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REVIEW

Induction of Permeability of the Inner Membrane of Yeast Mitochondria

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Abstract—The current view on apoptosis is given, with a special emphasis placed on apoptosis in yeasts. Induction of a nonspecific permeability transition pore (mPTP) in mammalian and yeast mitochondria is described, particularly in mitochondria from *Yarrowia lipolytica* and *Dipodascus* (*Endomyces*) *magnusii* yeasts, which are aerobes possessing the fully competent respiratory chain with all three points of energy conservation and well-structured mitochondria. They were examined for their ability to induce an elevated permeability transition of the inner mitochondrial membrane, being subjected to virtually all conditions known to induce the mPTP in animal mitochondria. Yeast mitochondria do not form Ca^{2+}-dependent pores, neither the classical Ca^{2+}/P_i-dependent, cyclosporin A-sensitive pore even under deenergization of mitochondria or depletion of the intramitochondrial nucleotide pools, nor a pore induced in mammalian mitochondria upon concerted action of moderate Ca^{2+} concentrations (in the presence of the Ca^{2+} ionophore ETH129) and saturated fatty acids. No pore formation was found in yeast mitochondria in the presence of elevated phosphate concentrations at acidic pH values. It is concluded that the permeability transition in yeast mitochondria is not coupled with Ca^{2+} uptake and is differently regulated compared to the mPTP of animal mitochondria.

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Key words: mitochondria, yeasts, *Yarrowia lipolytica*, *Dipodascus* (*Endomyces*) *magnusii*, Ca^{2+}, inorganic phosphate, saturated fatty acids, pH, membrane potential, swelling, pore, apoptosis

Studies over the past decade have led to the recognition that mitochondria, playing an important role in energy conversion and integrated cellular metabolism, are, at least in animal cells, also crucial in initiating apoptosis (or necrosis). Apoptosis is considered now as a highly coordinated mechanism of programmed cell death aimed at precise eliminating of self-destructive, unused, damaged, virus-infected, weakened, cell cycle-completed, and potentially dangerous cells [1]. According to the recommendation of the Nomenclature Committee on Cell Death, apoptosis is distinguished from other forms of cell death (necrosis, autophagy, mitotic catastrophe, and others) by characteristic morphological and biochemical markers including externalization of phosphatidylserine to the outer leaflet of the plasma membrane, in some cases release of cytochrome *c* from mitochondria into the cytosol, activation of cysteine proteases (caspases), generation of reactive oxygen species, blebbing of the cytoplasmic membrane, shrinking of the cell volume, breakage of nuclear DNA in internucleosomal sites, chromatin condensation on the periphery of the nucleus with further nucleus fragmentation, and cell fragmentation leading to formation of apoptotic bodies. Apoptosis is now a major focus of interest for researchers (since 2000, the number of publications on apoptosis is increasing exponentially and now it accounts for ~2% of all publications on biology). This interest is determined by the fact that apoptosis is a key obligatory mechanism of development (ontogenesis), morphogenesis, anticancer defense in metazoan organisms, in some cases, however, intensifying severe damage of the organism, causing large-scale cell death (e.g. in septic shock, infarction, insult, and neurodegenerative diseases).

Abbreviations: CsA, cyclosporin A; ETH129, specific Ca^{2+}-ionophore N,N,N′N′-tetra(cyclohexyl)diamide of diglycolic acid; mPTP (mitochondrial permeability transition pore), nonspecific permeability of the inner mitochondrial membrane; P_i, inorganic phosphate; YMUC, yeast mitochondrial unspecific channel.

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Until recently, there has been a general believe that apoptosis is uniquely metazoan, as in genomes of unicellular organisms genes encoding apoptotic factors analogous to those of metazoan have not been found and because there was reasonable skepticism about the physiological appropriateness and evolutionary advantages of apoptosis in unicellular organisms, including yeasts. The discovery of apoptosis-like death of yeast cells comes from experiments where pro- and anti-apoptotic factors of animals were heterologously expressed in the yeast *Saccharomyces cerevisiae* [2, 3]. In these experiments, yeast cells either died, showing a set of characteristic physiological markers of apoptosis, or survived, depending on the expression of pro- or anti-apoptotic factors, respectively. In 1997, a temperature-sensitive *S. cerevisiae* strain carrying a mutation in the *CDC*48 gene was described. Under non-permissive temperatures, mutant cells died, displaying markers of apoptosis commonly observed in higher eukaryotes such as exposure of phosphatidylserine on the outer leaflet of the plasma membrane, chromatin condensation, and cell fragmentation [4]. In 2002, in *S. cerevisiae* the first proapoptotic protein involved in apoptosis in yeast was identified, i.e. metacaspase (cysteine-containing protease) termed yeast metacaspase-1 (Yca1p) [5], a functional analog of caspases of animal cells.

