Achievements and perspectives in yeast acetic acid-induced programmed cell death pathways

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Abstract
The use of non-mammalian model organisms, including yeast Saccharomyces cerevisiae, can provide new insights into eukaryotic PCD (programmed cell death) pathways. In the present paper, we report recent achievements in the elucidation of the events leading to PCD that occur as a response to yeast treatment with AA (acetic acid). In particular, ROS (reactive oxygen species) generation, cyt c (cytochrome c) release and mitochondrial function and proteolytic activity will be dealt with as they vary along the AA-PCD time course by using both wild-type and mutant yeast cells. Two AA-PCD pathways are described sharing common features, but distinct from another with respect to the role of ROS and mitochondria, the former in which YCA1 acts upstream of cyt c release and caspase-like activation in a ROS-dependent manner and the latter in which cyt c release does not occur, but caspase-like activity increases, in a ROS-independent manner.

Introduction
The term PCD (programmed cell death) describes a highly heterogeneous process regulated by distinct but sometimes overlapping pathways including apoptosis, autophagic cell death, and necrosis according to their morphology [1]. In mammals, apoptosis is a major cell-suicide programme occurring in a variety of physiological processes, including tissue homeostasis, embryonic development, and in the immune response. Defects in apoptosis regulation can result in human pathologies including neurodegeneration and in cancer, thus requiring a detailed investigation as to how apoptosis occurs. In this regard, the high degree of conservation of genes and proteins between Saccharomyces cerevisiae and higher eukaryotes has made yeast a model system suitable to study biochemical processes and to identify regulatory pathways occurring in apoptosis.

Since the discovery of a yeast mutant exhibiting apoptosis-like hallmarks [2], including chromatin condensation, genomic DNA fragmentation and phosphatidylserine externalization, many papers have shown that yeast can undergo processes leading to PCD in response to different stimuli [3]. In particular, PCD in S. cerevisiae cells was suggested to reflect the altruistic role of some yeast cells to increase the fitness of the whole cell population [4,5].

A variety of yeast genes and proteins, orthologues of mammalian apoptosis regulators, were shown to play a role in yeast PCD triggered by a variety of either endogenous or exogenous stimuli, including harsh chemical treatment, gene mutations, aging, and heterologous expression of human pro-apoptotic proteins [3,6]. Accordingly, some processes occurring in mammalian apoptosis take place, sometimes with some difference, in yeast PCD. They include ROS (reactive oxygen species) generation, mitochondrial dysfunction, with a special interest in the release of cyt c (cytochrome c) and caspase-like proteolysis activation [7–12].

The involvement of mitochondria in yeast PCD has largely been recognized. They are the major source of the ROS and release crucial pro-death factors, including AIF (apoptosis-inducing factor), EndoG (endonuclease G) and cyt c (reviewed in [13,14]). However, the mechanisms by which mitochondria (as well as other cell components) mediate commitment and execution of yeast PCD remain obscure, thus requiring further investigation. Moreover, there is the possibility that PCD can also occur in the absence of the above processes.

To investigate PCD, we used yeast cells undergoing PCD induced by AA (acetic acid) [15]; certain key biochemical processes have been detected as occurring as a function of time after the PCD induction. In particular, in the present paper, we review increases in our knowledge of ROS generation, proteolytic activities, cyt c release and mitochondrial oxidative phosphorylation. To do this, in addition to wild-type yeast, we resorted to using a number of yeast mutant strains either lacking or overexpressing certain regulatory genes (Table 1).

How yeast PCD occurs in response to AA
AA is a weak acid that displays increased antimicrobial action at low pH in its undissociated state [16]. AA in undissociated form can enter the cell via diffusion through the plasma membrane, and in the cytoplasm, where pH is higher, dissociates leading to intracellular acidification [17].

Key words: cyt c, mitochondrion, programmed cell death (PCD), reactive oxygen species (ROS), Saccharomyces cerevisiae, YCA1

Abbreviations used: AA, acetic acid; AIF, apoptosis-inducing factor; cyt c, cytochrome c; COX, cyt c oxidase; mPTP, mitochondrial permeability transition pore; PCD, programmed cell death; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Depending on its concentration, AA added to exponentially growing S. cerevisiae cells at pH 3.00 was shown to induce death exhibiting either typical apoptosis-like features (AA-PCD) [15,18] or ultrastructural alterations typical of necrosis [15]. In this review, we shall deal with the former death process.

Progressive loss of yeast cell viability was shown after AA-PCD induction with cell death accomplished in 200 min; accordingly, a simultaneous increase in the percentage of cells showing DNA fragmentation occurred with a maximum at 150 min [19,20]. As expected, AA-PCD cells also showed chromatin condensation, and cell death was prevented by CHX (cycloheximide) [19].

