Farnesol-induced cell death in the filamentous fungus \textit{Aspergillus nidulans}

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Abstract

Farnesol (farnesol), a non-sterol isoprenoid produced by dephosphorylation of farnesyl pyrophosphate, has been shown to inhibit proliferation and induce apoptosis. We have been using \textit{Aspergillus nidulans} and farnesol as a model system and cell death stimulus, respectively, aiming to understand by which means filamentous fungi are driven towards cell death. Here, we review some of our findings about farnesol-induced cell death in \textit{A. nidulans}.

Introduction

Farnesol (farnesol), a non-sterol isoprenoid produced by dephosphorylation of farnesyl pyrophosphate, has been shown to inhibit proliferation and induce apoptosis in neoplastic cell lines and also to be effective in chemoprevention and chemotherapy in several in vivo cancer models (for a review, see [1]). In fungi, farnesol plays a role as an extracellular quorum-sensing molecule in the dimorphic fungus \textit{Candida albicans} [2,3]. Farnesol inhibits the yeast mycelium dimorphic transition and induces \textit{C. albicans} to grow as actively budding yeasts [4]. In \textit{Saccharomyces cerevisiae}, farnesol blocks growth by raising the concentration of mitochondrial ROS (reactive oxygen species) [5,6]. Farnesol has no effect on the filamentous fungus \textit{Aspergillus nidulans} hyphal morphogenesis, but it triggers morphological features characteristic of apoptosis through ROS accumulation [7,8]. In mammalian cells, farnesol-mediated cell death can be reduced by the addition of phorbol esters [9,10]. Phorbol esters are non-metabolizable structural mimics of DAG (diacylglycerol) that bind to and activate proteins containing C1 domains, such as PKC (protein kinase C) [11]. There is an increase of farnesol-sensitivity when PKC inhibitors are added, suggesting a role for PKCs as the C1 domain-containing proteins that regulate farnesol-sensitivity [9,10,12]. PC (phosphatidylcholine), PA (phosphatidic acid) and DAG were able to prevent induction of apoptosis by farnesol in mammalian cells [10]. Fain et al. [12] showed the involvement of the \textit{S. cerevisiae} PKC1 pathway in farnesol-sensitivity, showing that Pkc1 relocalized to the mitochondria upon farnesol addition and inactivation of the non-essential and non-redundant member of the Pkc1 signalling pathway, BCK1, resulted in farnesol-sensitivity. Furthermore, expression of activated alleles of \textit{PKC1}, \textit{BCK1} and \textit{MKK1} [MAPK (mitogen-activated protein kinase) kinase 1] increased tolerance to farnesol and \text{H}_{2}\text{O}_{2}.

Apoptosis-like cell death occurs in filamentous fungi during aging, reproduction and exposure to antifungal compounds (for a review, see [13,14]). Although there are several fungal homologues from genes involved in apoptosis in mammalian cells, it is not clear how they work and if their apoptotic function is conserved. We have been using \textit{A. nidulans} and farnesol as a model system and cell death stimulus, respectively, aiming to understand by which means filamentous fungi are driven towards cell death [7,8,15,16]. Here, we review some of our findings about farnesol-induced cell death in \textit{A. nidulans}.

Involvement of the mitochondria

Our initial strategy to identify genes involved in cell death induced by farnesol was to perform competitive microarray hybridizations using RNA obtained from germlings of the wild-type strain that was either exposed or not exposed to farnesol [8]. Statistical analysis of the dataset identified a total of 534 genes that displayed modulation. We observed decreased mRNA abundance of several genes involved in RNA processing and modification, transcription, translation, ribosomal structure and biogenesis, amino acid transport and metabolism. Genes involved in the ergosterol biosynthesis had decreased mRNA accumulation when \textit{A. nidulans} was exposed to farnesol. We observed increased mRNA expression of genes encoding a number of mitochondrial proteins including the AIF (apoptosis-inducing factor)-like mitochondrial oxidoreductase, the mitochondrial ATPase inhibitor and the cytochrome \textit{c} peroxidase respectively. We also observed the increased expression of several genes

Key words: \textit{Aspergillus nidulans}, cell death, endoplasmic reticulum, farnesol, reactive oxygen species (ROS).

