTA Ptet –*$hog1^{T174A-Y176F}$*-SAT1*	*hog* $^{T174A-Y176F}$-	Morales-Menchen et al. (2018)

Table 2
Primers used for Q-PCR analysis.

Gene	Primer's name	Sequence
ACT1	o-ACTQTup	TGGTGGTTCTATCTTGGCTTCA
	o-ACTQTlw	ATCCACATTTGTTGGAAAGTAGA
TPS1	o-TPS1up-QT	TCGCAAGGGTGTCTTGATCTTAT
	o-TPS1lw-QT	AACAATCAAGGCACCATTAAGTGA
TPS2	o-TPS2up-QT	TTGCTGTTGGTCCTGCATCA
	o-TPS2lw-QT	GGCGAGGTTCGTTCAAATGT

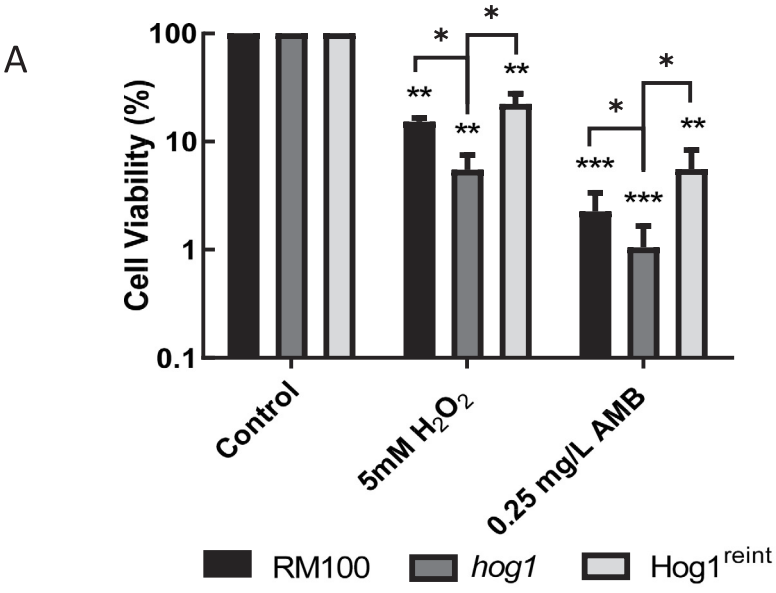
in this opportunistic yeast (Alonso-Monge et al., 2003), we aimed to analyze the role of Hog1 in the susceptibility to AMB. Firstly, the MIC_{100} values for AMB were determined for wild type, *hog1* mutant and the re-integrant Hog1$^+$ strains, following the CLSI protocol. The *hog1* null mutant displayed half of the MIC_{100} (0.06 mg/L) compared to the parental RM100 and the Hog1$^+$ reintegrant (0.12 mg/L). The quality control required by the CLSI procedure was included to ensure the validity of the methodology. This control strain was *C. parapsilosis* ATCC® 22019 being the corresponding MIC_{100} for AMB 0.25 mg/L. These concentrations are within the range previously reported for other *C. albicans* genetic backgrounds from clinical or laboratory origin (Campoy and Adrio, 2017). Then, cell viability was quantified in exponentially-growing cells treated with a toxic dose (0.25 mg/L) of AMB during 1 h at 37 °C. Cells were removed by centrifugation and the cell viability measured by spreading on YPD plates for CFUs counting. In this assay, a positive control of oxidative sensitivity (5 mM H_2O_2) was introduced. As expected, *hog1* null cells showed a significant loss of viability after addition of H_2O_2 (Fig. 1A). Interestingly, the *hog1* mutant also displayed an increased sensitivity to AMB compared to its parental and the reintegrant strains. This difference was statistically significant (expressed as percentage of viability; Fig. 1A).

The susceptibility to AMB was further confirmed by spotting 10-fold cell suspension on solid YPD medium supplemented with 0.25 mg/L AMB (Fig. 1B). The growth was clearly impaired in the *hog1* mutant compared to the other two strains (Fig. 1B upper row). These data indicates that the MAP kinase Hog1 is relevant in the *C. albicans* response against AMB exposure.

3.2. AMB induces intracellular ROS production through the electron transport chain

In order to elucidate the putative contribution of Hog1 in the generation of internal oxidative stress in response to AMB, the endogenous accumulation of ROS was quantified by flow cytometry. RM100 and *hog1* mutant cells were exposed to AMB for 1 h at 37 °C and intracellular ROS production was measured by using dihydrofluorescein diacetate (DHF) (see Methods). The basal ROS levels were higher in the *hog1* null mutant compared to their parental cells (Mean Fluorescent Intensity 14.50 *versus* 6.58) (Fig. 2A, left panels) in agreement with previous reports (Alonso-Monge et al., 2009a). The treatment with 0.25 mg/L AMB for 1 h significantly increased the amount of intracellular ROS produced (Fig. 2A, right panels), in agreement with previous researches showing that this antifungal caused oxidative stress in *C. albicans*.

