Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*

Rui D. Silva[1¶], Roberto Sotoca[2¶], Björn Johansson[1], Paula Ludovico[3], Filipe Sansonetty[4], Manuel T. Silva[5], José M. Peinado[2] and Manuela Côrte-Real[1*]

[1]Departamento de Biologia-Centro de Biologia Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal; [2]Department of Microbiology, Faculty of Biology, Universidad Complutense, 28040 Madrid, Spain; [3]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga, Portugal; [4]Laboratório de Citometria, Instituto de Patologia e Imunologia Molecular da Universidade do Porto (IPATIMUP), 4200-465 Porto, Portugal; [5]Imunobiologia, Instituto de Biologia Molecular e Celular (IBMC), 4150-180 Porto, Portugal.

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[¶]These authors contributed equally to this work.

*Corresponding author:
email: mcortereal@bio.uminho.pt
Tel.: 351-253604314
Fax: 351-253678980

Abstract

During the last years, several reports described an apoptosis-like programmed cell death process in yeast in response to different environmental aggressions. Here, evidence is presented that hyperosmotic stress induces in *Saccharomyces cerevisiae* a cell death process accompanied by morphological and biochemical indicators of apoptotic programmed cell death, namely chromatin condensation along the nuclear envelope, mitochondrial swelling and reduction of cristae number, production of reactive oxygen species and DNA strand breaks, with maintenance of plasma membrane integrity. Disruption of *AIF1* had no effect on cell survival, but lack of Yca1p drastically reduced metacaspase activation and decreased cell death indicating that this death process was associated to activation of this protease. Supporting the involvement of mitochondria and cytochrome *c* in caspase activation, the mutant strains *cyc1*Δ *cyc7*Δ and *cyc3*Δ, both lacking mature cytochrome *c*, displayed a decrease in caspase activation associated to increased cell survival when exposed to hyperosmotic stress. These findings indicate that hyperosmotic stress triggers *S. cerevisiae* into an apoptosis-like programmed cell death that is mediated by a caspase-dependent mitochondrial pathway partially dependent on cytochrome *c*.

Introduction

Apoptosis, one of the most common expressions of programmed cell death (PCD), has been observed in *Saccharomyces cerevisiae* aged cells (Herker *et al*., 2004; Laun *et al*., 2001), after heterologous expression of human pro-apoptotic genes, such as Bax (Ligr *et al*., 1998) or after mild treatment with stress agents. Yeast cells die and show typical markers of apoptosis after exposure to low doses of hydrogen peroxide (Madeo *et al*., 1999), acetic acid (Ludovico *et al*., 2001), UV radiation (Del Carratore *et al*., 2002), α mating-type pheromone (Severin and Hyman, 2002), salt (Huh *et al*., 2002), aspirin (Balzan *et al*., 2004) and by low sugar concentrations in the absence of additional nutrients (Granot *et al*., 2003). Initial screens of the *S. cerevisiae* complete genome sequence have revealed few homologs of mammalian apoptotic regulators. Though recently, orthologs of key regulators such as the metacaspase (*YCA1*/*MCA1*) (Madeo *et al*., 2002), the HtrA2-like protein (Fahrenkrog *et al*., 2004), or the yeast apoptosis inducing factor (Aif1p) (Wissing *et al*., 2004) have been observed in yeast. Fannjiang *et al*. (2004) also reported that Dnm1p, the *S. cerevisiae* homolog of the human mitochondrial fission protein Drp1p, was involved in yeast apoptosis.

In addition to the existence of Aif1p, HtrA2-like protein, Yca1p and Dnm1p, evidence has been provided for cytochrome *c* (cyt *c*)-associated mitochondrial involvement in yeast apoptosis. Yamaki *et al*. (2001) showed that cell death in the *asf1*/*cia1* deficient mutant, accompanied with predominant apoptotic features, was associated to decrease in mitochondrial membrane potential, dysfunction of mitochondrial ATPase complex and release of cyt *c*. Ludovico *et al*. (2002) observed in yeast cells undergoing apoptosis induced by acetic acid, cyt *c* release and a mitochondrial dysfunction pattern identical to that described in *S. cerevisiae* cells expressing Bax (Manon *et al*., 1997) including

major alterations in the respiratory chain namely, decrease in the amount of cyt *c* and reduction of the cytochrome *c* oxidase (COX) activity.

To date, there are no reports of apoptosis in yeasts induced by hyperosmotic stress. However, it has been recognised that in mammalian cells, hyperosmotic stress induces apoptosis and is involved in several pathological states such as ischemia, septic shock and diabetic coma (Wright and Rees, 1998; Galvez *et al*., 2001). Moreover, Chan *et al*. (1999) reported that during hyperosmotic shock–induced apoptosis in several cell types, $p21^{Cdc42/Rac}$-activated kinase (PAK2) is cleaved and activated via a caspase-dependent mechanism, and suggested the involvement of oxidative stress in the induction of this process.

