Formic acid induces Yca1p-independent apoptosis-like cell death in the yeast Saccharomyces cerevisiae

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Abstract
Formic acid disrupts mitochondrial electron transport and sequentially causes cell death in mammalian ocular cells by an unidentified molecular mechanism. Here, we show that a low concentration of formic acid induces apoptosis-like cell death in the budding yeast Saccharomyces cerevisiae, with several morphological and biochemical changes that are typical of apoptosis, including chromatin condensation, DNA fragmentation, externalization of phosphatidylserine, reactive oxygen species (ROS) production, loss of mitochondrial membrane potential and mitochondrial destruction. This process may not be dependent on the activation of Yca1p, the yeast caspase counterpart. In addition, the cell death induced by formic acid is associated with ROS burst, while intracellular ROS accumulate more rapidly and to a higher level in the YCA1 disruptant than in the wild-type strain during the progression of cell death. Our data indicate that formic acid induces yeast apoptosis via an Yca1p-independent pathway and it could be used as an extrinsic inducer for identifying the regulators downstream of ROS production in yeast.

Introduction
Apoptosis is an active cell death process that plays an essential role in maintenance of cellular homeostasis by removal of mutated, damaged or dispensable cells in multicellular eukaryotic organisms (Kerr et al., 1972; Ellis et al., 1991). In the past decades, apoptosis in multicellular eukaryotes has been extensively investigated and its mechanism is well documented (Nijhawan et al., 2000). Results of past studies have provided evidence that apoptosis occurs in unicellular organisms as well metazoas (Raff, 1998).

Because the finding of typical apoptotic cell death phenotypes in a CDC48 mutant in 1997 (Madeo et al., 1997), the budding yeast Saccharomyces cerevisiae has emerged as a genetically tractable model system to study apoptotic programmed cell death (PCD) (Madeo et al., 2004). Many orthologues of mammalian apoptosis-related proteins have been identified and characterized in yeast (Madeo et al., 2002; Wissing et al., 2004; Farenkrog et al., 2004; Qiu et al., 2005; Li et al., 2006), indicating that the apoptotic PCD pathway is conserved in unicellular organisms and multicellular eukaryotes, although a simpler process could exist in the former. Indeed, apoptotic PCD in yeast follows a series of typical apoptotic morphological changes resembling that in mammalian cells, including translocation of phosphatidylserine at the outer layer of the cytoplasmic membrane, DNA fragmentation, chromatin condensation, cell shrinkage and plasma membrane blebbing (Ludovico et al., 2005). Apoptotic PCD of budding yeast cells can be induced by several extrinsic and intrinsic signals, such as chemical reagents, drugs and physical stress (Madeo et al., 2004).

Formic acid (methanoic acid) is a weak acid capable of causing oxidative stress. Low concentrations of formic acid are widely used as a major ingredient of antiseptics. On the other hand, formic acid has been shown to be a toxic metabolite of methanol, it is a commonly used organic solvent that has been long known to be a selective human neurotoxin (Roe, 1955). Formic acid causes both the metabolic acidosis and ocular toxicity by affecting the retina and optic nerve cells, ultimately leading to blindness, a common and permanent consequence of methanol intoxication (Roe, 1955). Formic acid has been demonstrated in vitro to induce
mammalian cell death by inhibiting the activity of cytochrome oxidase, the terminal electron acceptor of the electron transport chain that is involved in ATP synthesis, resulting in depletion of ATP and subsequent cell death due to reduction of energy levels so that essential cell functions cannot be maintained (Nicholls, 1975; Nicholls et al., 1976). Inhibition of cytochrome oxidase also increases the production of cytotoxic reactive oxygen species (ROS), leading to cell death due to damage in ROS in cell compartments (Richter et al., 1995). Antioxidants such as catalase and glutathione/glutathione peroxidase may play a role in the protection of ocular cells from formic acid toxicity (Treichel et al., 2004).

Here, we report that a low concentration of formic acid induces apoptosis-like cell death in cells of the budding yeast *S. cerevisiae*, and this active process is accompanied by ROS burst, although it likely independent of Yca1p, the metacaspase orthologue of yeast, under experimental conditions. The properties of ROS production in wild-type and Yca1p mutant yeast strains are evaluated. To the best of our knowledge, this is the first study to unravel the mode of action of formic acid in inducing apoptosis in yeast. Furthermore, our study provides a model system to investigate the regulation cascade downstream of ROS production during Yca1p-independent cell death in yeast.