### ROS generation

Although oxidative stress has been shown to play a role in yeast PCD [7], whether ROS directly trigger PCD, or are generated as a result of the PCD cascade, and both the nature and the source of ROS remain to be elucidated.

Yeast treatment with AA was shown to cause early intracellular H$_2$O$_2$ accumulation with maximum H$_2$O$_2$ levels found at 15 min after PCD induction, as measured through oxidation of 2,7-dichlorodihydrofluorescein diacetate to dichlorofluorescein. Then the H$_2$O$_2$ levels decrease after 60 min when H$_2$O$_2$ is undetectable. On the other hand, accumulation of superoxide anion is observed only later in a PCD process (90 min) [21]. The observed difference in the time course of H$_2$O$_2$ and superoxide anion is in favour of a different role for the two ROS during AA-PCD.

The level of intracellular ROS is under the control of the antioxidant system, including SOD (superoxide dismutase) and catalase, which can scavenge superoxide anion and H$_2$O$_2$ respectively. Activities of the SOD and catalase have been assayed in yeast en route to AA-PCD: the SOD activity increases after AA addition (maximum at 15 min) and decreases afterwards; contrarily, the catalase activity is undetectable during the AA-PCD [19]. Whether the catalase undergoes enzyme inactivation and/or degradation in the AA-PCD cells remains to be established. Note that, unlike with mouse cell lines in which autophagy occurs as a result of selective catalase degradation [22], autophagy proved to be absent in AA-PCD [23].

Catalase plays a protective role under several stress conditions in S. cerevisiae [24]. Accordingly, AA-PCD was prevented in cells overexpressing cytosolic catalase T (encoded by the CTT1 gene, see Table 1), in which a lower level of H$_2$O$_2$ was found with respect to the control cells [25]. In cells overexpressing cytosolic Cu–Zn SOD, encoded by the SOD1 gene (Table 1), the AA-PCD was exacerbated and H$_2$O$_2$ levels were higher than in the control cells. Confirmation of the protective role of the catalase in S. cerevisiae AA-PCD is that yeast cells develop an adaptive response to AA-PCD when exposed to extracellular acidification at pH 3.0 [19]. Under these conditions, both high SOD and catalase activities with low levels of both superoxide anion and H$_2$O$_2$ were found [21]. All together, these findings are in favour of a role of H$_2$O$_2$ as a second messenger needed to start the PCD cascade triggered by AA. Such a conclusion is substantiated by the prevention of cyt c release and caspase-like proteolysis activation due to the antioxidant N-acetyl-L-cysteine [26] (see below).

Where ROS are generated and in what manner AA leads to the intracellular superoxide and H$_2$O$_2$ generation remains to be ascertained. Certainly mitochondria are the major source of ROS in the AA-PCD [8]. Moreover, it has been proposed that the occurrence of intracellular acidification, following AA treatment, causes superoxide protonation to HO$_2^*$ which is one of the most aggressive ROS. In this death cascade, the protein Ysp2p, localized into mitochondria, was shown to act downstream of ROS and to play a major role in mediating mitochondrial thread-to-grain transition en route to the PCD [27]. Such a process was found to be a crucial step in mammalian apoptosis and in yeast PCD induced by AA or H$_2$O$_2$ [28]. Note that the impairment of COX (cyt c oxidase) that occurs en route to AA-PCD [8,20] could itself cause the increase of ROS generation [29].

### Cyt c release and mitochondrial function

After the first evidence of cyt c release in yeast in AA-PCD cells [8], in a series of experiments carried out to ascertain how cyt c was released en route to the AA-PCD, it was shown that

<table>
<thead>
<tr>
<th>Mutated gene(s)</th>
<th>Mutation details</th>
<th>H$_2$O$_2$ level$^a$</th>
<th>Cyt c release</th>
<th>Caspase-like activity$^a$</th>
<th>AA-PCD death rate$^a$</th>
<th>References</th>
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<tr>
<td>None (wild-type)</td>
<td></td>
<td>++</td>
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<td>[18,20,24,25,40,41]</td>
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<td>SOD1</td>
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<tr>
<td>CTI1</td>
<td>Overexpression</td>
<td>+</td>
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<td>YCA1</td>
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<td>CYC1/CYC7</td>
<td>Knockout</td>
<td>+ + n.d.</td>
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<tr>
<td>YCA1/CYC1/CYC7</td>
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cyt c release starts 60 min after the initiation of AA-PCD and is complete by 150 min. Later, the released cyt c is degraded perhaps by unidentified proteases [20].