We further analyzed if this polyene-induced ROS production is caused by mitochondrial respiration. For this purpose, growing cells were pre-treated during 1 h at 37 °C with a non-toxic dose of rotenone



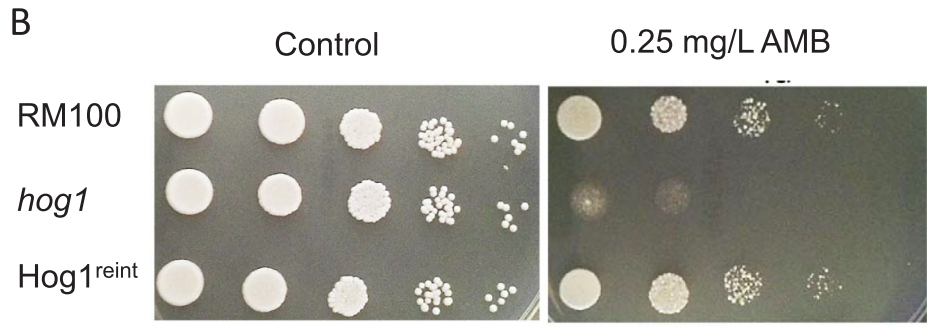


Fig. 1. The *hog1* mutant displays an enhanced sensitivity to AMB treatment. (A) Growing cells from the strains RM100 (black histograms), *hog1* (dark grey histograms) and Hog1^{reint} (light grey histograms) were treated with 5 mM H_2O_2 or 0.25 mg/L AMB during 1 h in liquid YPD at 37 °C. The cells were then collected and spread on YPD plates and cell viability determined by CFU counting. The data shown are the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 represent statistically significant differences with respect to an untreated control according to Mann Whitney *U* test or between the strains indicated with the bracket. (B) Ten-fold cell suspensions were spotted on YPD plates containing 0.25 mg/L AMB. Plates were incubated at 37 °C for 24 h.

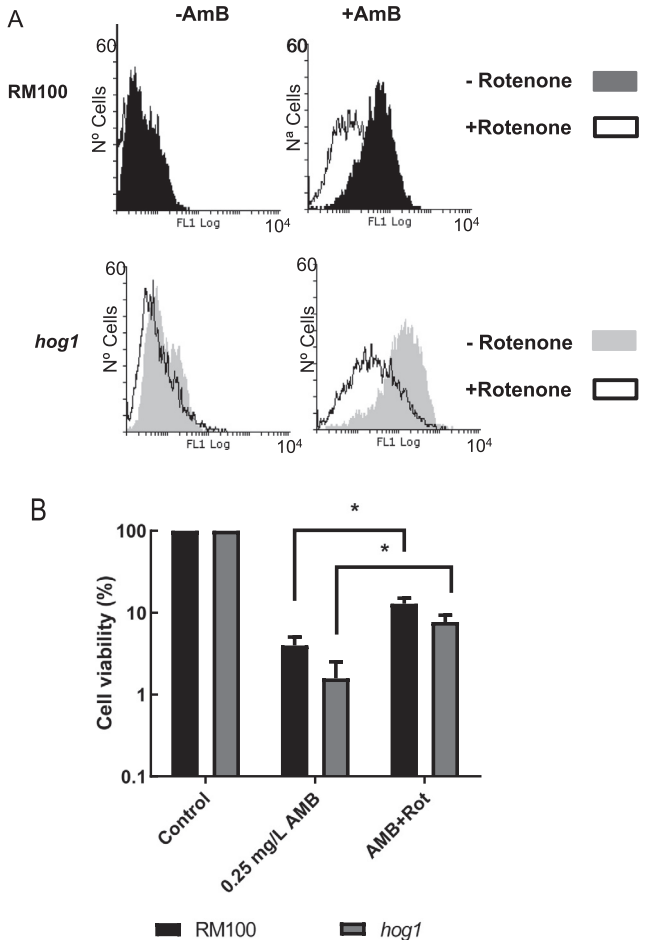


Fig. 2. Quantification of intracellular ROS production after AMB treatment and protective role of rotenone. Equivalent number of exponentially growing cells was incubated for 1 h in the presence or absence of rotenone (0.156 mM) at 37 ˚C, followed by 1 h of treatment with AMB (0.25 mg/L). (A) Intracellular ROS were quantified using DHF by flow citometry in RM100 (upper row histograms) and *hog1* (lower row histograms) strains incubated without AMB (left panels) or with AMB (right panels) in the absence (unfilled histograms) or presence of rotenone (filled histograms). The histograms display the cell number with respect to the green fluorescence intensity (FL1). (B) Cell viability was simultaneously determined in identical samples by CFU counting. The data shown are the mean ± SD of three independent assays. *P < 0.05 represent statistically significant differences between treatment with AMB and AMB plus rotenone for each strain according to Mann Whitney *U* test.