**Materials and methods**

The yeast strain W303-1a (MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100) and its isogenic knockout mutant W303-1a (yca1Δ) (a gift from Dr Stephen J. Kron of the University of Chicago) were used throughout this work. Yeast cells were grown in YPD medium (1% yeast extract, 2% Bacto peptone and 2% glucose) or selective synthetic and complete media (Guthrie & Fink, 1991). Liquid cultures were incubated in a mechanical shaker (200 r.p.m.) at 30 °C. Plasmid pYES-mtGFP (Westermann & Neupert, 2000), which expresses a mitochondrial targeted green fluorescent protein (GFP) by lactose induction, was transformed into the yeast cells using the LiAc protocol (Guthrie & Fink, 1991).

**Formic acid treatments and cell death assessment**

Yeast cells in the mid-exponential phase or stationary phase were resuspended in 20 mL of YPD containing 0, 20, 40, 50, 60, 70, 80 or 120 mM formic acid. The final density of the cells was $1 \times 10^7$ cells mL$^{-1}$. After incubation at 30 °C for 40 min with shaking, the cells were harvested and spread on YPD plates for survival rate assay by CFU counts. At least 200 cells were counted for each data point. All assays were repeated three times with similar results for each time point. Similar experiments were also performed to compare the survival rates of cells of the W303-1a and W303-1a (yca1Δ) strains, in which formic acid was added to a final concentration of 30 and 60 mM and the treatment time was 40 and 80 min, respectively.

**Transmission electron microscopy analysis**

Yeast cells ($1 \times 10^7$) from different treatments were observed under an electron microscope as previously described (Madden et al., 1997). Micrographs were recorded with a JEOL JEM 100C × II electron microscope at a magnification of $\times 14000$.

**Annexin-V-fluorescein-isothiocyanate (FITC) and propidium iodide (PI) staining**

Cells were stained using the Annexin-V-FITC kit (Bender Co.) and PI according to the manufacturer’s instructions with minor modifications. Briefly, cells from different treatments were washed twice with phosphate-buffered saline (PBS) and then resuspended in 250 μL of binding buffer. Aliquots of 195 μL of cells were taken and incubated with 5 μL of AnnexinV-FITC at room temperature for 10 min and harvested and then resuspended in 190 μL of binding buffer containing 1 μg mL$^{-1}$ of PI. The samples were observed immediately using a confocal microscope (Leica TCS-SP2).

**4′,6-diamino-2-phenylindole (DAPI) staining**

To assess chromatin condensation and fragmentation, samples were stained with DAPI (KPL Co.). Yeast cells were harvested, fixed with 3.7% formaldehyde for 30 min, and washed three times with PBS. After incubation in 50 μL of a solution containing 0.05 μg of DAPI for 10 min at room temperature without light, the samples were washed with PBS three times and dropped onto a gelatin-coated slide. Image acquisition by a confocal microscope was performed as described for Annexin-V-FITC and PI staining.

**Rhodamine 123 (Rh123) staining**

The assessment of mitochondrial membrane potential was performed using Rh123 (Alexis) staining. After formic acid treatment, cells were washed three times with PBS and incubated with Rh123 at 0.2 μg mL$^{-1}$ for 10 min in the dark. These cells were then analyzed using a fluorescence activated cell sorter (FACS) (Vantage™ SE, BD Biosciences) equipped with a 488-nm argon laser. Emission signals were detected at a wavelength of 530 nm to determine the FL-1 parameter. The data were analyzed using CELLQUEST DATA software.

**TUNEL assay**

The DNA strand breaks were identified by TUNEL assay using the TdT mediated dUTP nick end labeling (TUNEL)
assay kit (R&D Co.) according to the manufacturer’s instructions. In brief, yeast cells were fixed with 3.7% formaldehyde, followed by the TUNEL-kit protocol: cells were dehydrated with ethanol and permeabilized with Cytonin and were dropped onto gelatin-coated slides. The slides were rinsed with water and labeled with the Labeling Reaction Mix containing B-dNTP, TdT Enzyme and 1 × TdT Labeling Buffer. Finally, the slides were rinsed with water, clarified by Methyl Green and dehydrated by ethanol and Xylenes, and the cells were observed with a microscope under natural light.