Here we report that hyperosmotic stress is also able to trigger *S. cerevisiae* into a PCD process associated with characteristic apoptotic markers namely, chromatin condensation along the nuclear envelope, reactive oxygen species (ROS) production, DNA strand breaks and metacaspase activation. Further evidence is provided supporting the involvement of mitochondria and a role for the yeast metacaspase Yca1p in the hyperosmotic induced-apoptosis.

Results

S. cerevisiae dies in response to hyperosmotic stress

Exposure of *S. cerevisiae* cells, strain PYCC3507, to hyperosmotic stress caused by high glucose or sorbitol concentrations resulted in cell death (Fig. 1A). Moreover, in medium with 70% (w/w) glucose cell death was shown to be temperature- and growth phase-dependent (Table 1). The results obtained showed that, at 28 or 35ºC, stationary cells (that possess fully active respiring mitochondria and display a higher

Table 1

mitochondrial mass) exhibited higher death rates in comparison to exponential cells. Additionally, cell death was enhanced at higher temperatures (Table 1) and by aeration at 28ºC, achieved by mechanical shaking (data not shown). Subsequently, PYCC3507 stationary phase cells exposed to 60% (w/w) glucose or 60% (w/w) sorbitol in yeast morphology broth (YMB) at 28ºC with aeration were used to characterize cell death mode under hyperosmotic conditions.

Huh *et al*. (2002) reported that ionic imbalance caused by high concentrations of NaCl induced a lysigenous apoptosis in *S. cerevisiae*. The possibility of cell death imparted by incubation under hyperosmotic conditions being due to cellular lysis was analysed by determining the percentage of cells with ultrastructural lytic alterations. The values obtained (5.9% for treated cells and 0.7% for the control) discard that possibility (Fig. 2E). Propidium iodide (PI) staining (see below) supported the same interpretation. All these data pointed to a metabolic dependence of the death process and suggested that an active rather than an accidental process was underlying *S. cerevisiae* cell death under hyperosmotic stress.

Characteristic markers of apoptosis accompany *S. cerevisiae* hyperosmotic stress-induced cell death

Preservation of plasma membrane integrity is one of the characteristic markers of apoptotic death. PI exclusion is most frequently used to assess that parameter. Dead or dying cells with compromised membrane incorporate this dye that stains nucleic acids (Haugland, 2000). Loss of *S. cerevisiae* proliferative capacity in response to hyperosmotic stress caused by 60% (w/w) glucose was not accompanied by significant loss of plasma membrane integrity. After 12 and 24 hours incubation, about 10 and 30% respectively, lost their membrane integrity suggesting that most cells were dying by

Figure 1

apoptosis rather than by necrosis. (Fig. 1B; third column). This increase in the percentage of cell with damaged membranes likely results from secondary necrosis, the terminal stage of yeast apoptosis (reviewed in Ludovico *et al*., 2005). To assess whether the cell death induced by high glucose concentration is apoptotic, several apoptotic markers were investigated. Nuclear alterations along treatment of PYCC3507 *S. cerevisiae* cells with 60% (w/w) glucose were monitored by staining with 4,6-diamido-2-phenyl-indole (DAPI). Cell staining with this dye allowed visualizing well-defined nucleus in control cells (0 hours treatment, Fig. 2A). In contrast, treated cells displayed, very early after exposure to 60% (w/w) glucose, loose nuclei with kidney or ring shaped condensed chromatin characteristic of apoptotic cells (Fig. 2A). Fig. 1B shows that most cells with characteristic apoptotic nuclear alterations revealed by DAPI staining have preserved membrane integrity as shown by PI staining. This was confirmed by DAPI/PI double staining (data not shown). Electron microscopy analysis further confirmed the occurrence of nuclear alterations by showing chromatin condensation along the nuclear envelope in treated cells whereas nuclei of untreated cells were homogeneous in shape and density (Fig. 2B). Treated cells also exhibited mitochondrial ultrastructural changes namely, swelling and reduction of cristae number (Fig. 2C). In addition, TUNEL-positive cells displaying a nuclear green fluorescence were detected along exposure to 60% (w/w) glucose (Fig. 2D) indicating the occurrence of DNA strand breaks. The percentage of cells displaying TUNEL-positive staining increased along time reaching a maximum at 12 hours (Fig. 1B; second column). On the other hand, TUNEL-positive cells were rare in control cells (Fig. 1B and 2A).

