Oxidative and amphotericin B-mediated cell death in the opportunistic pathogen *Aspergillus fumigatus* is associated with an apoptotic-like phenotype

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When protoplasts of the opportunistic fungal pathogen *Aspergillus fumigatus* were treated with low but toxic levels of hydrogen peroxide (0–1 mM) or amphotericin B (0–5 μg ml⁻¹), loss of cell viability and death were associated with a number of phenotypic changes characteristic of apoptosis. The percentage of protoplasts staining positive with annexin V-FITC, an indicator of the externalization of phosphatidylserine and an early marker of apoptosis, rose to ~55% within 1 h. This was followed by a similar increase in apoptotic DNA fragmentation detected by the TUNEL assay, and led to a loss of cell permeability and death in ~90% of protoplasts, as indicated by the uptake of propidium iodide. The development of an apoptotic phenotype was blocked when protoplasts were pre-treated with the protein synthesis inhibitor cycloheximide, indicating active participation of the cell in the process. However, no significant activity against synthetic caspase substrates was detected, and the inclusion of the cell-permeant broad-spectrum caspase inhibitor Z-VAD-fmk did not block the development of the apoptotic-like phenotype. Higher concentrations of H₂O₂ (1–8 mM) and amphotericin B (1 μg ml⁻¹) caused protoplasts to die without inducing an apoptotic phenotype. As predicted, the fungistatic antifungal agent itraconazole, which inhibits growth without causing immediate cell death, did not induce an apoptotic-like phenotype.

INTRODUCTION

The incidence of life-threatening invasive aspergillosis in immunocompromised hosts has increased dramatically over the last two decades (Groll et al., 1996; Vogeser et al., 1997; Latgé, 1999; Denning et al., 2002). *Aspergillus fumigatus* is the most common aetiologic agent of invasive aspergillosis, being responsible for approximately 90% of human infections (Derouin, 1994). The incidence of invasive aspergillosis varies between 1 and 19% for patients who have undergone solid organ transplantation (Patel & Paya, 1997; Verweij & Denning, 1997), and patients with leukaemia, AIDS and granulomatous disease are also at risk (Brown et al., 1998; Denning, 1998; Kaizer et al., 1998). In contrast, the disease is rarely found in immunocompetent hosts (Karim et al., 1997). *A. fumigatus* is a common, widespread saprophytic fungus, and environmental surveys indicate that humans inhale several hundred *A. fumigatus* conidia per day (Hospenthal et al., 1998). Ingestion and killing of spores by alveolar macrophages represent the first line of defence against infection, mainly by non-oxidative mechanisms, whilst neutrophils employ an oxidative respiratory burst to kill germinating conidia that have escaped macrophage engulfment (Schaffner et al., 1986; Levitz et al., 1986; Morgenstern et al., 1997; Roilides et al., 1998; Latgé, 2001). Invasive aspergillosis has a high mortality and morbidity rate, with only 34% of patients showing a favourable response to antifungal therapy (Denning, 1996). The fungicidal agent amphotericin B is widely used to treat invasive aspergillosis, although it can have serious side-effects (Clements & Peacock, 1990; Pathak et al., 1998).

Previously, we reported that the viability of *A. fumigatus* decreases rapidly when the organism enters stationary phase in liquid culture, and that this is associated with the appearance of an apoptotic-like phenotype (Mousavi & Robson, 2003). Although programmed cell death and the underlying mechanisms are well documented in mammalian cells (Strasser et al., 2000; Hengartner, 2001), there have been few studies on the mechanism of cell death in fungi, and in filamentous fungi in particular (Umar & Van Griensven, 1997; Raju & Perkins, 2000; Lu et al., 2003; Cheng et al., 2003). In the