(0.156 mM, Mesa-Arango et al., 2014), which is a specific inhibitor of complex I in the electron transport chain, and immediately exposed to 0.25 mg/L AMB during 1 h. After that, the intracellular ROS production was quantified by flow cytometry. Rotenone led to a lower degree of ROS accumulation in both strains in comparison with identical cells pre-incubated in the absence of the inhibitor (Fig. 2A). Cell viability was simultaneously determined by CFUs counting in samples harvested and spread on YPD plates (Fig. 2B). It is worth noting that rotenone had a noticeable protective effect on cell viability in both strains exposed to the antifungal. Thus, RM100 and *hog1* mutant cells preincubated with rotenone showed a larger percentage of cell survival upon AMB addition respect to non-treated cells (Fig. 2B). Therefore, the production of endogenous ROS caused by AMB treatment is associated with a functional electron transport chain.

Table 3
Trehalose accumulation on AMB-treated cells and effect of the presence of rotenone. Exponentially growing cells of the two *C. albicans* strains used in this study were preincubated for 1 h with 0.156 mM rotenone (or not) and immediately treated with AMB 0.5 mg/L. Samples for trehalose determination were harvested after an additional 1 h. Results represent the mean ± SD of three independent measurements.

Strains	Trehalose [nmol trehalosa (mg wet weight)$^{-1}$] ± SD (Fold induction)			
	Control	Control + Rotenone	AMB	AMB + Rotenone
RM100	2.3 ± 0.2 (1.0)	2.1 ± 0.2 (0.9)	23.8 ± 2.9 (10.0)	4.1 ± 0.5 (1.8)
hog1	3.6 ± 0.4 (1.0)	1.9 ± 0.2 (0.5)	25.4 ± 3.3 (7.0)	6.5 ± 0.7 (1.8)

3.3. AMB induces synthesis of intracellular trehalose through increasing TPS2 transcription

The synthesis of intracellular trehalose was also quantified, since it is well-established that this non-reducing disaccharide plays a crucial protective role against oxidative stress in several pathogenic yeasts (Alvarez-Peral et al., 2002; Gonzalez-Parraga et al., 2011). As shown in Table 3, a strong storage of trehalose was evident in AMB-treated parental cells (≈10-fold increase on the control levels) at 37 ˚C. Regarding the homozygous *hog1* mutant a more modest trehalose increase was recorded (≈7-fold increase), although the final content stored by both strains was rather similar or even higher in the *hog1* mutant (25.4 *versus* 22.08 mmol trehalose/mg wet weight). Remarkably, the presence of rotenone completely prevented trehalose accumulation, suggesting that either the synthesis of trehalose is Hog1-independent but dependent on the electron transport chain or is a consequence of intracellular ROS accumulation. In addition, rotenone also caused a reduction in the basal level of trehalose displayed by the *hog1* mutant.

Of note is the fact that the AMB-triggered synthesis of trehalose must be caused, at least in part, by the large rise recorded in mRNA expression of *TPS2* gene (coding for trehalose phosphatase) detected in both strains at 15 and 30 min after AMB addition (Fig. 3, lower row). Surprisingly, the expression of *TPS1* gene (coding for trehalose synthase) followed a distinct pattern. As shown in Fig. 3 (upper row), AMB did not cause any relevant change in the transcription of *TPS1* in parental cells. By contrast, an evident induction in *TPS1* gene expression was detected in the *hog1* mutant at 15 min after being exposed to AMB that was followed by a progressive decrease. These data suggest that Hog1 differentially controls the expression of *TPS1* mRNA in response to AMB treatment, which could explain the distinct basal content of trehalose detected in the *hog1* mutant (Table 3 and Gonzalez-Parraga et al., 2010).