Detection of ROS production
To detect ROS production in W303-1a cells and their isogenic YCA1 disruptant, 5 μg mL⁻¹ dihydrorhodamine 123 (DHR123, Sigma-Aldrich) was added to yeast cells (1 × 10⁷) in YPD and incubated at 37 °C for 15 min in darkness. The cells were then rinsed once with YPD and resuspended in 1 mL of YPD containing various concentrations of formic acid. After incubation at 30 °C for 40 min, the cells were washed once with PBS. ROS generation of treated cells was monitored under the confocal microscope. The process of ROS generation was also determined by FACS at different time points. To do this, 1 mL of W303-1a yeast cells and their isogenic YCA1 disruptant were incubated with 20 μM of the fluorescence probe, 2’,7’-dichlorofluorescin diacetate (H₂DCFH-DA, Cayman Co.) for 15 min at 30 °C. The cells were then treated with formic acid. Samples were harvested at different time points and analyzed immediately by FACS (Vantage™ SE, BD Biosciences) equipped with a 488-nm argon laser. Emission signals were detected at the wavelength of 530 nm. The data were analyzed by CELLQUEST DATA software.

Results

Low concentrations of formic acid induces apoptosis-like cell death in S. cerevisiae
Treatment of the wild-type yeast strain, W303-1a, with different concentrations (20–120 mM) of formic acid for 40 min at 30 °C resulted in dose-dependent cell growth arrest (Fig. 1). The percentage of viability, estimated by CFU counts, decreased with increased formic acid concentration. Furthermore, the cell arrest rate increased rapidly when the incubation temperature increased from 30 to 35 °C after incubation with various concentrations of formic acid for 40 min (data not shown). On the other hand, the cells in the stationary phase were more resistant to formic acid treatment than were those in the mid-exponential phase (Fig. 1), indicating that the cell survival rate is dependent on the temperature and the growth-phase with formic acid treatment. As reported previously, too high or too low doses of the extrinsic inducer will make it difficult to characterize the process of apoptosis (Ludovico et al., 2001; Reiter et al., 2005). Thus, in this study, we chose the cells in the mid-exponential phase incubated with 60 mM formic acid for 40 min (the survival rate of cells was around 30% under this condition) with aeration shaking at 120 r.p.m. in a water shaker at 30 °C to study the mode of cell death.

We first assessed the hallmarks of cells undergoing apoptosis-like PCD by investigating the alterations of their nuclei during treatment with formic acid. First, DAPI, the dye that specifically stains nuclei, was used to monitor the nuclear alterations. As shown in Fig. 2a, the shape of the nuclei of most of the cells treated with formic acid were ring-like, indicating the chromatin condensation characteristic of apoptosis, whereas the nuclei of the control cells were rounded (Fig. 2a, right panel). Chromatin fragmentation occurred as the progression of cell death was detected by TUNEL assays (Fig. 2b). TUNEL-positive cells were abundant after treatment with 60 mM formic acid for 40 min (Fig. 2b, left panel). On the contrary, most of the cells were not stained by TUNEL in the untreated control (Fig. 2b, right panel). The ultrastructural alterations of apoptotic cells were further confirmed by electron microscopy. As shown in Fig. 2c, the chromatin in the nuclei of the apoptotic cells were condensed to one side of the nuclear envelope, demonstrating DNA strand breakage (Fig. 2c, middle panel), whereas the chromatin of untreated cells or of boiled cells were homogeneous in the nuclei with intact envelopes (Fig. 2c, left panel) or it was destroyed completely (Fig. 2c, right panel), respectively. Furthermore, the intactness of the cell membrane shown in Fig. 2c (middle panel) and by PI staining clearly demonstrated a nonlytic cell death process in the yeast cells under formic acid stress.

Fig. 1. Cell death upon formic acid treatment in yeast cells. Cell survival (% of CFU on YPD agar plates) of yeast in stationary (squares) and mid-exponential (circles) phases treated with various concentrations of formic acid for 40 min. Values are mean ± SEM of three independent experiments.
Formic acid-induced cell death is accompanied by alterations of plasma membrane integrity

Annexin-V-FITC plus PI staining was used to examine the externalization of phosphatidylserine in *S. cerevisiae* after treatment with formic acid. The cells treated with 60 mM formic acid were green in color, indicating that the cells were stained by FITC but not by PI (Fig. 3a), demonstrating the intactness of the plasma membrane of the apoptotic cells. Living cells that were not undergoing apoptosis could not be stained by either FITC or PI (Fig. 3b).