Abbreviations: PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.
yeast *Saccharomyces cerevisiae*, expression of the mamma-
lian pro-apoptotic protein Bax induces an apoptotic-like
phenotype, which is suppressed by simultaneous over-
expression of Bcl-XL, a member of the mammalian anti-
apoptotic Bcl-2 family (Liggr et al., 1998). Moreover, death in *S. cerevisiae* during the starvation phase, and following
 treatment with hydrogen peroxide, has also been shown to
be associated with an apoptotic-like phenotype and to
involve a metacaspase, a protease related to the mamma-
lian caspase family (Madeo et al., 2002). Treatment of *S.
cerevisiae* with toxic levels of acetic acid is also associated
with an apoptotic-like phenotype (Ludovico et al., 2001),
and was subsequently shown to involve the mitochondrion
and cytochrome c release (Ludovico et al., 2002), in a similar
manner to that seen in mammalian cells (Hengartner,
2000). Recently, Cheng et al. (2003) reported that the
antifungal sphingoid long-chain base phytosphingosine
induces an apoptotic phenotype in *Aspergillus nidulans* in
a pathway that did not appear to involve metacaspase
activity (Cheng et al., 2003).

In this study, we report that cell death in *A. fumigatus*,
induced either by hydrogen peroxide (oxidative death) or
by treatment with the widely used antifungal agent amphi-
terin B, is associated with the induction of an apoptotic-
like phenotype, suggesting that these agents cause death in
*A. fumigatus* by inducing a primitive form of apoptosis.
Moreover, unlike entry into the stationary phase, the
development of the apoptotic-like phenotype induced by
these two fungicidal agents was not blocked by the broad-
spectrum caspase inhibitor Z-VAD-fmk, suggesting the
presence of two apoptotic-like pathways in *A. fumigatus*.

**METHODS**

**Organism and growth conditions.** *A. fumigatus* AF10, a clinical
isolate (ATCC 90240), was stored in 20% (v/v) glycerol at −80°C.
Cultures were grown on modified Vogel’s medium (Vogel, 1956),
with 1% (v/v) glucose replacing 2% (w/v) sucrose. For liquid
cultures, *A. fumigatus* was grown in 50 ml modified Vogel’s medium
supplemented with 50 mM MES (pH 5.5) and 0.15% (w/v) of the
polyacrylate polymer Junylon PW 110 (Honeywell and Stein, UK) to
induce filamentous growth (Trinci, 1983), in 250 ml conical flasks,
and inoculated with 1 ml conidial suspension (≈1 × 10⁸ conidia ml⁻¹). Flasks were incubated with shaking (250 r.p.m.) at 37°C,
and growth of the cultures was followed by measuring optical
density (540–560 nm) in an EEL colorimeter (Trinci, 1972). Viability
was measured by determining c.f.u. ml⁻¹ on modified Vogel’s
medium solidified with 1.5% (w/v) agar. Spore suspensions were
prepared from 5-day-old cultures grown on Sabouraud’s glucose
agar in 250 ml tissue culture flasks at 37°C, harvested by gentle
agitation with 0.01% (v/v) Tween 80 and filtered through two
layers of lens tissue.

**Effect of H₂O₂, amphotericin B and itraconazole on mycelial
growth.** To study the effect of H₂O₂, amphotericin B and itracona-
zole on the growth and viability of *A. fumigatus*, various concentra-
tions of H₂O₂ (from 30%, v/v, stock solution), amphotericin B
(from 1 mg ml⁻¹ stock) or itraconazole (from 1 mg ml⁻¹ stock)
were added to early exponential phase cultures (OD ≤ 1-5 EEL units),
and viability monitored by determining c.f.u. ml⁻¹ at 30 min inter-
vals. For H₂O₂-treated mycelia, 0.25% (w/v) catalase (Sigma) was
added after sampling to remove H₂O₂ from the sample, whilst for
amphotericin B or itraconazole treatments, mycelia were washed
brieﬂy in sterile deionized water. To determine if DNA degradation
had occurred, mycelium was frozen in liquid nitrogen, ground to a
fine powder in a mortar and pestle, and genomic DNA extracted
according to Reader & Brody (1985). DNA was run on a 1.5% (w/v)
agarose gel in TPE buffer (0.09 M Tris/phosphate, pH 8.0, 2
mM EDTA) and visualized following ethidium bromide staining
(0–4 µg ml⁻¹ in TPE buffer).