Comparison of the results regarding TUNEL and PI staining (Fig. 1B) revealed a great discrepancy. After 12 hours incubation, about 50% of the cells were TUNEL positive but only about 10% lost their membrane integrity. Although it is not possible to assess

at the individual level whether TUNEL-positive cells still maintain their membrane integrity, this comparison suggested that the majority of the cells with DNA strand breaks had preserved membrane integrity.

Since it is known that high glucose concentration causes several metabolic changes in the yeast cell it was addressed whether this was the cause of apoptosis induction rather than the reduced value of water activity. For this purpose the response of *S. cerevisiae* to 60% (w/w) sorbitol was analysed. Incubation of cells in YMB with 60% (w/w) sorbitol (corresponding to a water activity similar to the one obtained in YMB with 60% (w/w) glucose) also results in cell death. The PI staining kinetics was similar to the one obtained in YMB with 60% (w/w) glucose (data not shown). Moreover, under these conditions, cell death was accompanied by caspase activation as detected by flow cytometry (data not shown) and by fluorescence microscopy (Fig. 2F).

In summary, the data presented above support the interpretation that hyperosmotic stress trigger *S. cerevisiae* into a PCD with an apoptotic phenotype sharing common features to mammalian apoptosis.

ROS are produced during apoptosis induced by hyperosmotic stress

Production of ROS in *S. cerevisiae* cells dying due to exposure to 60% (w/w) glucose was monitored with dihydroethidium (DE) or 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). DE passively diffuses into the cell and, in the presence of the superoxide anion, is oxidized to ethidium that intercalates within nucleic acids, staining the cell with a bright red fluorescence. H_2DCFDA also enters the cell and once inside a live cell is hydrolysed by intracellular esterases to H_2DCF. This compound is oxidised by ROS and leads to emission of green fluorescence (Haugland, 2000).

Figure 3

Most yeast cells from untreated samples did not show any fluorescence after staining with either DE or H2DCFDA (Fig. 3 A and B). On the other hand, some cells from yeast suspensions exposed to 60% (w/w) glucose for 3 hours, displayed a red fluorescence after staining with DE (Fig. 3A) or a green fluorescence after staining with H2DCFDA (Fig. 3B). The observed staining patterns with the two selected fluorophores indicate that ROS production is an early event of the hyperosmotic stress-induced apoptosis of *S. cerevisiae*.

Mitochondria participate in the hyperosmotic stress-induced apoptosis

Indication of mitochondrial involvement in apoptosis induced by hyperosmotic stress was given by the observation of mitochondrial ultrastructural alterations and significant ROS production, described above. Further confirmation of this hypothesis was achieved by the study of two *S. cerevisiae* BY4741 mutant strains, lacking nuclear encoded mitochondrial proteins, namely the *cyc1*Δ *cyc7*Δ strain, deleted in the genes encoding isoform 1 and 2 of cyt *c*, and the *cyc3*Δ strain lacking the cytochrome *c* heme lyase, essential for the covalent binding of the heme group to isoform 1 and 2 of apocytochrome *c* (Reilly and Sherman, 1965; Dumont *et al*., 1987) These two strains share the lack of mature cyt *c*. The kinetics of cell death induced by incubation of wild-type strain BY4741 in SC (synthetic complete medium) with 60% (w/w) glucose (Fig. 4) is similar to that described above for strain PYCC3507 in YMB broth with 60% (w/w) glucose or sorbitol (Fig. 1A). The two BY4741 mutant strains were found to be more resistant to death induced by hyperosmotic stress, comparatively to the wild-type strain (Fig. 4). However, in *cyc1*Δ *cyc7*Δ and *cyc3*Δ strains cell death was not completely abolished. Both mutants, likewise the wild-type strain, displayed DNA strand breaks, detected by TUNEL (data not shown), as well as caspase activation (Fig.

Figure 4

5B). On the other hand the *aif1*Δ mutant strain did not differ from the wild type strain regarding cell survival in response to hyperosmotic stress (Fig. 4).