Some features of cyt c release in yeast PCD have already been elucidated in some detail: we have shown that en route to AA-PCD cyt c release occurs from intact coupled mitochondria, thus excluding a role of the yeast mitochondrial unselective channel [30,31] in cyt c release. Note that, similarly, in cerebellar granule cell apoptosis no opening of the mPTP (mitochondrial permeability transition pore) occurs in cyt c release and that mPTP is dispensable for apoptosis to occur [32]. The involvement of the ADP/ATP carrier for mitochondrial outer membrane permeabilization and cyt c release has been reported [33]. As mentioned above, the dynamic network interconnecting mitochondria has been shown to disintegrate during AA-PCD [28]. The proteins mediating mitochondrial fission and fusion, which are phylogenetically conserved, were proposed to be involved in the release of mitochondrial proteins, including cyt c, during yeast PCD [34].

Although cyt c precedes caspase-like proteolytic activation en route to AA-PCD (see below), to date there is no evidence that the released cyt c is used for the formation of a yeast functional homologue of the apoptosome shown in mammalian cells to have a role in the initiation and execution of apoptosis [35,36]. Since mitochondria proved to be coupled when cyt c has already been released, the question arises as to the role of the released cyt c in the AA-PCD. In this regard, it has been shown that the released cyt c functions both as a ROS scavenger and a respiratory substrate [20]. This is consistent with the antioxidant functions proposed for cyt c in the apoptotic cascade [36,37] together with its role in supplying energy for AA-PCD execution.

It should be noted that mitochondrial dysfunction occurs en route to AA-PCD with a gradual uncoupling, which is complete 150 min after AA-PCD induction, as shown by the decrease of the respiratory control index from 3 to 1 [20]. Collapse of the membrane potential, with a decrease in COX activity and in the amounts of cytochromes a+a3 were also observed [8,20]. Recently, vacuolar protease Pep4p was shown to be released into the cytosol and to have a role in mitochondrial degradation in yeast cells undergoing AA-PCD, together with the ADP/ATP carrier [23].

Proteolytic activities

Like bacteria and plants, yeast contain a gene encoding a metacaspase, named YCA1, which has a distant amino acid sequence similarity to mammalian caspases [11,38]. YCA1-encoded metacaspase shows cleavage specificity different from that of caspases, since it can hydrolyse proteins after arginine or lysine residues, but not after aspartate [39]. Nonetheless, both target substrates and the precise function of the yeast metacaspase in PCD are still unknown. On the other hand, the mammalian caspase 6 and 8 substrates can also be efficiently cleaved in yeast [12,40]. This caspase-like proteolytic activity was shown to be induced en route to PCD in H2O2-stressed yeast cells [11].

We have shown that YCA1 participates in yeast AA-PCD: caspase-like activity, dependent in part on YCA1, was found to be specifically activated en route to AA-PCD in a late phase (200 min). However, caspase-like activity inhibition does not increase cell viability upon AA-PCD induction, showing that YCA1 participates in the AA-PCD independently from the caspase-like activity [41,42]. The YCA1-independent caspase-like activities have also been measured en route to yeast AA-PCD [42,43]. Our results and those from other laboratories show the existence of YCA1-dependent and YCA1-independent yeast PCD pathways [44].

Note that in addition to the caspase-like proteolytic activity, a proteasome transient activation was shown to be necessary for the AA-PCD to occur [45].

The role of ROS, cyt c and YCA1 in AA-PCD

The AA-PCD picture emerging from the above reported findings is as follows. Early ROS production occurs in AA-PCD in a manner modulated by catalase and SOD. Release of cyt c starts at 60 min of AA-PCD, when 60% of cells are still alive, and is complete at 150 min. Caspase-like activity together with the percentage of cells showing DNA fragmentation increases, with a maximum at 150 min with complete loss of cell viability at 200 min.

With the aim of gaining further insights into whether and how cyt c and YCA1 metacaspase interrelate and regulate AA-PCD, we used YCA1 and/or CYC1 and CYC7-lacking cells (Table 1). CYC1 and CYC7 encode the two isoforms of cyt c in yeast. Knockout cells lacking cyt c and/or YCA1 undergo the AA-PCD with typical apoptotic hallmarks, but with a death rate slower than that of the wild-type. In knockout yeast cells, although AA induces an early burst of H2O2, it activates a ROS-independent AA-PCD pathway [26,42].

Deletion of YCA1 was shown to lead to high intracellular ROS level and to a large accumulation of oxidized proteins upon PCD induction with formic acid or H2O2 respectively [46,47]. Δyca1 cells were shown to accumulate deleterious mutations with time [5]. However, the basic mechanism responsible for this remains to be established. Since no cyt c release occurs in these cells, it indicates the involvement of YCA1 in the cyt c release during the AA-PCD. Further investigation is needed to confirm the YCA1 role in this process. This, together with the evidence of AA-PCD occurrence in cyt c-lacking cells clearly shows that cyt c release is dispensable for AA-PCD to occur [42].