Abbreviations used: AIF, apoptosis-inducing factor; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia mutated- and Rad3-related; CHI, cell wall integrity; DAG, diacylglycerol; DnaK, endonuclease G; ER, endoplasmic reticulum; EFK, electron transport chain; FOH, farnesol; Hsp90, heat-shock protein 90; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MMS, methyl methanesulfonate; mRFP, monomeric red fluorescent protein; NF-κB, nuclear factor-κB; NOD, NOD-like receptor; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NQO, NAD(P)H quinone oxidoreductase; PC, phosphatidylcholine; PA, phosphatidic acid; PAK, p21-activated kinase; PKC, protein kinase C; PKC, protein kinase C; ROS, reactive oxygen species; UPR, unfolded protein response.

*Owing to exceptional unforeseen circumstances, this speaker was unable to give this presentation at the meeting. This paper is included in the interests of completeness of the meeting.

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The fungus is either exposed to FOH or to oxidative stress agents [8]. The nucleic acid from Delta1 mutant strain were fully condensed at 10 and 100 μM FOH, contrasting with approximately 1% and 96% in the wild-type strain respectively. A. nidulans AifA is important for decreasing the effects of oxidative stress caused by FOH and menadione. Surprisingly, when germlings were exposed to FOH, AifA::GFP (green fluorescent protein) did not translocate to the nucleus but instead remained in the cytoplasm. Our results strongly suggest that cell death caused by FOH in A. nidulans is not acting via AIF, i.e., it does not involve the translocation of AIF from the mitochondrion to the nucleus. Other authors have suggested an oxidoreductase role for AIF in mitochondria based on in vitro NADH oxidase activity. Although it is still controversial, AIF has also been proposed to act as a putative ROS scavenger [17]. AIF::knoout and Δ-knockdown cells exhibit a reduction in the protein expression level of the Complex I subunits p17, p20 and p39, coupled to a reduction in Complex I activity [18,19], and have a partial Complex III defect [18]. S. cerevisiae cells lacking AIF1p exhibit reduced growth on media containing only non-fermentable carbon sources, but since S. cerevisiae has no equivalent of Complex I, this indicates that the AIF deficiency influences the mitochondrial biogenesis [20]. Loss of AIF directly influences the mitochondrial biogenesis by increasing ROS and the addition of exogenous antioxidants lessen the Complex I deficiency caused by AIF reduction [21]. These results suggest that a Complex I deficiency increases the generation of ROS and consequently increases the susceptibility of cells to apoptotic stimuli. What is currently controversial is the role that AIF plays during this process, i.e. whether AIF determines the resistance of cells to oxidative stress because of its antioxidant properties or indirectly by its effects on the respiratory chain [20].

Complex I–Complex IV form a functional ETC (electron transport chain), referred as the cytochrome ETC. In contrast, internal and external NADH dehydrogenases as well as alternative oxidases can form a functional ETC, referred to as the alternative ETC [22]. The importance of these pathways in terms of the physiology of the cell is not very well understood. It has been suggested that these pathways are involved in the prevention of ROS formation [23,24]. However, some other authors have shown that they may increase ROS production and cause cell death [25,26]. The fungi have extensive redundancy within their respiratory chains and the presence of these non-proton-pumping alternative NADH dehydrogenases contrasts between different fungi [27]. S. cerevisiae lacks Complex I and has three NADH dehydrogenases: NDE1, NDE2 (both external) and NDI (internal) [27,28]. However, their specific function is not clear. During normal respiration in animals, superoxide anion is generated and Complex I is strongly implicated in this generation, due to the low redox potential required for one electron reduction of dioxygen to superoxide [27]. It is generally believed that the presence of internal and/or external alternative dehydrogenases should enable NADH oxidation and decrease the production of ROS. We have previously shown that when A. nidulans was exposed to FOH, a gene (ndeA) encoding an NADH-ubiquinone oxidoreductase had increased mRNA expression [8,16]. We characterized in more detail the function of this gene and two other mitochondrial genes: another external and an internal NADH-ubiquinone oxidoreductase (ndeB and ndiA) respectively [16]. All three genes have increased mRNA accumulation in the presence of FOH and other oxidative stressing conditions [16]. The ΔndeA and ΔndeB deletion mutant strains showed lower viability than the wild-type when exposed to 10 μM FOH (approximately 8% and 65%) in the ΔndeA and ΔndeB mutant strains compared with 92% in the wild-type strain, [16]. Surprisingly, the double mutant displays 60% viability when exposed to 10 μM FOH. These results suggest that there are other mitochondrial pathways (for instance, the internal NADH alternative dehydrogenase and/or the alternative oxidase) that could compensate for the deletion of the external NADH alternative dehydrogenases. In fact, there is an increase of 34% in the alternative oxidase activity in the double mutant ΔndeA ΔndeB when compared with the wild-type strain [16]. Interestingly, the alternative oxidase gene (aoxA) also has increased mRNA accumulation upon FOH incubation [16]. The ΔndiA deletion strain showed approximately 80% viability when exposed to 10 μM FOH (compared with 92% in the wild-type). Again, as observed previously, this increased survival could be due to increased compensatory pathways that will help the cells to survive in the presence of FOH, such as the increased external NADH dehydrogenase activity shown above for the ΔndiA mutant strain.