**Analysis of apoptotic markers.** Terminal deoxynucleotidyl
transferase-mediated dUTP nick end labelling (TUNEL; TdT-FragEL
DNA fragmentation detection kit, Oncogene Research Products)
and annexin V-FITC (Oncogene Research Products) were used as
markers of apoptosis, and uptake of propidium iodide (PI) was
used as a marker of cell-membrane integrity, as previously described
(Mousavi & Robson, 2003). In order to detect the expression of
these apoptotic markers, the cell wall was first removed by digesting
mycelium from mid-exponential-phase growth with Novozyme, as
previously described (Mousavi & Robson, 2003), and protoplasts
were then treated with various concentrations of H₂O₂, amphotericin B
or itraconazole for up to 6 h. Following treatment, protoplasts
were washed twice by centrifugation (1500 g) for 10 min and resus-
pended in an equal volume of regeneration buffer (0–1 M phosphate
buffer, pH 7.0–7.9 M sorbitol). In the case of H₂O₂-treated proto-
plasts, 0.25% (w/v) catalase was added prior to washing. To deter-
mine protoplast viability, protoplasts were regenerated by spreading
gently over the surface of modified Vogel’s medium solidified with
1.5% (w/v) agar (supplemented with 0.9–9 M sorbitol), and plates
incubated at 37°C until colonies became visible. To inhibit meta-
caspase activity or protein synthesis, respectively, 25 µg ml⁻¹ of the
broad-spectrum caspase inhibitor Z-VAD-fmk (Calbiochem) or
50 µg ml⁻¹ cycloheximide (Sigma) was added to the protoplast
suspending buffer, 1 h prior to treatment with H₂O₂ or amphotericin B.
DAPI staining was used to determine the percentage of protoplasts
containing nuclei, as previously described (Mousavi & Robson,
2003).

**Caspase activity.** Intracellular caspase activity was determined
using a colorimetric assay based on the cleavage of a p-nitroaniline
dye from the C-terminus of specific peptide substrates (Caspase
Colorimetric Substrate/Inhibitor Quantipak, Calbiochem). Mycelium
was ground in liquid nitrogen and the biomass resuspended in ice-
cold lysis buffer (50 mM HEPES, pH 7.4, 1 mM DTT, 0.5 mM
EDTA and 0.1% (v/v) CHAPS), centrifuged at 1500 g for 10 min,
and the caspase activity of the supernatant against substrates for
caspase-1, -3 and -8 determined according to the manufacturer’s
instructions. Protein concentration was determined according to

**RESULTS**

**Growth of *A. fumigatus* treated with H₂O₂ and
amphotericin B**

Exponentially growing *A. fumigatus* cultures were treated with
various concentrations of H₂O₂ (0–3.6 mM; Fig. 1) and
amphotericin B (0–3 µg ml⁻¹; Fig. 2) 16 h after ino-
culation. Growth was monitored by measuring the optical
density of the cultures (Figs 1a and 2a), and viability
determined by measuring c.f.u. ml⁻¹ (Fig. 2a, b). Growth was not
affected by the addition of 0.1 mM H₂O₂, whereas 1.2 and
1.8 mM H₂O₂ caused a cessation of growth for 5 h, after
which growth resumed. When cultures were treated with
concentrations of 2.4 mM H₂O₂ and above, growth ceased

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immediately and did not recover. As shown in Fig. 1(b),
the number of colonies was not affected by concentrations
up to 0.1 mM H₂O₂, whereas 1.2 and 1.8 mM H₂O₂
initially caused a drop in cell viability for the first 4 h, from
which there was subsequent recovery. Treatment for 2 h
with 3.0 mM H₂O₂ and above was lethal. Cultures treated
at amphotericin B concentrations less than 1 mM
had no effect on growth, whereas addition of 2 µg ml⁻¹ or
more inhibited growth within 1 h (Fig. 2a). Fig. 2(b)
shows that exposure to low concentrations of amphotericin B (0.5 or 1 µg ml⁻¹) had no effect on cell viability,
whereas cell viability was abolished after treating with 2 µg
amphotericin B ml⁻¹.