Finally, different effects on caspase-like proteolytic activation (see above) have been observed in AA-PCD depending on the variety of yeast mutations. Specifically, an early and extra activation of caspase activity has been observed in cyt c-lacking cells which, on the other hand, display increased survival [26,42]. Thus this caspase-like activity has no direct role in PCD execution; other yet unidentified proteases must be involved in AA-PCD.
Figure 1 | Yeast AA-PCD cascade in glucose grown cells

AA enters yeast cells by diffusion through the plasma membrane. In the cytosol, AA dissociates into acetate and protons causing intracellular acidification. Alternative PCD pathways are induced by AA: a ROS-dependent (blue lines) and a ROS-independent (pink lines) pathway. H2O2 accumulates early in both the pathways. In the ROS-dependent pathway SOD activity increases at 15 min. YCA1 acts upstream of cyt c (c) release from mitochondria to the cytosol; released cyt c acts as an electron donor (c(red)) to mitochondrial respiratory chain and as superoxide anion (O2•−) scavenger (c(ox)) and is degraded by unidentified proteases in a late phase; mitochondrial functions progressively decline as judged by decrease in mitochondrial membrane potential (ΔΨ), respiratory control index (RCI) and COX activity; caspase-like activity increases in a late phase with a complete loss of cell viability at 200 min. In the ROS-independent AA-PCD pathway, cyt c is not released into the cytosol but the caspase-like activity increases in a late phase (see text for further details).

Conclusions and perspectives

All these findings are in favour of the existence of two alternative death pathways induced by yeast cell treatment with AA: the ROS-dependent and ROS-independent AA-PCD, they will be described separately (Figure 1). In both cases, the undissociated form of AA enters cells via diffusion through plasma membrane causing intracellular acidification.

In the ROS-dependent AA-PCD pathway, yeast cells loose viability 200 min after challenge with a lethal concentration of AA. A specific and early intracellular high level of H2O2 is detected at 15 min and then decreases and is undetectable at 60 min. The proteasome activation starts 60 min after the AA-PCD induction, with a maximum at 90 min, and decreases at 150 min. YCA1 acts upstream of the ROS-dependent release of cyt c which starts at 60 min of the AA-PCD when 60% of cells remain alive and completes at 150 min. The released cyt c functions as a ROS scavenger and an electron donor. At a late stage, there is a gradual decrease in mitochondrial coupling with a decrease in ΔΨ and an impairment of COX; the released cyt c is degraded at 200 min. Caspase-like activity progressively increases up to a maximum at 200 min and is partially ROS-dependent.

Alternatively, after the early burst of intracellular H2O2 accumulation, AA-PCD can proceed via a ROS- and YCA1-independent pathway, in which the death rate is slower than that of the ROS-dependent pathway [26], cyt c is not released, but still a late caspase-like activity increase is observed which is not affected by H2O2 scavengers, such as N-acetyl-L-cysteine.

The role that mitochondria play in the ROS-independent PCD pathway in which no cyt c release takes place remains to be seen. However, Aif1p, a S. cerevisiae orthologue of AIF involved in caspase-independent mammalian cell death, is required for the AA-PCD to occur; in particular, en route to death Aif1p moves from mitochondria to the nucleus. Its function in PCD has been shown to be partially dependent on YCA1 [48]. Thus, in the two AA-PCD pathways, mitochondria play a different role. In this regard, further studies are required.

In Nature, S. cerevisiae can be found in acidic environments such as rotten fruit and other decomposed plant materials. Moreover, the gradual acidification of the culture medium due to accumulation of AA in aged yeast cell cultures as a result of glycolysis might contribute to a quorum-sensing mechanism [49]. In this context, the capability of S. cerevisiae to cope with AA stress ([50] and references therein) or to succumb to AA-PCD should be assumed as physiological behaviour. Note that the experimental system used to study AA-PCD consists of exponentially growing, i.e. actively dividing, yeast cells. The balance between proliferation and apoptosis must be strictly maintained to sustain tissue homeostasis. An...
imbalance between these two processes can result in either unwanted tissue atrophy or tissue growth in mammals. Thus understanding the complex role of mitochondria in integrating cell adaptation and death pathways in response to AA stress is a challenge for future investigations. *Saccharomyces* will continue to serve as an ideal eukaryotic model organism to unravel mechanisms involved in degenerative processes, thus answering fundamental questions such as those regarding the different response to apoptotic stimuli of cells in a population, depending on variation in cellular environment, as well as cell adaptation and cell death in response to stress.

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