3.4. Hog1 becomes phosphorylated in the presence of AMB in C. albicans.

In order to get a better understanding of the implication of Hog1 in the fungicidal action of this polyene, we have also studied whether AMB induced Hog1 phosphorylation. Exponentially-growing wild type cells were exposed to different AMB concentrations and samples were taken at different time points for immunoblotting analyses. According to the results presented in Fig. 4A, the addition of AMB triggered a clear phosphorylation of Hog1, which was already observed after 5 min at 0.2 and 0.5 mg/L, being further increased at 15 min and reaching a maximum at 30 min. A lower concentration of AMB (0.1 mg/L) required a longer exposure (15 min) in order to record a conspicuous signal of Hog1 phosphorylation (Fig. 4A). Thus, the activation of Hog1 in cells exposed to AMB seems to be dose-dependent.

Additionally, Hog1 phosphorylation was analyzed in exponentially growing wild type cells exposed to 0.15 mM rotenone during 1 h.

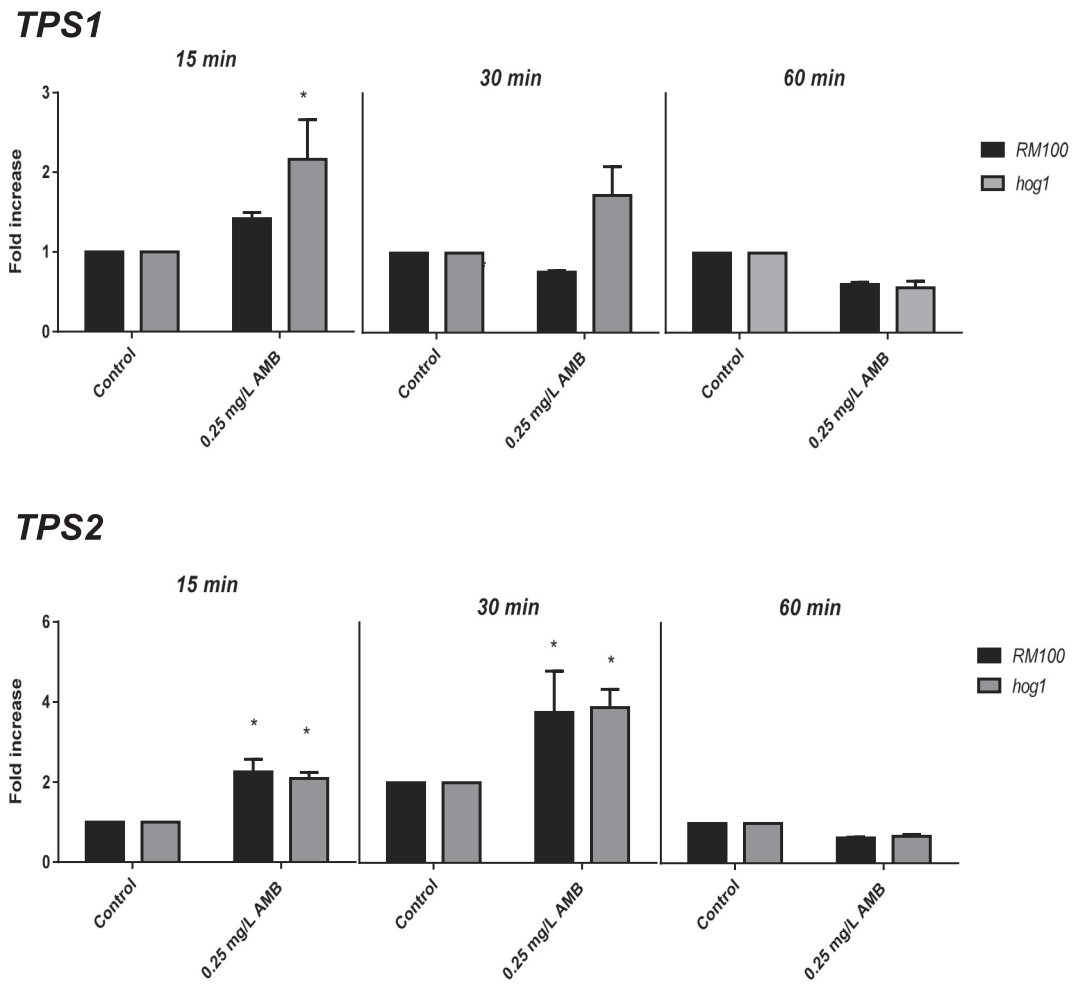


Fig. 3. Quantification of *TPS1* and *TPS2* gene expression after treatment with AMB. Growing cells of the strains under analysis were split in two and AMB was added to one of them. Samples were taken at 15, 30 and 60 min post-antifungal addition and processed for RT-PCR-Q. *TPS1* and *TPS2* mRNA transcripts were measured and expressed as fold increase respect to untreated cells at the same time point for each strain. Data are the mean ± SD of three independent experiments. *P < 0.05 represent statistically significant differences with respect to an untreated control according to Mann Whitney *U* test.