Apoptosis induced by hyperosmotic stress is mediated by a partially cyt *c*-dependent Yca1p activation

To examine whether the yeast metacaspase Yca1p has a role in hyperosmotic stress-induced cell death, cell survival of a strain lacking Yca1p was firstly assessed. The results (Fig. 5A) showed that absence of the metacaspase increased cell viability consistent with the occurrence of a metacaspase-dependent active cell death process. In order to further confirm metacaspase involvement in this apoptotic process, cells were incubated with the FITC-labelled pan-caspase inhibitor VAD-fmk that binds to the active site of caspases, allowing flow cytometric determination of cells with active enzymes (Madeo *et al*., 2002). Treatment for 2 or 6 hours led to an increase in the number of cells with active metacaspase in the wild-type strain (Fig. 5B) with about 50% of cells with active metacaspase after 6 hours. Consistently, only slight metacaspase activation was observed in *YCA1* disrupted cells (Fig. 5B). Comparison of the levels of caspase activation in the wild type strain after 2 hours and *YCA1* null strain after 6 hours incubation in presence of 60% (w/w) glucose, showed that, for similar values of cell survival, metacaspase activation is three times higher in the wild type strain. Moreover, exposure of the wild-type strain to 60% (w/w) glucose in the presence of zVAD-fmk caused a drastic decrease in the percentage of cells with active caspase (data not shown). In order to ascertain the role of cyt *c* in metacaspase activation induced by hyperosmotic stress, strains *cyc1*Δ *cyc7*Δ and *cyc3*Δ were also monitored for this protease. Metacaspase activation was detected in these two mutant strains under

Figure 5

hyperosmotic stress, although at a much lower rate than the one observed with the wild type strain (Fig. 5B).

Discussion

An increasing number of reports show that different molecules, at a given concentration range, are able to trigger *S. cerevisiae* into a PCD process sharing features common to those of mammalian apoptosis (for a review see Madeo *et al*., 2004 and Ludovico *et al*., 2005). Here we show that cell death induced by hyperosmotic stress in a *S. cerevisiae* osmosensitive strain is not a lytic process and is temperature-, aeration- and growth phase-dependent, indicating that cells were dying by a metabolic process. That death induced by hyperosmotic stress in the *S. cerevisiae* strain PYCC3507 is apoptotic is concluded from the occurence of chromatin condensation, DNA strand breaks, ROS production, metacaspase activation and preservation of plasma membrane integrity. Some of these apoptotic markers were also observed in *S. cerevisiae* BY4741, a strain with a different genetic background. Yeast and plant cells exposed to hyperosmotic NaCl concentrations die by an apoptotic process (Huh *et al*., 2002). However, this apoptotic process is a consequence of an ion disequilibrium rather than of a hyperosmotic stress. Hence, the present paper is the first report on yeast apoptosis induced by hyperosmotic stress.

Evidences regarding mitochondria involvement in yeast apoptosis in response to different stimuli have been obtained (Ludovico *et al*., 2002; Severin and Hyman, 2002; Wissing *et al*., 2004). Here we present several results indicating that mitochondrial function maybe required for cell death induced by hyperosmotic stress. Absence of

holocytochrome *c* in *cyc1*Δ *cyc7*Δ and *cyc3*Δ mutant strains enhances cell survival. This can have two possible explanations. First, this could be due to the lack of cyt *c*, second to retrograde response due to respiratory deficiency. However, under the apoptotic inducing conditions used, namely, high glucose concentrations (Liao *et al.*, 1991) and presence of glutamate in the medium (Liu and Butow, 1999), retrograde response is repressed. These evidences indicate that the retrograde pathway is not involved and that mature cyt *c* is important for the execution of the death program induced by hyperosmotic stress. Additionally, we found that this death process is accompanied by ROS production and mitochondrial ultrastructural alterations. These alterations were also detected in apoptosis induced by low doses of acetic acid in *S. cerevisiae* and *Zygosaccharomyces bailii* (Ludovico *et al*., 2002; Ludovico *et al*., 2003). Likewise swelling and reduction in cristae number, have been found in several instances of mammalian apoptotic cell death (reviewed by Vieira *et al*., 2000). Brown (1975) had already reported that high glucose concentrations provoked structural and functional mitochondrial alterations in a sensitive *S. cerevisiae* strain including decrease in cristae number, respiration rate, NADH oxidase activity and cyt *c* content. Taking into account these results it is conceivable that the mitochondrial ultrastructural alterations seen in cell death induced by hyperosmotic stress represent the same mitochondrial molecular events described by Brown (1975). Moreover, because of the absence of mitochondrial respiration in the *cyc1*Δ *cyc7*Δ and *cyc3*Δ strains, it could be argued that the decreased ability of those strains to develop the hyperosmotic-induced apoptosis would be due to the failure in mitochondrial ATP generation. Nevertheless, this is not the case because cells of wild-type strain were able to commit apoptosis even when mitochondrial ATP synthesis was inhibited by oligomycin (data not shown). A similar response was observed for acetic acid-induced apoptosis (Ludovico *et al*., 2002).