Since that time numerous cases of apoptosis-like death of yeast cells (investigations were done primarily on *S. cerevisiae*) caused by various environmental stimuli and intercellular defects [6-15], as well as a result of replicative or chronological aging [16-18], have been described. Apoptosis implicated not only metacaspase(s), but also HtrA2-like protein [19], AIF and AMID (AIF-homologous mitochondrion-associated inductor of death) [20], Dnm1p [21], a homolog of animal Drp1p responsible for fragmentation of mitochondria in man, cytochrome *c* [22, 23], endonuclease G [24, 25], Rho5 GTPase (under apoptosis induced by reactive oxygen species) [26], and so far the only antiapoptotic factor Bir1p [27]. We found that the yeast *Y. lipolytica*, whose genome is well known [28], contains the same apoptotic factors as the yeast *S. cerevisiae* (table).

Collectively, these data are a solid indication of similarity of several elements in the mechanisms underlying apoptosis in yeast cells and animals. Intensive current studies have helped to clarify the physiological importance and appropriateness of apoptosis for yeast cells. Now it is clear, that in the wild, microorganisms including yeasts preferably live in multicellular communities (e.g. biofilms, colonies) [29-31]. Some mechanisms (formation of specific aromatic alcohols) mediating social interactions between yeasts have been described. Yeast

Components that might be involved in formation of an unspecific pore in *Y. lipolytica* mitochondria and apoptotic factors revealed in this yeast species

Protein	Genes	Degree of homology
Cytochrome *c*	*CYC1*	70-80% homology with two isoforms of cytochrome *c* of *S. cerevisiae*
Mitochondrial porin	OR YALI0F17314g	42% homology with the *S. cerevisiae* mitochondrial porin
Cyclophilin D	*CPR6*	46, 48, and 56% of homology with cyclophilin D of *S. cerevisiae*, man, and horse, respectively
Translocase of adenine nucleotides (ANT1, ANT2, ANT3)	*YlAAC1*, *AAC2*, *AAC3*	70-80% homology with all three forms of *S. cerevisiae* ANTs
Metacaspase 1	OR YALI0F04059g	60% homology with metacaspase of *S. cerevisiae*
Endonuclease G	OR YALI0D05071g	66% homology with endonuclease G of *S. cerevisiae*
HtrA protease (Nma111p in *S. cerevisiae*)	OR YALI0F31603g	49% homology with Nma111p of *S. cerevisiae*
Rad9p	OR YALI0E31273g	30% homology with Rad9p of *S. cerevisiae*
Alg2p	*ALG2*	42% homology with Alg2p of *S. cerevisiae*
Ysp1p	OR YALI0A09020g	30% homology with Ysp1p of *S. cerevisiae*

Note: For identification of apoptotic factors in *Y. lipolytica* we used the SRS (UniProt) system (to get general information on a protein and its location on the chromosome) and the BlastP system (to reveal homology in protein structure).

colonies can be considered as a multicellular organism that undergoes a sort of differentiation coupled to apoptosis. For yeast cells advantages are obvious when apoptosis is induced by pheromones or aging. Elimination of infertile (i.e. incapable of mating) cells can offer advantages for diploid cells better (as compared to haploid cells) adapted to changing environmental conditions. Removal of old and damaged cells under starvation enhances the chance for the rest of the population to survive and sporulate, therefore increasing probability for clones to survive. Moreover, the increased production of reactive oxygen species increases the likelihood for generation of genetic variants that can adapt to continuously changing conditions. Therefore, altruistic cell death via the activation of highly conservative, self-destructive, enzymatic machinery confers an advantage on the population, enhancing its genetic diversity both via sexual reproduction and somatic mutations. At the same time, apoptosis, limiting longevity, would favor genetic conservatism.

Thus, much progress toward studying and understanding of apoptosis in yeasts has been achieved. Moreover, it has been recognized that yeasts, relatively simple unicellular organisms, vigorously growing on simple and inexpensive media, having small and well-characterized genome, relatively easily changing their physiological and genetic status, are extremely promising models for functional analysis not only of known pro- and anti-apoptotic factors, but also for revealing novel ones (see, for example, [8, 25, 32]).