followed by the addition of AMB to 0.25 mg/mL. Samples were taken at different time points (Fig. 4B). Immunoblot analysis showed that the MAPK Hog1 became phosphorylated in the presence of AMB both in the presence or absence of the inhibitor of the electron transport chain; therefore, a reduction in the intracellular ROS does not impair Hog1 phosphorylation. Since AMB also causes alterations in the cellular membrane and loss of selective permeability, intracellular glycerol was quantified in order to determine if AMB triggered osmotic stress. There was not a significant increase in the intracellular glycerol amount in cells treated with AMB (Supplementary Fig. 1). All together, these data suggest that the immediate activation of Hog1 is, at least partially, independent of the osmotic stress or intracellular ROS caused by AMB.

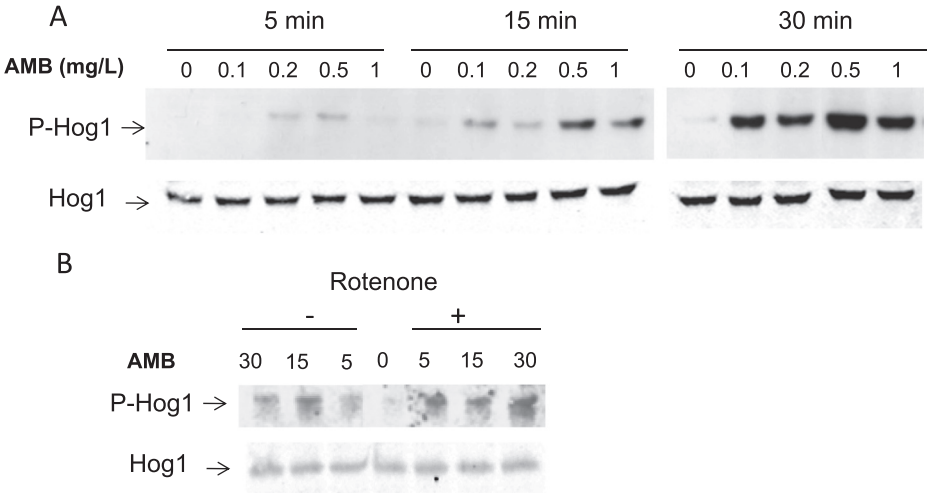


Fig. 4. Hog1 phosphorylation in response to AMB. (A) Parental cells were challenged with the indicated concentrations of AMB at 37 °C in YPD liquid medium. The samples were then harvested at different time points and processed for immunoblotting. (B) Exponentially growing cells were exposed (or not) to 0.15 mM rotenone for 1 h; then, AMB to 0.25 mg/L was added and samples were collected at the time points indicated (in min) and processed for immunoblotting. Hog1 phosphorylated form was detected using anti-phospho p38 antibody (P-Hog1). Total Hog1 protein was detected with an anti-ScHog1 antibody and used as load control.

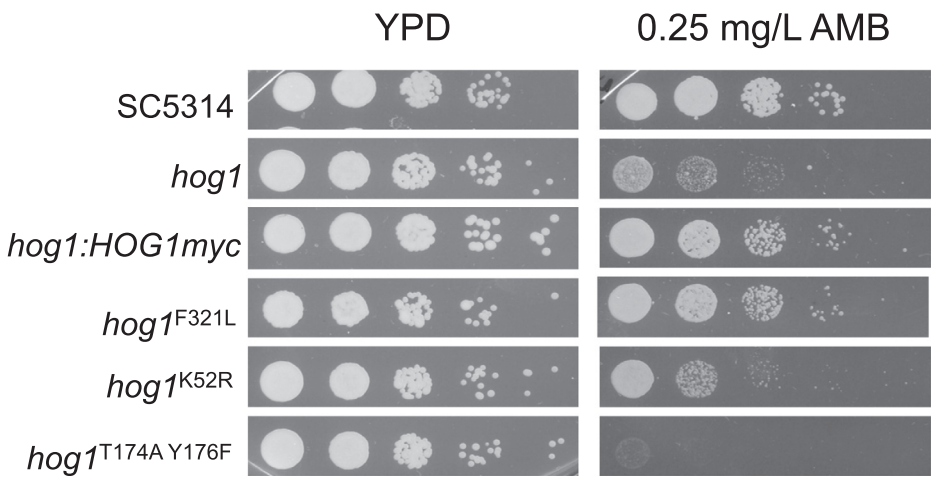


Fig. 5. Susceptibility to AMB of a set of *hog1* mutant. Ten-fold cell suspensions of the indicated strains were spotted on YPD plates supplemented (or not) with 0.25 mg/L AMB. Plates were incubated at 37 °C for 24 h.