In many mammalian cells, apoptosis is often associated
with the action of specific endonucleases that attack nuclear DNA in the internucleosomal linker regions,
resulting in double-stranded, low-molecular-mass oligo-
nucleosomal DNA fragments in multiples of about 180 to
200 bp, which are visible after electrophoresis as a DNA
‘ladder’ (De Mario et al., 1997). Fig. 3(a) shows that incubation of mycelium with 3 mM H₂O₂ did not cause
DNA fragmentation after 2 h; however, exposure for 4 h
and above resulted in complete loss of intact DNA and the
appearance of fragmented DNA as a smear (Fig. 3a). A
DNA ladder was not visible. Exposure of mycelium to 3 µg
amphotericin B ml⁻¹ for up to 4 h had no visible effect on
DNA integrity; however, complete loss of intact DNA
occurred after 8 h exposure, and fragmented DNA was
visible as a smear. As with H₂O₂ treatment, a DNA ladder
was not visible (Fig. 3b).

**Effect of H₂O₂ and amphotericin B on protoplast viability**

The effect of various concentrations of H₂O₂ and amphotericin B on protoplast viability is shown in Figs 4(a) and
4(b), respectively. Protoplast viability was unaffected by
0.01 mM H₂O₂, whereas, following the addition of 0.1 mM
H₂O₂, less than 50% of viable protoplasts survived after
1 h, and less than 20% after 2 h exposure. No protoplasts
were viable 2 h after treatment with 1.8 mM H₂O₂ (Fig. 4a).
When exposed to 0.25 µg amphotericin B ml⁻¹, more than
70% of protoplasts retained viability after 2 h, whereas less
than 20% of protoplasts were viable 1 h after the addition of 0.5 mg ml$^{-1}$. No protoplasts remained viable 2 h after the addition of 1 or 2 mg amphotericin B ml$^{-1}$ (Fig. 4b and data not shown).

**H$_2$O$_2$- and amphotericin B-induced death of A. fumigatus is associated with an apoptotic phenotype**

To assess whether H$_2$O$_2$- or amphotericin B-induced cell death in A. fumigatus displayed characteristics associated with apoptosis, we determined the percentage of protoplasts (containing nuclei) that stained positive with annexin V-FITC, TUNEL and PI, following addition of H$_2$O$_2$ and amphotericin B (Fig. 5a, b, respectively). Annexin V binds specifically to phosphatidylserine (PS), and is widely used to detect the exposure of PS on the outside of the plasma membrane, which occurs early in the apoptotic process (Champagne et al., 1999; Martinet et al., 1999). Protoplasts prior to treatment showed a low level of staining (<7%) with annexin V-FITC, TUNEL and PI, following addition of H$_2$O$_2$ and amphotericin B (Fig. 5a, b, respectively). Annexin V binds specifically to phosphatidylserine (PS), and is widely used to detect the exposure of PS on the outside of the plasma membrane, which occurs early in the apoptotic process (Champagne et al., 1999; Martinet et al., 1999). Protoplasts prior to treatment showed a low level of staining (<7%) with annexin V-FITC, TUNEL and PI, following addition of H$_2$O$_2$ and amphotericin B (Fig. 5a, b, respectively).

H$_2$O$_2$ at 0.1 mM induced apoptotic-like nuclear degradation in ~55% of protoplasts (TUNEL-positive), but at the higher concentration of 1.8 mM, this number fell to ~20%. Treatment with 0.1 mM H$_2$O$_2$ increased PI staining to ~17%; however, 1.8 mM led to ~80% of the protoplasts staining positive with PI.