Our results suggest that A. nidulans has two external and one internal alternative dehydrogenases and they (and probably the alternative oxidase too) could play an important role in response to the great amount of ROS produced during FOH-induced cell death. These results emphasize the importance of alternative respiratory pathways for A. nidulans when the mitochondrial environment is extensively damaged by ROS. In summary, our study shows a cooperative involvement of alternative respiratory pathways and AifA in FOH-induced cell death.

Besides producing most of the cellular energy in eukaryotes, mitochondria have also been involved in PCD, through the generation of ROS and the release of mitochondrial proteins [29]. One of the steps in apoptosis is the fragmentation of mitochondria [30]. When germlings were exposed to 100 μM FOH, they showed a mitochondrial network fragmentation process as observed by an intense mitotracker punctuated fluorescent distribution in the cytoplasm of the germlings [8]. In S. cerevisiae cells undergoing
an apoptotic process induced by acetic acid, translocation of Cyc1p (encoding cytochrome c) to the cytosol was observed [31]. Another factor of the intrinsic pathway is the EndoG (endonuclease G) that has been described as a mitochondrial endonuclease that digests both DNA and RNA [32,33]. Upon apoptosis induction, translocation of mammalian EndoG and its S. cerevisiae homologue NUC1 to the nucleus coincides with large-scale DNA fragmentation [33–35]. Recently, we described a homologue of EndoG in the filamentous fungi by investigating if the A. nidulans EndoG homologue, named nucENDOG, is being activated during FOH-induced cell death [36]. There is a low nucA mRNA accumulation when A. nidulans germlings were exposed to oxidative stressing agents, such as H2O2 and paraquat and DNA-damaging agents [hydroxyurea, MMS (methyl methanesulfonate) and bleomycin]. The nucA deletion strain has no apparent growth or conidiation defects and did not show increased susceptibility to FOH and we were not able to observe any other phenotype of susceptibility to several agents that cause cell death [36]. However, the nucA deletion mutant is more sensitive to 4-NQO (4-nitroquinoline oxide), and its quiescent and germinating conidia are slightly more sensitive to UV light. ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia mutated- and Rad3-related) are paralogous PIKKs (phosphoinositide 3-kinase-related kinases) that orchestrate the DNA damage response in eukaryotic cells [37]. The double ΔnucA ΔatmAATM was shown to be more sensitive to MMS and 4-NQO, whereas the double mutant ΔnucA and ΔatmAATM mutations are more sensitive to MMS, 4-NQO and menadione. NucA::mRFP (monomeric red fluorescent protein) accumulation was detected along the cytoplasm but not in the nuclei when NucA::mRFP germlings were exposed or not to FOH. The same results were observed when NucA::mRFP was exposed to different concentrations of 4-NQO. Cell fractionation studies showed that NucA::mRFP localizes to the mitochondria when NucA::mRFP germlings were exposed or not to FOH [36]. These results indicate that NucA is genetically interacting with these two genes aiming to repair DNA damage caused by these genotoxins. Taken together, all these results suggest that NucA does not seem to be involved in cell death but it plays a role in the DNA damaging response in A. nidulans.