3.5. Kinase activity and Hog1 phosphorylation are required to survive to AMB

Finally, we analyzed the sensitivity to AMB displayed by a set of *hog1* mutants (Fig. 5), which were constructed in different *C. albicans* backgrounds (Morales-Menchen et al., 2018). The *hog1* mutant HI3-21 (Prieto et al., 2014) was the parental strain where the set of *HOG1* mutants were constructed. As expected, this *hog1* mutant (the HI3-21 strain) was also more sensitive to AMB (Fig. 5), indicating that this phenotype depends on the presence of Hog1 but not on the genetic background. As an additional control, a reintegrant *HOG1*$^{+}$ strain was introduced harboring a native *HOG1* copy under the control of Tet promoter and integrated in the genome (named as *hog1:HOG1myc* in the Fig. 5). The same strategy was used to generated the *hog1* mutants' collection (Morales-Menchen et al., 2018). The phosphomimetic Hog1 version (*hog1*^{F321L}) behaved as the parental and reintegrant strains, concluding that this mutation did not enhance the resistance to AMB. The *hog1* mutant carrying a kinase dead version (*hog1*^{K52R}) behaved as the *hog1* defective mutant, while the non-phosphorylatable *hog1* (*hog1*$^{T174A\text{-}Y178F}$) was even more sensitive to AMB than the *hog1* mutant. Thus, a functional kinase activity is relevant to generate an appropriate response to AMB, but Hog1 phosphorylation seems to be even more important, as suggests the increased sensitivity to AMB displayed by the non-phosphorylatable mutant (Fig. 5).

4. Discussion

Intensive research carried out in the last years has revealed that the mechanism of action of the main antifungals used in medical practice is more complex than initially believed. Hence, in the case of AMB together with the classical target of ergosterol binding and pore formation (Gray et al., 2012; Sokol-Anderson et al., 1986), compelling evidences demonstrate that generation of intracellular oxidative stress through the synthesis of ROS is also a contributory factor to the fungicidal action of this polyene. This endogenous ROS formation is dependent on tricarboxylic-acid-cycle and the respiratory-electron-chain and is a common feature of the fungicidal action exerted by some antibiotics, such as AMB, miconazole and ciclopirox (Belenky et al., 2013), despite the fact that these compounds do not share chemical structure or target within the fungal cell.

Given the role of HOG pathway in signaling oxidative stress (Alonso-Monge et al., 2003), we have examined the hypothetical implication of this signaling pathway in the *C. albicans* response to AMB. Mutants lacking the *HOG1* gene are clearly more sensitive to AMB and Hog1 becomes phosphorylated in the presence of this antifungal (Figs. 1 and 4). Hog1 phosphorylation as well as its kinase activity are important to cope with AMB exposure as demonstrated by the assay performed with non-phosphorylatable or kinase–deficient *hog1* mutants (Fig. 5). Collectively, these data strongly support that Hog1 mediates the *C. albicans* response to AMB. The relevance of Hog1 phosphorylation in the response to oxidative and osmotic stress has unequivocally been demonstrated (Chang et al., 2016; Cheetham et al., 2011; Morales-Menchen et al., 2018) while kinase activity only played a significant role upon addition of compounds that alter the cell wall integrity (Morales-Menchen et al., 2018). Our data indicate that in the defensive response of *C. albicans* to AMB, both activities have a relevant role and highlight the importance of phosphorylation, since kinase activity should depend on the phosphorylation status.

Our results support previous work in other laboratories demonstrating that AMB triggers oxidative stress in *C. albicans* by increasing the intracellular synthesis of ROS, which must be the outcome of respiratory electron chain (Fig. 2). Indeed, the specific inhibition of electron transport chain complex I by rotenone caused a marked decrease of ROS production together with a concomitant improvement in the percentage of cell viability (Fig. 2). Here, we demonstrate that this phenomenon is Hog1-independent, since the accumulation of intracellular ROS induced by AMB was also detected in the *hog1* mutant strain and is prevented by rotenone (Fig. 2). In this case, AMB-induced intracellular-ROS increase is dependent on the electron transport chain in both wild type and *hog1* mutant strains. It is noteworthy that the phosphorylation of Hog1 in AMB-treated cells was not prevented by rotenone suggesting that Hog1 activation is not a direct consequence of the increase of intracellular ROS.