Several reports describe Yca1p involvement in yeast apoptosis in response to different stimuli (Madeo *et al*., 2002; Herker *et al*., 2004; Wadskog *et al*., 2004; Wissing *et al*., 2004). Here we show that deletion of *YCA1* reduced cell death in response to hyperosmotic stress. Moreover, cell death was accompanied by metacaspase activation and the deletion of Yca1p consistently resulted in a drastic decrease of metacaspase activation. Recently, Wysocki and Kron (2004) reported that FITC-VAD-fmk, the substrate we used for detection of metacaspase activation, binds nonspecifically to dead cells. These authors claimed that staining with fluorochrome-conjugated caspase inhibitors is subjected to artifacts. Yet this does not appear to happen under our conditions. Firstly, our results show the presence of caspase positive/PI negative cells (Fig. 1F). Secondly the results presented above show that the percentage of cells with active metacaspase is not strictly correlated with the percentage of dead cells (e.g. after 2 hours treatment, the wild type strain displayed approximately the same cell survival as the *yca1*Δ strain at 6 hours, but metacaspase activation was three times higher). All these evidences suggest that unspecific labelling by FITC-VAD-fmk is not occurring and further reinforce the involvement of Yca1p in hyperosmotic stress-induced apoptosis.

Participation of cyt *c* and Yca1p in different scenarios of yeast apoptosis has been reported before. However, a link between these two proteins had never been shown. The results presented above showing that, under hyperosmotic stress, there was a decrease in the percentage of cells with active metacaspase in the mutant strains *cyc1*Δ *cyc7*Δ and *cyc3*Δ in comparison with the wild type strain, point to a causal relationship between cyt *c* and metacaspase activation. Hindrance of ROS production often results in lack of activation of mammalian effector caspases and could be a reason for the observed decrease in metacaspase activation in *cyc1*Δ *cyc7*Δ and *cyc3*Δ strains (Baker *et al*.,

2000). However, this may not be the case in our study since cyt *c* is not esssential for ROS production (Severin and Hyman, 2002; Pozniakovsky *et al*., 2005), which can occur in the absence of an intact mitochondrial respiratory chain (Heeren *et al*., 2004). Our result with *cyc1*Δ *cyc7*Δ and *cyc3*Δ strains is the first indication in favour of the interpretation that cyt *c*, at least in yeast apoptosis induced by hyperosmotic stress, is important for metacaspase activation and probably acts upstream of this protease in the apoptotic process. However, the observation that the two cyt *c* mutant strains tested still present caspase activation, although delayed in comparison to the wild type strain, indicates that cyt *c* is not indispensable for the activation of this protease but appears critical for a rapid onset of the activation process.

Although our results show that metacaspase Yca1p and cyt *c* are key factors in the apoptotic process, it should be stressed that their absence only reduces but not abolishes the apoptotic death. Since *AIF1* has been shown to be involved in apoptosis induced by H_2O_2 and chronological aging (Wissing *et al*., 2004) its involvement in apoptosis induced by hyperosmotic stress was also studied. However, lack of Aif1p had no effect on cell survival indicating the existence of alternative cell death pathways.

As a whole, these results indicate that, under hyperosmotic stress, *S. cerevisiae* cells triggers a PCD with an apoptotic phenotype that is partially mediated by a metacaspase- and mitochondria-dependent pathway. Furthermore, several of the nuclear and mitochondrial changes that accompany apoptosis induced by hyperosmotic stress in *S. cerevisiae*, appear to be common to those described in *S. cerevisiae* undergoing apoptosis following Bax expression (Manon *et al*., 1997; Ligr *et al*., 1998) and in *Z. bailii* cells under apoptosis induced by acetic acid (Ludovico *et al*., 2003) suggesting that a common mitochondrial apoptotic pathway can be activated in yeast in response to different signals.

Several implications in the biotechnological field can be anticipated from this study. A significant number of yeast species has been described as usual contaminants of high sugar foods and these species are usually osmotolerant. Understanding the mechanism that triggers apoptotic cell death under hyperosmotic stress in osmosensitive strains and how osmotolerant yeast are able to prevent the activation of this cell death program will allow the development of new preservation strategies for conservation of high sugar foods. Moreover, the elucidation of the cytotoxic effects induced by high glucose concentrations can contribute to the optimisation of the industrial fermentative yeast performance under hyperosmotic stress.

It has been recognised that several pathological states such as ischemia, septic shock and diabetic coma can be associated to hyperosmotic changes (Wright and Rees, 1998; Galvez *et al*., 2001), and that hyperosmotic stress induces apoptosis in human cell lines (Matthews *et al*., 1997; Edwards *et al*., 1998; Chan *et al*., 1999; Hoover *et al*., 2000; Mockridge *et al*., 2000; Morales *et al*., 2000). As in the case with *S. cerevisiae*, apoptosis induced by hyperosmotic stress in mammalian cells is caspase-dependent and seems to involve oxidative stress (Chan *et al*., 1999). Thus, the genetically tractable yeast appears as a promising model to unravel apoptotic mechanisms occurring in human pathological conditions associated to hyperosmotic stress.