Western-blot analysis showed that A. nidulans CycA is not translocated to the cytoplasm upon exposure to FOH [36]. Our results showed that CycA::mRFP is predominantly located to the mitochondria, even when CycA::mRFP germlings were exposed to FOH. Interestingly, during A. nidulans FOH-induced cell death not only is cytochrome c not released to the cytoplasm but also caspases are activated [18]. This could reflect a particular feature of the FOH-induced cell death or a more general observation related to cell death in filamentous fungi linked to the fact that regulation and expression of these genes involved in apoptosis could have undergone a rewiring process in mammals during their evolution. However, in S. cerevisiae cells undergoing an apoptotic process induced by acetic acid, translocation of CytC to the cytosol and ROS production were observed [31]. The behaviour of A. nidulans CycA during different cell-death stimuli remains to be investigated.

**Engagement of the CWI (cell wall integrity) pathway and the unfolded protein response**

FOH-induced apoptosis in human lung carcinoma cells analysed by microarray analysis revealed that many genes implicated in ER (endoplasmic reticulum) stress were highly up-regulated, suggesting that FOH-induced apoptosis in these cells is coupled to the activation of an ER stress response pathway and the UPR (unfolded protein response) [1]. The precise mechanism by which FOH induces ER stress has not been elucidated yet [1]. Increased levels of PkaC expression enhance the A. nidulans FOH-induced cell death and the influence of PkaC on cell death seems to be specifically related to FOH [15]. The overexpression of PkaC increases the susceptibility of the alcA::pkaC mutant strain to agents that disrupt ER homeostasis, such as 2-DG (2-deoxy-D-glucose) and DTT (dithiothreitol). FOH led to induction of the A. nidulans UPR as judged by the enhancement of the mRNA accumulation of genes involved in ER stress and the UPR, such as bipA, pdiA, pdiB, the transcription factor Hsf1 and the chaperones Hsp90 (heat-shock protein 90) and Hsp104. In addition, FOH increases the differential splicing of the haca transcription factor, a central event for the UPR activation, and this is also modulated by PkaC [15].

Two-component systems (histidine-to-aspartate phosphorelay) are signal transduction mechanisms involved in several stimuli, including oxidative stress responses [38,39]. A. nidulans has a histidine kinase (NikA) and two response regulators (SskA and SrrA) implicated in oxidative stress responses [40,41]. In A. nidulans, the HogA/MAPK cascade plays important roles downstream of the NikA–SskA phosphorelay [42–44]. In this species, it is known that NapA and AtfA are transcription factors activated by the MAPK cascade that are involved in stress responsive transcriptional regulation to oxidative stress [45,46]. There are four MKK in A. nidulans: MpkA–C and SakA/HogA (for a review, see [47]). Our work suggests that SsrA, SskA, AtfA and MKKs are involved in the signal transduction in response to the effects caused by FOH [15].

In S. cerevisiae, PKCl is involved in the regulation of cell construction via the reactivation of MPK1 and activation of the transcription factor Rlm1p, which regulates the expression of genes whose products are important in cell wall biosynthesis [48]. Fujioka et al. [49] demonstrated that A. nidulans rlmA and mpkA are functional orthologues of S. cerevisiae RLM1 and MPK1. Considering that the ΔmpkA mutant is more resistant to FOH [15], we speculated whether FOH activates the CWI pathway. The agsA (encoding one of the A. nidulans α-1,3-glucan synthases), rlmA and mpkA genes showed increased mRNA accumulation upon
A. nidulans exposure to different FOH concentrations, and their mRNA accumulation is modulated by PkcA. Increasing FOH concentrations also induced A. nidulans MpkA phosphorylation, a hallmark of the CWI pathway. FOH exposure has an additive lethal effect which strongly indicates that there is a connection between PkaC, CWI and UPR in A. nidulans. It is well known that FOH increases ROS production in A. nidulans [7,8], and pcka mRNA accumulation is increased in the presence of oxidative stress [15]. Thus it is possible that the connection between PkaC and UPR could be mediated through ROS formation upon FOH exposure, by not only activating PkaC activity but also the UPR. The environment of the ER is oxidizing, supporting the formation of intra- and inter-chain disulfide bonds that serve to stabilize the folding and assembly of nascent proteins [50]. Each disulfide bond that forms during oxidative folding should produce a single ROS [50]. Increased ROS production through FOH will create an intense oxidized environment in the ER with increased production of misfolded proteins, activating the UPR.

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References


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