The role of mitochondria in the choice between cell life and death relies on the fact that mitochondria are not only the major intracellular generators of reactive oxygen species (frequently potent triggers of apoptosis), but also the place (normally in the intermembrane space) of some pro-apoptotic factors. Rupture of the outer mitochondrial membrane is accompanied by the release of pro-apoptotic factors, which in turn induces and enhances a reaction cascade ultimately leading to the cell death. For animal mitochondria, two major mechanisms for release of mitochondrial apoptotic factors from the intermembrane space have been suggested. One involves recruitment, activation, conformational rearrangement, and insertion in the mitochondrial outer membrane of pro-apoptotic Bax, a member of the Bcl-2 family proteins. Bax insertion in the mitochondrial outer membrane is a very complicated process (see, for example [33, 34]) modulated by the mitochondrial complex of translocases of the outer membrane [35] and requires cardiolipin [36].

The other mechanism relies on increased mitochondrial conductance due to opening of some pores in the inner mitochondrial membranes:

– a nonspecific, Ca^{2+}/P_i-dependent, cyclosporin A (CsA)-sensitive pore (known as the mitochondrial permeability transition pore, mPTP), a megachannel with diameter of 2.6-2.9 nm, enabling free passage of molecules of <1.5 kDa [37-39]. Although the exact molecular identity of the mPTP remains uncertain, current evidence implicates cyclophilin D located in the matrix and two inner membrane proteins, the adenine nucleotide translocase (ANT) and the phosphate carrier, in the mPTP formation [39]. The ANT plays rather a regulatory role than a role as the main transmembrane pore-forming component, as it has recently been declared. This function is fulfilled by the phosphate carrier. According to the proposed model [39], Ca^{2+}- and cyclophilin D-induced conformational changes in the structure of the phosphate carrier are responsible for pore opening. Opening of the Ca^{2+}/P_i-dependent pore in animal mitochondria is greatly enhanced by elevated concentrations of inorganic phosphate and free Ca^{2+} in the matrix, adenine nucleotide depletion, deenergization of mitochondria, oxidative stress, and agents (atractyloside and carboxyatractyloside) stabilizing the ANT in the "*c*" conformation. Acidification of the mitochondrial matrix, high transmembrane potential, Mg^{2+}, ADP, spermine, antioxidants, and CsA and its analogs favor pore closing [37];

– a non-protein pore formed by Ca^{2+} in the presence of saturated fatty acids (palmitate and stearate) [40, 41] and distinguished from the classical pore (mPTP) by its insensitivity to CsA and P_i, a nonselective permeability to divalent cations, and an ability to be spontaneously closed. Opening of this pore requires energization of the inner mitochondrial membrane;

– a CsA-sensitive pore induced by high extramitochondrial phosphate concentrations at acidic pH values [42, 43].

Pore opening is accompanied by dissipation of the membrane potential (as a result of free passage of protons across the inner membrane), loss of the energy-transducing function of mitochondria, high-amplitude swelling of mitochondria, and hence, rupture of the outer membrane, and release of proapoptotic factors located in the intermembrane space. Release of cytochrome *c* from mitochondria into the cytosol initiates the irreversible stage of apoptosis. However, it was reported that release of cytochrome *c* from the intermembrane space could occur without massive swelling of mitochondria and rupture of the outer membrane [44, 45]. Increasing evidence has evolving that release of cytochrome *c* is a complicated, finely regulated multistep process [46, 47] involving cristae structural remodeling and induction of nonspecific permeability transition of the outer mitochondrial membrane [47]. Inasmuch the cristae structural remodeling was inhibited by CsA, an inhibitor of the mPTP, a crosstalk between these processes was postulated, but the precise mechanism underlying this relationship is uncertain.

Recently particular interest of researchers has been attracted to another way of permeabilization of the inner mitochondrial membrane in animal mitochondria, i.e. to the K^+-channel of the inner mitochondrial membrane inhibited by ATP (mito-K_{ATP}-channel) [48, 49].

In yeasts, some of the abovementioned apoptotic factors are located in the mitochondrial intermembrane space, like in the animal mitochondria. However, the longstanding principal question of how apoptotic factors are released from yeast mitochondria and how these apoptotic factors initiate apoptosis in yeast cells lacking proteins of the Bcl-2 family (the yeast genome does not contain genes encoding these proteins) remains open. We are still in the dark about the role of cristae structural remodeling in release of apoptotic factors from yeast mitochondria. Information on an mPTP-like pore in yeast mitochondria has been until recently scarce, fragmentary, and contradictory.