A similar pattern was observed when protoplasts were treated with amphotericin B (Fig. 5b). When treated with 0.25 mg ml$^{-1}$ amphotericin B, ~15% stained positive with annexin V-FITC. This rose to ~50% when treated with 0.5 or 1 mg amphotericin B ml$^{-1}$. When treated with 0.5 mg amphotericin B ml$^{-1}$, the proportion of TUNEL-positive protoplasts increased to ~65%; however, the level of staining was less than 20% when treated with 1 mg ml$^{-1}$. The proportion of PI-positive protoplasts was less than 20% when treated with 0.25 or 0.5 mg amphotericin B ml$^{-1}$, but rose to ~85% when treated with 1 mg amphotericin B ml$^{-1}$.

To test if the apoptotic-like phenotype was induced by agents that inhibited growth, without being fungicidal, we used the fungistatic azole itraconazole, which in the short term inhibits growth, without killing the cell (Lamb et al., 1999). Treatment of protoplasts with 10 μg itraconazole ml$^{-1}$, a concentration that inhibited protoplast regeneration without causing death, did not cause a significant...
increase in annexin V-FITC, TUNEL or PI-positive staining (data not shown).

Influence of \( \text{H}_2\text{O}_2 \) on the appearance of apoptotic markers over time

To study the development of the apoptotic phenotype over time, we treated protoplasts with 0·1 mM \( \text{H}_2\text{O}_2 \) and monitored the proportion of protoplasts staining positive for annexin V-FITC, TUNEL and PI over 6 h (Fig. 6). Initially, prior to treatment, the proportion of protoplasts staining positive for any of the markers was <10%. Within 1 h of treatment with 0·1 mM \( \text{H}_2\text{O}_2 \), the proportion of protoplasts staining positive with annexin V-FITC rose to ~45%, and remained approximately constant thereafter. The proportion of TUNEL-positive protoplasts increased slightly to ~15% after 1 h exposure to \( \text{H}_2\text{O}_2 \), but had risen to 50% after 2 h and continued to increase to a maximum of ~65% after 4 h, before decreasing to ~40% after 6 h. PI-positive protoplasts increased slightly to ~10% after 1 h, increased to ~25% after 2 h, and thereafter rose steadily to reach a maximum of ~90% after 5 h.

Blocking protein synthesis prevents the development of an apoptotic-like phenotype

Programmed cell death and the development of an apoptotic phenotype is dependent on the active participation of the cell and the synthesis of novel proteins. To determine whether the development of the apoptotic-like phenotype, following treatment with \( \text{H}_2\text{O}_2 \) or amphotericin B, was dependent on novel protein synthesis, protoplasts were treated with the protein synthesis inhibitor cycloheximide for 1 h, prior to the addition of \( \text{H}_2\text{O}_2 \) or amphotericin B.
Pre-incubation with cycloheximide completely blocked the increase in TUNEL-positive protoplasts following treatment with either H2O2 or amphotericin B, but had no significant effect on the proportion of PI-positive protoplasts induced by 1-8 mM H2O2 or 1 μg amphotericin B ml⁻¹.

**Caspase activity**

To determine whether caspases contribute to cell death, following treatment with H2O2 or amphotericin B, a colorimetric assay based on the cleavage of p-nitroaniline dye from the C-terminal of specific peptide substrates was performed (Thornberry, 1998). Total protein was extracted from the mycelium at various time points up to 6 h, following the addition of 3 mM H2O2 or 3 μg amphotericin B ml⁻¹ to the culture, and assayed for activity against substrates specific for caspases-1, -3 and -8. No significant increase in activity against the caspase substrates was observed (data not shown). Moreover, addition of the broad-spectrum caspase inhibitor Z-VAD-fmk to protoplasts, prior to treatment with 0-1 mM H2O2 or 0-5 μg amphotericin B ml⁻¹, did not block the subsequent increase in the percentage of TUNEL-positive or PI-positive protoplasts (data not shown).

**DISCUSSION**

Apoptosis is controlled by a complex regulatory network, which can be activated by external signals (e.g. reactive oxygen species, ROS; Madeo et al., 1999; Bustamante et al., 2000) and internal processes (e.g. replication failures or developmental programmed cell death; Okuno et al., 1998).