The *hog1* mutant is also able to promote the storage of endogenous trehalose in response to AMB addition (Table 3), probably due to an increase in the expression of genes involved in trehalose synthesis (Fig. 3). In accordance, the AMB-induced accumulation of trehalose appears to be Hog1-independent. However, Hog1 may have a repressive role in the synthesis/accumulation of trehalose, since the basal levels of trehalose are always higher in *hog1* cells (Table 3 and Gonzalez-Parraga et al., 2010) and the expression of *TPS1* gene induced by AMB is also up-regulated in *hog1* null mutant (Fig. 3). Taken together, this suggests that the oxidative stress generated by the polyene AMB is induced in both wild type and *hog1* mutant cells, but *C. albicans* requires this MAPK to sense and face the antifungal. Since AMB also affects the cellular membrane and alterations in this structure could be perceived by fungal cells as an osmotic challenge (Morales-Menchen et al., 2018) triggering event, the intracellular glycerol was quantified in *C. albicans* cells exposed to 0.25 mg/L AMB. No significant accumulation of glycerol was detected when wild type *C. albicans* cells were treated with this polyene

(Supplementary Fig. 1) indicating that no osmotic stress response was induced at least in the analyzed conditions. The damage generated by AMB at the membrane level may result in a general cellular failure to enable glycerol accumulation.

Although Hog1 is involved in the response to oxidative stress (Alonso-Monge et al., 2003), mutants defective in this MAPK are still able to respond to oxidative challenges (Dantas Ada et al., 2015; Enjalbert et al., 2006). A transcriptomal analysis showed that the response to 5 mM H_2O_2 displayed by the *hog1* mutant was comparable to the wild type strain (Enjalbert et al., 2006). Similarly, the *hog1* mutant was more susceptible to phagocytes, although this mutant retained the capacity to increase the expression of genes involved in the defense against oxidative and nitrosative stress (Arana et al., 2007). In this sense, other signaling pathways and transcription factors are involved in the response to oxidative stress that could be responsible for this (Dantas Ada et al., 2015). Mkc1, the cell wall integrity MAPK, becomes phosphorylated in response to oxidative stress by Hog1-dependent mechanisms (Arana et al., 2005; Navarro-García et al., 2005) and is also phosphorylated in presence of AMB (Navarro-García et al., 2005). The response to oxidative stress is complex and therefore involves different elements within the cell.

AMB clearly induces oxidative stress in *C. albicans* cells and the presence of an intact HOG pathway increases the survival of fungal cells under these conditions. This could open up new therapeutic approaches as the specific combination of AMB with compounds that inhibit Hog1 signaling could enhance the fungicidal action of AMB while simultaneously allowing a reduction of the therapeutic dose, which has the potential to minimize nephrotoxic side effects caused by this antifungal.

In the search for novel, potent and safer antifungals, it would be useful to elucidate the putative circuits and signaling pathways involved in sensing and responding to a specific compound. In this work, we have demonstrated that the Hog1 MAPK becomes phosphorylated in cells exposed to AMB and this phosphorylation together with the kinase activity are required to deal with AMB challeng in *C. albicans*. This antifungal generates an archetypical oxidative stress response by increasing intracellular ROS and trehalose accumulation, which also occurs in *hog1* defective mutants. Further research is needed in order to better understand the contribution of the oxidative stress to the AMB-induced fungal dead.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2019.103302.