Experimental procedures

Yeast strains, plasmids and growth conditions

The yeast strains used in this study are listed in Table 2. The *cyc7*Δ Y10280 strain (EUROSCARF) was transformed with a DNA fragment carrying the HphMX4 (hygromycin) resistance gene from the vector pAG32 (Goldstein and McCusker, 1999). Table 2

Chromosomal DNA was prepared from the resulting hygromycin resistant and G418 sensitive strain and used as template for PCR using primers GTT ATA GCG CCC CTT ATT GAA TTA T and TTC CTC TAC TGC TAC TAA GAA CGG A, amplifying a fragment containing the *cyc7*::hphMX4 gene. The *cyc1*Δ strain Y06846 (EUROSCARF) was transformed with the PCR product and the correct integration into the *CYC7* locus was confirmed with colony PCR. The resulting *cyc1*Δ *cyc7*Δ strain was unable to grow on media with glycerol as carbon source.

S. cerevisiae PYCC3507 strain was grown for 2 days in slants of yeast morphology agar (YMA; yeast extract, 3 g/l, malt extract, 3 g/l, proteose peptone, 5 g/l and agar, 20 g/l) and was inoculated in 250 ml flasks containing 100 ml of YMB (yeast extract, 3 g/l, malt extract, 3 g/l and proteose peptone, 5 g/l) supplemented with 1% (w/w) glucose. The cultures were incubated in a mechanical shaker (150 rpm) at 28ºC.

The BY4741-based strains were pre-grown for 24 hours in glass tubes containing SC medium consisting of 0.17% yeast nitrogen base (Difco), 0.5% ammonium sulfate, 80 mg/L of all amino acids (except 20 mg/L adenine, 400 mg/L leucine and 8 mg/L para-aminobenzidine) and supplemented with 2% (w/w) glucose as carbon source (SCD). After 24 hours cells were harvested and transferred to 50 ml flasks containing 10 ml of SCD. The cultures were incubated on a mechanical shaker (200 rpm) at 30ºC.

Cell death assays

Exponential and stationary cells of *S. cerevisiae* PYCC3507 strain grown in YMB with 1% (w/w) glucose were centrifuged and suspended to a final concentration of $5x10^7$ cells/ml in 50 ml flasks containing 30 ml of YMB supplemented with 70% (w/w) glucose and incubated at 28ºC without agitation. Loss of cell viability was assessed by c.f.u. counts after 3 days incubation at 30ºC on YMA plates supplemented with 18%

(w/w) glucose. No further colonies appeared after this incubation period. Death rates were also assessed at 4 and 35ºC and the effect of aeration (150 rpm) on cell death was tested at 28ºC.

Further characterization of cell death induced by high glucose concentrations was performed with early stationary cells. The cells were harvested and inoculated to a final concentration of $2x10^7$ cells/ml in 100 ml flasks containing 25 ml of YMB supplemented with 60% (w/w) glucose or 60% (w/w) sorbitol. The treatment was carried out for 24 hours at 28ºC with mechanical shaking (150 rpm).

To study the role of cyt *c*, Yca1p and Aif1p, BY4741-based strains were used. Early stationary cells, grown in SCD medium, were inoculated to a final concentration of $2x10^7$ cells/ml in 50 ml flasks containing 5 ml of SC medium with 60% (w/w) glucose. The treatment was carried out for 6 hours at 30ºC with mechanical shaking (200 rpm). Loss of cell viability was assessed as described above.

Transmission electron microscopy analysis

To analyse nuclear and lytic ultrastructural alterations, cells from different treatments were harvested, suspended in 2.5% (v/v) glutaraldehyde in 40mM phosphate/magnesium buffer, pH 6.5 and fixed overnight at 4ºC. After cell wall digestion with lyticase of the pre-fixed yeast cells (Ludovico *et al*., 2001), protoplasts were washed and fixed with aqueous 2% (w/v) osmium tetroxide (2 hours) followed by postfixation (30 minutes) with 1% (w/v) aqueous uranyl acetate (Silva *et al*., 1987). Percentage of lytic cells was determined by analysing 152 control cells and 167 cells exposed for 12 hours to 60% (w/w) glucose.