During the course of this work, H2O2 was used to generate oxidative stress to mimic killing of cells by the respiratory burst, whilst cell death caused by amphotericin B was investigated, as it is still the most widely prescribed antifungal agent for the treatment of aspergillosis, despite its nephrotoxicity (Wingard et al., 1999; Patterson et al., 2000). Analysis of chromosomal DNA from H2O2- or amphotericin B-treated mycelia by agarose electrophoresis showed smearing of the DNA. However, evidence of a DNA ladder pattern, which is found in many apoptotic systems as the result of internucleosomal DNA cleavage (Hofmann et al., 1999; Bustamante et al., 2000), was not found. DNA laddering was also absent during cell death in the stationary
phase in *A. fumigatus* (Mousavi & Robson, 2003), and was also absent during cell death in *S. cerevisiae* and in *Pichia pastoris* (Martinet et al., 1999; Madeo et al., 1999). Moreover, DNA smearing was also reported as a late response in *A. nidulans*, following treatment with phytosphingosine (Cheng et al., 2003). Apoptotic cell death in certain mammalian cells has also been shown to occur in the absence of a DNA ladder (Oberhammer et al., 1993; Knapp et al., 1999).

A more sensitive test of apoptotic DNA fragmentation is based on labelling the free 3'-OH termini, which are exposed during apoptotic DNA cleavage. The TUNEL assay, which relies on the incorporation of biotinylated or fluorescein-labelled dUTP, catalysed by terminal deoxynucleotidyl transferase (TdT), enables DNA breakage to be visualized in individual cells undergoing apoptosis and has become one of the most widely used indicators of apoptosis (Gavrieli et al., 1992; Frohlich & Madeo, 2000). Previously, we demonstrated that cell death during the stationary phase in *A. fumigatus* was associated with a marked increase in the proportion of TUNEL-positive nuclei indicating apoptotic-like cleavage of the DNA (Mousavi & Robson, 2003), and TUNEL staining was also reported during cell death in *A. nidulans* treated with phytosphingosine (Cheng et al., 2003). In this study, lower concentrations of H2O2 and amphotericin B (0.1 mM and 0.5 μg ml⁻¹, respectively) increased the proportion of TUNEL-positive protoplasts indicating apoptotic-like DNA fragmentation, whereas higher concentrations (1.8 mM and 1 μg ml⁻¹, respectively) caused a lower increase in TUNEL-positive protoplasts (Fig. 5). This decrease in TUNEL-staining at higher concentrations correlates with a large increase in PI-staining, indicating loss of membrane permeability and necrotic death. The induction of necrosis by high concentrations of numerous cytotoxic substances, and apoptosis at lower concentrations, is a well known phenomenon (Lieberthal & Levine, 1996), and has also been reported in *S. cerevisiae* treated with H2O2 and acetic acid (Madeo et al., 1999; Ludovico et al., 2001).

In mammalian cells, an earlier indicator of apoptosis is the translocation of PS from the inner to the outer leaflet of the cytoplasmic membrane (Champagne et al., 1999), and can be detected with FITC-labelled annexin V, which specifically binds to PS (Martinet et al., 1999). As with TUNEL staining, treatment with low concentrations of H2O2 or amphotericin B led to a large increase in annexin V-FITC staining. Moreover, this increase occurred prior to the increase in TUNEL staining (Fig. 6), and this early increase in PS translocation has also been reported in *A. nidulans* (Cheng et al., 2003).