On one hand, it was found [50] that ethanol-respiring mitochondria from *S. cerevisiae* (lacking a uniport-like system for Ca^{2+} uptake [51]) accumulated large amounts of Ca^{2+} in the presence of the Ca^{2+}-ionophore ETH129 (being inserted into the membrane, it forms a Ca^{2+}-selective channel) without pore opening. On the other hand, a channel with high peak conductance of 1-1.5 nS revealed in *S. cerevisiae* mitoplasts by patch-clamp could be equivalent to an mPTP [52]. Wild-type *S. cerevisiae* cells were very resistant to high concentrations of Ca^{2+}, P_i, or *t*-butyl peroxide, but the membrane potential $\Delta\Psi$ collapsed in permeabilized *S. cerevisiae* spheroplasts in the presence of hydrophobic dithiol-binding reagent phenylarsine oxide and very high (0.5 mM, i.e. far from physiological) Ca^{2+} concentrations [53]. Spheroplasts from a mutant *S. cerevisiae* strain lacking thioredoxin reductase generated higher peroxide concentration and exhibited diminished viability in comparison with the wild strain and displayed a rapid decrease in the membrane potential in response to the Ca^{2+} overload and oxidative stress. Similar results were obtained by the same authors when yeast cells were grown in the presence of a catalase inhibitor. It was concluded that thioredoxin reductase and catalase, being complementary to each other, preserved cell viability, protecting cells against the Ca^{2+}-induced damage of the inner mitochondrial membrane, thus suggesting a possible induction in mitochondria of *S. cerevisiae* a Ca^{2+}-dependent mPTP-like pore. However, in [53] the needed control experiments describing effect of prooxidants under conditions when Ca^{2+} was omitted were missing. Besides, only the two parameters taken together, i.e. the collapsed membrane potential and high-amplitude swelling of mitochondria are indicative of pore formation. On spheroplasts, mitochondrial swelling and release of intramitochondrial substances were not measured, and this measurement is, in principle, highly conjectural. Under conditions of the Ca^{2+} overload combined with oxidative stress, dissipation of the membrane potential can be due not only to opening of a mPTP-like pore, but also to activation of Ca^{2+}-dependent lipases leading to accumulation of free fatty acids, and ultimately, activation of fatty acid-dependent Ca^{2+}/H^+-antiporter [54]. The final result will be uncoupling of mitochondria owing to futile Ca^{2+}-cycling.

All our attempts to induce the Ca^{2+}/P_i-dependent pore in tightly-coupled, de-energized, or adenine nucleotide-depleted yeast mitochondria from *Y. lipolytica* and *D.* (*Endomyces*) *magnusii* have failed [55], even when the incubation medium was supplemented with agents (phenylarsine oxide, for example) known to promote mPTP opening in mammalian mitochondria [37]. Both yeast strains used are aerobes containing well-structured mitochondria and fully competent respiratory chain with three invariantly functioning energy conservation points, including complex I [56-60], that can be involved in induction of the canonical Ca^{2+}/P_i-dependent pore [37]. In the presence of the Ca^{2+} ionophore ETH129, moderate Ca^{2+} concentrations caused pronounced membrane depolarization (uncoupling) with no swelling of mitochondria, suggesting functioning of the futile Ca^{2+} cycling under these conditions, presumably as a result of activation of a fatty acid-dependent Ca^{2+}/H^+ antiporter [54]. The (Ca^{2+} + ETH129)-induced decline in the $\Delta\Psi$ was insensitive to CsA, Mg^{2+}, and ADP, inhibitors of the canonical Ca^{2+}/P_i-dependent pore in mammalian mitochondria [37], and partially rescued by P_i and ATP [55]. The recoupling effect of ATP was specific, being almost totally abolished and prevented by atractyloside, carboxyatractyloside, and bongkrekic acid [55], inhibitors of the of adenine nucleotide translocase. The absence of response in yeast mitochondria to ETH129 + Ca^{2+} was not simply due to structural limitations, since high-amplitude swelling occurred in the presence of alamethicin, which being incorporated into mitochondrial membranes forms channels with a diameter of 1 nm that are permeable to low-molecular-weight compounds.

We also found that in yeast mitochondria from *Y. lipolytica* and *D. magnusii*, in contrast to mammalian mitochondria, concerted action of saturated fatty acids and Ca^{2+} did not induce a pore [55]. Fatty acids (palmitic, stearic, and pentadecanoic) added to yeast mitochondria decreased the membrane potential and increased the respiration rate in state 4, which is indicative of uncoupling, but did not change the organelle volume, thus definitely indicating lack of pore (megachannel) formation in yeast mitochondria under these conditions [55].