To analyse mitochondrial ultrastructure, cells were prefixed with glutaraldehyde as above, fixed with aqueous 2% (w/v) potassium permanganate (1 hour), and postfixed

with uranyl acetate as above. Dehydration was performed in ethanol. After 100% ethanol washes, the samples were transferred to 100% propylene oxide, and infiltrated with 50% (v/v) propylene oxide and 50% (v/v) Epon (TAAB Laboratories) for 30 minutes and with 100% Epon overnight. Cells were transferred to gelatin capsules with 100% Epon and incubated at 60°C for 48 hours before cutting thin sections and staining with uranyl acetate and lead citrate (Silva *et al*., 1987). Micrographs were taken with a Zeiss EM 10C electron microscope.

DAPI and PI staining

Nuclear staining protocol with DAPI (Sigma) was adapted from Madeo *et al*. (1997). Yeast cells were collected, ressuspended in 3.7% formaldehyde for 30 minutes, washed three times with PBS and incubated with 2 µg/ml of DAPI. After 10 minutes at room temperature cells were washed with PBS and mounted on a slide with a drop of anti-fading agent Vectashield (Vector laboratories, Inc.).

PI staining was used to monitor cell membrane integrity as previously described (Ludovico *et al*., 2003). Along exposure to 60% (w/w) glucose in YMB, 300 µl of yeast samples were taken and incubated with 1.5 µl of a stock solution (1mg/ml) of PI (Sigma) for 10 minutes at room temperature. Cell suspensions in YMB with 60% (w/w) glucose boiled for 5 minutes were used as positive control whereas cells not subjected to hyperosmotic glucose (YMB with 1% (w/w) glucose) were used as a negative control.

The samples were observed under a Leitz Laborlux S epifluorescence microscope equipped with a 50 W mercury lamp and appropriate filter setting. The digital images were acquired with a 3CCD colour video camera (SONY, DXC-9100P), a frame

grabber (IMAGRAPH, IMASCAN/Chroma-P) and software for image archival and management (AxioVision 3.0, Carl Zeiss Vision GmbH)

Assessment of ROS production

ROS production was detected with DE (Molecular Pobes) or H_2DCFDA (Molecular Pobes), essentially as described by Madeo *et al*. (1999). Before treatment with glucose (60%, w/w), cells were preloaded with 10 μg/ml of DE or 40 μg/ml of H_2DCFDA for 45 minutes at 30ºC. After preloading with the probe cells were transferred to YMB with 60% (w/w) glucose to induce hyperosmotic stress and ROS production was monitored by epifluorescence microscopy. Cells not subjected to hyperosmotic stress were used as a negative control. Microscope and image acquisitions were performed as described for DAPI and PI staining.

TUNEL assay

DNA strand breaks were detected by the "In Situ Cell Death Detection Kit, Fluorescein" (Roche Applied Science) using a protocol previously described (Ludovico *et al*., 2001) with slight modifications. Yeast cells were fixed with 3.7% (v/v) formaldehyde, the cell wall was digested with lyticase as referred above, and cells were applied to poly-lysine-coated slides. The slides were rinsed with PBS, incubated in permabilization solution (0.1%, v/v, Triton X-100 and 0.1%, w/v, sodium citrate) for 2 min in ice, rinsed twice with PBS and incubated with 10 μl TUNEL reaction mixture (terminal deoxynucleotidyl transferase 200 U/ml, FITC-labeled dUTP 10 mM, 25 mM Tris-HCl, 200 mM sodium cacodylate and 5 mM cobalt chloride) for 60 minutes at 37ºC. Finally the slides were rinsed three times with PBS and a coverslip was mounted with a drop of

anti-fading agent Vectashield (Vector Laboratories, Inc). Microscope and image acquisitions were performed as described for DAPI and PI staining.

Detection of metacaspase activity

Detection of active metacaspase was performed using the "CaspACE, FITC-VAD-fmk In Situ Marker" (Promega) and a protocol adapted from Madeo *et al*. (2002). Briefly, $1x10^6$ cells were washed in PBS, ressuspended in 100 µl staining solution containing 50 µM of FITC-VAD-FMK and incubated for 20 minutes at 30ºC. After incubation cells were washed once and ressuspended in PBS. Cells were subsequently incubated with 2 µg/ml of PI for 10 min at room temperature for double staining with PI.

Flow cytometric analysis was performed in an Epics® XL-MCL™ (Beckman Coulter) flow cytometer equipped with an argon-ion laser emitting a 488-nm beam at 15mW. Green fluorescence was collected through 488 blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass. Twenty thousand cells were analysed per sample at low flow rate. Data were analysed by WinMDI 2.8 software. Microscope and image acquisitions were performed as described for DAPI and PI staining.