Apoptosis requires the active participation of the cell in the synthesis of new proteins that contribute to cell death. Consequently, protein synthesis inhibitors can actively block apoptosis in cells, and this is widely used to demonstrate the participation of the cell during death (Hiraoka et al., 1997; Sanchez et al., 1997). In *S. cerevisiae*, cycloheximide has been shown to block the development of an apoptotic phenotype in response to various stimuli (Madeo et al., 1999), to block TUNEL staining in *A. fumigatus* following entry into the stationary phase, and to block TUNEL staining in *A. nidulans* following treatment with phytosphingosine (Cheng et al., 2003; Mousavi & Robson, 2003). When added prior to treatment with low concentrations of H2O2 or amphotericin B, cycloheximide blocked the development of a TUNEL-positive phenotype, whilst having no effect on the later increase in PI-positive staining that indicates loss of membrane integrity and cell death (Fig. 7). Thus, the development of an apoptotic-like phenotype requires protein synthesis and active participation of the cell. However, although entry into an apoptotic pathway can be prevented by blocking protein synthesis, subsequent cell death through a necrotic process is not prevented, as reported previously when *A. fumigatus* enters stationary phase (Mousavi & Robson, 2003).

A number of studies in yeast and mammalian cells have demonstrated that accumulation of ROS within the cytoplasm plays a central role in apoptotic-like cell death (Greenlund et al., 1995; Slater et al., 1995; Ligr et al., 1998; Madeo et al., 1999). It is possible that treatment with low concentrations of H2O2 or amphotericin B may trigger an apoptotic-like phenotype through the accumulation of ROS, and that continued accumulation in the cytoplasm ultimately causes physical damage and loss of cell integrity, as indicated by the increase in PI-positive staining. Treatment of protoplasts with low but toxic concentrations of the strong oxidizing agent sodium hypochlorite also induced an apoptotic phenotype similar to that observed with H2O2, suggesting that this is a general response to oxidative stress (results not shown). Previously, we reported an increase in intracellular activity toward caspase substrates as cultures entered the stationary phase. Moreover, the cell-permeant broad-spectrum caspase inhibitor z-VAD-fmk was able to block the increase in TUNEL and annexin V-FITC staining, suggesting a role for an upstream caspase-like activity in nuclear degradation and PS externalization (Mousavi & Robson, 2003). In *S. cerevisiae*, a metacaspase with caspase-like activity, YCA1, has been cloned and shown to mediate apoptosis induced by H2O2 and in chronologically aged cells (Madeo et al., 2002). However, in this study, no significant activity against caspase substrates was induced following H2O2 or amphotericin treatment. Moreover, inclusion of z-VAD-fmk prior to treatment did not block the development of an apoptotic-like phenotype, suggesting an alternative pathway to that involved in stationary-phase-induced cell death. Caspase-independent death has been described in other systems under specific conditions (De Mario et al., 1997; Brunet et al., 1998; Kroemer et al., 1998; Okuno et al., 1998), and the caspase inhibitor z-VAD-fmk found not to block the development of an apoptotic-like phenotype (Villa et al., 1997). In *A. nidulans*, a metacaspase homologue, casA, has been identified, and a ΔcasA disrupted strain shown still to undergo apoptosis in response to
phytosphingosine, suggesting death by a caspase-independent pathway (Cheng et al., 2003). As previously reported, A. fumigatus appears to contain two metacaspase homologues (Mousavi & Robson, 2003), both with a high level of identity to A. nidulans casA. Interrogation of the A. nidulans genome sequence at the Whitehead Institute also revealed a second metacaspase homologue in A. nidulans (data not shown). It is possible, therefore, that one metacaspase homologue is activated through starvation, upon entry into the stationary phase, and is active against caspase substrates, whereas the second may be activated by H$_2$O$_2$ and amphotericin B, but is inactive against the synthetic substrates tested in this study.

In this study, we have demonstrated that cell death induced by low but toxic concentrations of H$_2$O$_2$, or amphotericin B triggers the development of a protein-synthesis-dependent apoptotic-like phenotype. Thus, cell death in A. fumigatus in infected humans, as a consequence of phagocyte action or of treatment with the antifungal agent amphotericin B, may actively involve the participation of the fungal cells in their own death, through triggering an apoptotic-like pathway. Further work on the role of ROS, metacaspase and the mitochondrion will be needed to understand better the mechanisms underlying the apoptotic-like pathway(s) in A. fumigatus.

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