References

Akins, R.A., 2005. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. Med. Mycol. J. 43, 285–318.
Alonso-Monge, R., et al., 2009a. The Hog1 MAP kinase controls respiratory metabolism in the fungal pathogen *Candida albicans*. Microbiology 155, 413–423.
Alonso-Monge, R., et al., 1999. Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. J. Bacteriol. 181, 3058–3068.
Alonso-Monge, R., et al., 2003. The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*. Eukaryot. Cell 2, 351–361.
Alonso-Monge, R., et al., 2009b. Fungi sensing environmental stress. Clin. Microbiol. Infect. 15 (Suppl 1), 17–19.
Alvarez-Peral, F.J., et al., 2002. Protective role of trehalose during severe oxidative stress caused by hydrogen peroxide and the adaptive oxidative stress response in *Candida albicans*. Microbiology 148, 2599–2606.
Arana, D.M., et al., 2007. Differential susceptibility of mitogen-activated protein kinase pathway mutants to oxidative-mediated killing by phagocytes in the fungal pathogen *Candida albicans*. Cell. Microbiol. 9, 1647–1659.
Arana, D.M., et al., 2005. The Pbs2 MAP kinase kinase is essential for the oxidative-stress response in the fungal pathogen *Candida albicans*. Microbiology 151, 1033–1049.
Belenky, P., et al., 2013. Fungicidal drugs induce a common oxidative-damage cellular death pathway. Cell Rep. 3, 350–358.
Brajtburg, J., et al., 1990. Amphotericin B: current understanding of mechanisms of action. Antimicrob. Agents Chemother. 34, 183–188.
Campoy, S., Adrio, J.L., 2017. Antifungals. Biochem. Pharmacol. 133, 86–96.
Chang, W.H., et al., 2016. The conserved dual phosphorylation sites of the Candida albicans Hog1 protein are crucial for white-opaque switching, mating, and pheromone-stimulated cell adhesion. Med. Mycol. 54, 628–640.
Cheetham, J., et al., 2011. MAPKKK-independent regulation of the Hog1 stress-activated protein kinase in *Candida albicans*. J. Biol. Chem. 286, 42002–42016.
Dantas Ada, S., et al., 2015. Oxidative stress responses in the human fungal pathogen, *Candida albicans*. Biomolecules 5, 142–165.
Enjalbert, B., et al., 2006. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. Mol. Biol. Cell 17, 1018–1032.
Gillum, A.M., et al., 1984. Isolation of the *Candida albicans* gene for orotidine-5'- phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. Mol. Gen. Genet. 198, 179–182.
Gonzalez-Parraga, P., et al., 2010. Adaptive tolerance to oxidative stress and the induction of antioxidant enzymatic activities in *Candida albicans* are independent of the Hog1 and Cap1-mediated pathways. FEMS Yeast Res. 10, 747–756.
Gonzalez-Parraga, P., et al., 2011. Amphotericin B induces trehalose synthesis and simultaneously activates an antioxidant enzymatic response in *Candida albicans*. Biochem. Biophys. Acta 1810, 777–783.
Gray, K.C., et al., 2012. Amphotericin primarily kills yeast by simply binding ergosterol. Proc. Natl. Acad. Sci. U. S. A. 109, 2234–2239.
Herrero-de-Dios, C., et al., 2018. Redox regulation, rather than stress-induced phosphorylation, of a Hog1 mitogen-activated protein kinase modulates its nitrosative-stress-specific outputs. MBio 9.
Hoehamer, C.F., et al., 2010. Changes in the proteome of *Candida albicans* in response to azole, polyene, and echinocandin antifungal agents. Antimicrob. Agents Chemother. 54, 1655–1664.
Kultz, D., 1998. Phylogenetic and functional classification of mitogen- and stress-activated protein kinases. J. Mol. Evol. 46, 571–588.
Kultz, D., Burg, M., 1998. Evolution of osmotic stress signaling via MAP kinase cascades. J. Exp. Biol. 201, 3015–3021.
Martín, H., et al., 1993. Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37 degrees C. Mol. Gen. Genet. 241, 177–184.
Mesa-Arango, A.C., et al., 2014. The production of reactive oxygen species is an universal action mechanism of Amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug: AMPHORES study. Antimicrob. Agents Chemother.
Morales-Menchen, A., et al., 2018. Non-canonical activities of Hog1 control sensitivity of *Candida albicans* to killer toxins from *Debaryomyces hansenii*. Front. Cell Infect. Microbiol. 8, 135.
Navarro-García, F., et al., 2005. The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*. Microbiology 151, 2737–2749.
Oura, M., et al., 1955. A new antifungal antibiotic, amphotericin B. Antibiot. Annu. 3, 566–573.
Perlroth, J., et al., 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med. Mycol. 45, 321–346.
Pfaller, M.A., Castanheira, M., 2016. Nosocomial candidiasis: antifungal stewardship and the importance of rapid diagnosis. Med. Mycol. 54, 1–22.
Pfaller, M.A., Diekema, D.J., 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 20, 133–163.
Pfaller, M.A., Diekema, D.J., 2010. Epidemiology of invasive mycoses in North America. Crit. Rev. Microbiol. 36, 1–53.
Prieto, A.D., et al., 2014. The HOG pathway is critical for the colonization of the mouse gastrointestinal tract by *Candida albicans*. PLoS ONE 9, e87128.
San José, C., et al., 1996. The mitogen-activated protein kinase homolog *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*. J. Bacteriol. 178, 5850–5852.
Sangalli-Leite, F., et al., 2011. Amphotericin B mediates killing in Cryptococcus neoformans through the induction of a strong oxidative burst. Microbes Infect. 13, 457–467.
Sokol-Anderson, M.L., et al., 1986. Amphotericin B-induced oxidative damage and killing of *Candida albicans*. J. Infect. Dis. 154, 76–83.
Urrialde, V., et al., 2015. The Pho4 transcription factor mediates the response to arsenate and arsenite in Candida albicans. Front. Microbiol. 6, 118.