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Figure legends

Figure 1. Cell survival and apoptotic markers in the *S. cerevisiae* PYCC3057 strain during hyperosmotic stress. (A) Cell survival evaluated by c.f.u. (100% corresponds to the number of cells at time zero) in medium with 60% (w/w) glucose (empty circles) or with 60% (w/w) sorbitol (full circles). Values are mean ± SEM of a representative experiment of three independent experiments. (B) Percentage of cells displaying chromatin condensation (DAPI +), DNA strand breaks (TUNEL +) and loss of membrane integrity (PI +) as detected by DAPI staining, TUNEL assay and PI staining, respectively, after exposure to 60% (w/w) glucose. To determine the percentage of positive cells, at least 300 cells were evaluated for each parameter. Data are from a representative experiment of three independent experiments.

Figure 2. Nuclear and mitochondrial apoptotic alterations and metacaspase activation in *S. cerevisiae* PYCC3507 cells under hyperosmotic stress. (A) DAPI staining images of control cells (0 hours) and cells treated during 2 and 10 hours; bar 10 μm. (B) Electron microscopy images of control cells (0 hours) and cells treated during 12 hours, with fixation with glutaraldehyde-OsO_4-uranyl acetate. N–nucleus. Arrows mark chromatin condensation; bar 0.3 μm. (C) Electron microscopy images of control cells (0 hours) and cells treated during 12 hours, with fixation with glutaraldehyde-$KMnO_4$-uranyl acetate. Arrowheads mark swollen mitochondria; bar 0.3 μm. (D) TUNEL images of control and of cells treated for 8 or 12 hours. Left panel: phase contrast microscopy; right panel: fluorescence microscopy of the same cells; bar 10 μm. (E) Electron microscopy image showing ultrastructural lytic alterations, with fixation with glutaraldehyde-OsO_4-uranyl acetate; bar 0.3 μm. (F) Metacaspase activation image in

cells treated with 60% (w/w) sorbitol during one hour. Left panel: FITC filter; right panel: PI filter; bar 10 μm.

Figure 3. ROS production in *S. cerevisiae* PYCC3507 cells during hyperosmotic stress. ROS production detected by dihydroethidium (A) or H_2DCFDA (B) in control cells and cells treated for 3 hours. In A and B the upper panels show phase contrast microscopy; the lower panels show fluorescence microscopy of the same cells. Bar 10 μm.

Figure 4. Involvement of cyt *c* but not of Aif1p in hyperosmotic induced cell death. Cell survival evaluated by c.f.u. (100% corresponds to the number of cells at time zero) of the wild-type BY4741 strain and of three mutant strains lacking cyt *c* (*cyc1*Δ *cyc7*Δ), mature cyt *c* (*cyc3*Δ) or the yeast Aif1p (*aif1*Δ). Values are mean ± SEM of five independent experiments. * denotes values significantly different from the control ($P<0.05$; unpaired Student's *t*-test).

Figure 5. Metacaspase involvement in death induced by hyperosmotic stress. (A) Cell survival evaluated by c.f.u. (100% corresponds to the number of cells at time zero) of the wild-type BY4741 strain and of a strain deleted in the yeast metacaspase (*yca1*Δ). Values are mean ± SEM of five independent experiments and are significantly different (*$P<0.05$, **$P<0.001$; unpaired Student's *t*-test) from those obtained with the control. (B) Percentage of cells with active metacaspase of the wild-type BY4741 strain and three mutant strains (*cyc1*Δ *cyc7*Δ, *cyc3*Δ and *yca1*Δ) during hyperosmotic stress. Control cells (0 hours) and treated cells (2 and 6 hours) labelled for active metacaspase with FITC-VAD-fmk and analysed by flow cytometry. Data represent one of three independent experiments.

Table 1

Specific death rates (μ_d) of exponential and stationary cells of *S. cerevisiae* PYCC3507 in YMB 70% (w/w) glucose at different temperatures. The values are mean ± SD of two independent experiments.

	μ_d (days^{-1})[a]	
Temperature (ºC)	Exponential cells	Stationary cells
4	0.407 ± 0.0044	0.226 ± 0.0053
28	0.458 ± 0.0028	0.566 ± 0.0324
35	0.648 ± 0.0023	0.813 ± 0.0191

[a] The values of μ_d were estimated from the slope of the linear part of the semilogaritmic plot of the number of colony forming units as a function of incubation time.

Table 2
Yeast strains used in this study.

Strain	Genotype	Source
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
cyc1Δ cyc7Δ	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cyc1*::kanMX4 *cyc7*::hphMX4	This study
cyc3Δ	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cyc3*::kanMX4	Euroscarf
yca1Δ	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yca1*::kanMX4	Euroscarf
aif1Δ	MATa *his3Δ1 leu20 met15Δ0 ura3Δ0 aif1*::kanMX4	Euroscarf
PYCC3507		PYCC[a]

[a] Portuguese Yeast Culture Collection