**ROS are produced during apoptosis-like cell death induced by formic acid**

ROS play a pivotal role in the induction of apoptotic cell death in mammalian and yeast cells, and cause damage to cell structures during apoptosis (Madeo *et al.*, 1999; Kannan...
Formic acid induces yeast apoptosis-like cell death

Formic acid-induced yeast cell death decreases mitochondrial membrane electric potential and finally causes decomposition of mitochondrial filaments

To determine if the mitochondrial membrane electric potential of apoptotic cells dropped during formic acid treatment, yeast cells were incubated with Rh123, a fluorescent dye that specifically stains active mitochondria in living cells. As shown in Fig. 5, cells undergoing apoptosis could be only weakly stained by Rh123 (Fig. 5a), indicating the inactiveness of the mitochondria, whereas the mitochondrial potential of most untreated cells was much higher (Fig. 5a).

HO, a small uncharged hydrophobic molecule, is one of the most active forms of ROS. Diffusion of $O_2$ from mitochondria and formation of HO in cytoplasm leads to massive oxidative damage in all intracellular compartments (Chandel & Budinger, 2007) and finally causes destruction of mitochondrial filaments, a necessary event linked to the PCD cascade in mammalian and yeast cells (Frank et al., 2001; Fannjiang et al., 2004; Skulachev et al., 2004). To
determine such a ‘thread-grain’ transition of mitochondria in formic acid-induced cell death, yeast cells were transformed by the vector pYES-mtGFP, which expresses GFP fusion protein targeted to mitochondria (Westermann & Neupert, 2000). The transformants were then treated with formic acid to induce cell death. Mitochondrial fragmentation/degradation was observed under the fluorescence microscope in formic acid-treated cells with the fluorescent granular-like phenotype (Fig. 5b). The mitochondria of untreated cells, however, remained net-like (Fig. 5c).

Taken together, these results indicated that low concentrations of formic acid induced an apoptotic cell death process in yeast resembling that in mammalian cells.

**Low-concentration formic acid-induced cell death is Yca1p-independent**

Caspases are a group of cysteine proteases responsible for apoptosis in multicellular organisms. Yca1p is so far the only metacaspase homologue identified in yeast that has been demonstrated to mediate apoptosis triggered by several intrinsic and extrinsic inducers (Uren et al., 2000; Madeo et al., 2002; Silva et al., 2005). However, yeast apoptosis can also be triggered via a Yca1p-independent cascade (Wissing et al., 2004). To assess whether Yca1p plays a similar role in formic acid-induce apoptotic PCD in yeast, we treated wild-type and YCA1 null strains with 60 and 30 mM formic acid, respectively, for 40 and 80 min, respectively, survival rates were calculated by CFU counts. The error bars represent the SD calculated from at least three assays. The production of ROS in W303-1a (c) and W303-1a (yca1Δ) (d) after 40 min treatment with 60 mM formic acid is shown by DHR123 staining. Scale bar, 10 μm.

**Fig. 6.** Survival rates of yeast strains W303-1a and W303-1a (yca1Δ). Cells of W303-1a and W303-1a (yca1Δ) in the mid-exponential phase (1 x 10⁷ cells mL⁻¹) were resuspended in 20 mL of YPD liquid containing 60 mM (a) and 30 mM (b) formic acid, respectively. After treatment for 40 and 80 min, respectively, survival rates were calculated by CFU counts. The error bars represent the SD calculated from at least three assays. The production of ROS in W303-1a (c) and W303-1a (yca1Δ) (d) after 40 min treatment with 60 mM formic acid is shown by DHR123 staining. Scale bar, 10 μm.
The burst of ROS during cell death occurs earlier and at a higher level in the YCA1 null strain than in the wild-type strain

To characterize the role of ROS in the apoptosis-like PCD process further and to understand the influence of Yca1p on the ROS burst, we measured ROS production in wild-type strains and YCA1 disruptants at various time points during formic acid treatment. ROS production was clearly observed in yca1Δ cells within 20 min after treatment with 30 mM formic acid (Fig. 7, right panel). In the wild-type strain, the positive fraction of ROS increased slightly but constantly (Fig. 7, left panel), while the number of ROS-positive cells in the disruptant increased dramatically after 20 min (Fig. 7, right panel), indicating the rapid burst of ROS under formic acid stress. Similar results were also obtained by treating the cells with 60 mM formic acid with much more rapid ROS burst (data not shown).