We believe that all yeast mitochondria, regardless of the type of their energy metabolism, lack Ca^{2+}-dependent permeabilization. The lack of the Ca^{2+}-dependent permeabilization (pore) in mitochondria from *Y. lipolytica* and *S. cerevisiae* can be easily explained as they do not possess a natural uniporter to take Ca^{2+} up and therefore are unable to attain sufficient Ca^{2+} concentration in the mitochondrial matrix needed for pore induction. However, as we have showed previously [55, 61], mitochondria from the yeast *D. magnusii*, harboring a high-capacitive, finely regulated, effective Ca^{2+} uniporter [62-65] did not exhibit Ca^{2+}-dependent permeabilization. This could be due to some factors including lack in yeast mitochondria of certain components needed for the for-

mation of the mPTP; changed functions or properties of mitochondrial cyclophilin D, the CsA receptor; a higher sensitivity of yeast mitochondria to pore inhibitors (ADP, Mg^{2+}, and spermine), and, more likely, much more effective antioxidant system of yeast mitochondria as compared to animal mitochondria. Indeed, mitochondria from *Y. lipolytica* and *D. magnusii* were found to be more resistant to prooxidants (peroxide, *t*-butyl peroxide, menadione, diamide, phenylarsine oxide, and a combination of Fe^{2+} and ascorbate) than rat liver mitochondria.

In contrast to mammalian mitochondria [42, 43], mitochondria from *Y. lipolytica* and *D. magnusii* appeared to be very resistant to Ca^{2+} load in the presence of high (up to 10 mM) P_i concentrations at acidic pH values (to 6.0) even in hypotonic media. No collapse of the membrane potential and no swelling of mitochondria were observed under these conditions [55].

Prooxidants, added separately or in combination with each other, induced in mitochondria from *Y. lipolytica* and *D. magnusii* a low-conductance regulated channel permeable only for protons (experimental data will be published elsewhere in detail).

Finally, in *S. cerevisiae* the existence of YMUC (yeast mitochondrial unspecific channel) has been reported [66-76]. This channel, in contrast to the mito-K_{ATP}-channel of animal mitochondria, opened in response to ATP addition and closed upon ATP depletion [72]. YMUC exhibited a similar cutoff size to that of the mammalian mPTP and was active *in situ* [72].

We succeeded, to our knowledge for the first time, in revealing in mitochondria from *Y. lipolytica* and *D. magnusii* the existence of an ATP-dependent K^+-channel, closed, as in animal mitochondria, by ATP (experimental data will be published elsewhere in detail). ATP acted specifically and it could not be replaced by ADP or other di- and trinucleotides. Moreover, according to our preliminary results, other modulators of the mito-K_{ATP}-channel of animal mitochondria [77] exerted on the yeast mitochondria used the same effect and at comparable concentrations, allowing us to declare the presence in these of ATP-dependent K^+-channel of animal type (designated as yeast mito-K_{ATP}, ymito-K_{ATP}).

To summarize, we believe that in yeast mitochondria only ATP-dependent K^+-channels have been reliably detected. Exactly the opposite effect of ATP on mitochondria in *S. cerevisiae*, on one hand, and *Y. lipolytica* and *D. magnusii*, on the other hand, presumably reflects the principle difference in mechanisms underlying energy supply in these yeast species. *Saccharomyces cerevisiae* is a facultative anaerobe, whereas *Y. lipolytica* and *D. magnusii* are aerobes (*Y. lipolytica* is an obligate aerobe), with mitochondria being vitally important for supporting their life.

Assuming that the cytoplasmic ATP concentration is ~1 mM (K_m values for ATP of most ATP-dependent enzymes fall in this range), it would mean that under "normal" conditions the ATP-dependent K^+-channel of mitochondria from yeast *Y. lipolytica* and *D. magnusii* (quite possibly, from other yeasts of the aerobic type), inhibited by micromolar ATP, should be closed. However, we showed that the inhibitory (closing) effect of ATP was partially reversed by Mg^{2+} and inorganic phosphate. Therefore, it is conceivable that under physiological conditions (at intracellular Mg^{2+} and P_i concentrations ranging from 5 to 10 mM), ATP may have a key role in regulating the ymito-K_{ATP} channel. It seems very likely that a considerable decrease in ATP (as a result of unfavorable conditions, stresses for example) can serve as a signal for ymito-K_{ATP} opening and, ultimately, for triggering a reaction cascade causing yeast cell apoptosis. Conversely, in mitochondria of facultative anaerobes, a decrease in the intracellular ATP level would assist in closing of the ATP-dependent channel, thus ensuring transition to the more effective mitochondrial type of energy transduction.

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