Discussion

It has been reported that many cytotoxic substances can induce apoptotic PCD or necrotic cell death in yeast, depending on the concentration of the substance used (Madeo et al., 2004; Ludovico et al., 2005). Compared with the extensive and fruitful studies of Yca1p-dependent PCD, the existence of caspases other than Yca1p in yeast apoptosis has yet to be investigated, although the phenomenon of Yca1p-independent cell death in yeast has recently been reported (Farenkrog et al., 2004; Wissing et al., 2004; Wysocki & Kron, 2004; Ivanovska & Hardwick, 2005; Hauptmann et al., 2006; Purver & Hawkins, 2006; Almeida et al., 2007). In this study, we showed that a low concentration of formic acid induces cell death in the yeast S. cerevisiae and this process seems to be independent of Yca1p but it exhibits many typical markers of apoptosis like chromatin condensation, mitochondrial fragmentation, phosphatidylserine externalization and increased ROS production.

Low concentrations of hydrogen peroxide and acetic acid are two reagents that are commonly used to induce apoptotic cell death in yeast (Madeo et al., 1999; Ludovico et al., 2001). Hydrogen peroxide per se is a weak oxidant and kills yeast cells by producing ROS to oxidize the labile amino acid residues of proteins, nucleic acid and lipids, leading to the destruction of compartments in the cells. On the other hand, acetic acid is an end product of alcohol metabolism during yeast fermentation and it enters the cell by a process of membrane protein channel-facilitated diffusion (Mollapour & Piper, 2007). Accumulation of acetic acid in a cell will acidify the intracellular environment and finally result in cell death (Pinto et al., 1989) by the apoptotic or necrotic pathway (Ludovico et al., 2001). However, the downstream process of killing yeast cells by high doses of hydrogen peroxide and acetic acid, especially via the necrotic pathway, is still unclear. Different from the mode of action of acetic acid and H2O2 as inducers of apoptosis, the first step that formic acid takes in triggering apoptosis in yeast is probably by inhibiting the activity of cytochrome oxidase by formate, resulting in the rapid depletion of intracellular ATP and the burst of ROS and sequential cell death. Because the metabolism of formic acid in vivo does not produce ROS, it is noteworthy to point out that, in contrast to the mode of ROS production by H2O2, the ROS induced by formic acid is endogenously generated. This is especially important in evaluating the role of intracellular ROS in apoptosis by excluding the interference of extrinsic sources of ROS. Thus, formic acid can be used as a convenient and functional...
inducer of yeast apoptosis. Formic acid-induced apoptosis could provide an alternative to investigate the downstream specific-protein cascades involved in yeast apoptosis.

As mentioned above, inhibition of cytochrome oxidase also increases the production of cytotoxic ROS, leading to cell death due to the detrimental effects caused by ROS in cell compartments (Richter et al., 1995). In addition, caspase could be activated directly or indirectly by ROS and in turn trigger PCD (Madeo et al., 2004). However, the relationship between ROS generation and Yca1p activation is still not clear. Given the pivotal roles of ROS and caspases in PCD, we tried to clarify the relationship between ROS generation and Yca1p activation during the apoptosis process, although apoptosis induced by formic acid may not require Yca1p activation. In Fig. 6a and b, we can see that the survival rate of the Yca1p disruptant is slightly lower than that of the wild type at various time points of treatment and different doses of formic acid, although the differences are not large. This phenomenon was also observed in yeast apoptosis due to N-glycosylation defects (Hauptmann et al., 2006). Moreover, the level of intracellular ROS is higher in the disruptant than that in the wild-type cells (Fig. 7). Similarly interesting results were reported by Khan et al. (2005), as they quantitatively determined the level of oxidized proteins in yeast cells under H2O2 stress and showed that Yca1p disruptants accumulate higher amounts of intracellular oxidized proteins than wild-type cells do. These observations suggest a linkage between ROS production and Yca1p activation during apoptosis in yeast. This linkage remains to be further clarified.

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