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Cell Death Induced by Mild Physical Perturbations Could Be Related to Transient Plasma Membrane Modifications

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Abstract An understanding of membrane destabilization induced by osmotic treatments is important to better control cell survival during biotechnological processes. The effects on the membranes of the yeast *Saccharomyces cerevisiae* of perturbations similar in intensity (same amount of energy) but differing in the source type (heat, compression and osmotic gradient) were investigated. The anisotropy of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene was measured before and after each treatment to assess the reversibility of the membrane changes related to each treatment. Except for heat shock at 75°C, changes in membrane fluidity were reversible after the return to initial conditions, showing that two kinds of physical stress can be distinguished regarding the reversibility of membrane changes: high and mild energy stresses. With the application of osmotic gradients, anisotropy was assessed during treatment with five osmotic pressure levels from 30.7 to 95.4 MPa with two different yeast strains and related to the rate of cell death caused by each stress. The exposure of cells to increasing osmotic pressures involved a progressive lowering of membrane anisotropy during lethal perturbations. Osmotic stresses associated with reversible fluidity changes of increasing intensity in the membrane led to proportional death rates and time-dependant cell death of increasing rapidity during the application of the stress. Finally, a hypothesis relating the extent of membrane structural changes to the kinetic of cell death is proposed.

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Introduction

The cytoplasmic membrane is an active organelle composed of a highly dynamic lipid bilayer in which proteins such as enzymes or receptors are embedded (Singer & Nicolson, 1972). It maintains ion and solute gradients and regulates water and solute transfer. In yeasts, the cytoplasmic membrane is surrounded by a thick cell wall, mostly composed of mannoproteins and fibrous β1,3-glucan.

The cytoplasmic membrane is thought to be a primary target of physical stresses in microorganisms. In fact, membranes may undergo structural changes when exposed to variations in the thermodynamic conditions of their environments, such as changes in temperature and hydrostatic or osmotic pressure (Denich et al., 2003; Los & Murata, 2004). Exposure to high temperatures results in a continuous increase in membrane permeability caused by time-dependent changes in the lipid and protein components of the membranes (Bischof et al., 1995; Lepock, 2005). In the same way, Kato et al. (2002) showed that hydrostatic pressure causes reversible phase transitions and protein damage in biological membranes, which are amplified with increasing pressure. Osmotic perturbations of the environment also lead to massive damage to cellular membranes and proteins as protein aggregation and/or denaturation (Crowe et al., 1988; Prestrelski et al., 1993) and lateral phase separation in lipids (Crowe, Hoekstra & Crowe, 1992; Laroche & Gervais, 2003; Leslie et al., 1994).

However, if involvement of the plasma membrane in cell death during physical perturbations is strongly suspected, the mechanism remains unclear. In the case of osmotic stresses, some authors relate the rate of cell death to the magnitude of volume decrease after the osmotic shock (Martinez de Marañon, Marechal & Gervais, 1996; Vindelov & Arneborg, 2002) or to the level of cell envelope shrinkage (Adya, Canetta & Walker, 2006) and thus attribute cell death to mechanical effects. Protein denaturation is also described as a critical event for cells exposed to hypertonicity due to molecular crowding in the cell (Garner & Burg, 1994) and to the pH decrease in the cell interior (Vindelov & Arneborg, 2002). Finally, lipid phase transitions are known to modify the permeability of the membrane and could lead to a lethal leakage of cellular components (Laroche & Gervais, 2003).

This study aimed to clarify the relationship between the plasma membrane modifications induced by physical stresses of different nature and intensity and the associated cell death. Such lesions may consist of irreversible or reversible changes of membranes during stress application. We chose to study the overall fluidity of the membranes as a parameter to evaluate the membrane changes during and after different physical treatments. Only a few techniques allow the study of membrane fluidity *in vivo*. Fluorescent probes are excellent for use in living cells, and, since the early 1970s, fluorescence anisotropy has been used to evaluate membrane fluidity. The probe 1,6-diphenyl-1,3,5-hexatriene (DPH) has mainly been used to study membrane anisotropy in bacteria (Tymczyszyn, Gomez-Zavaglia & Disalvo, 2005; Vincent, England & Trevors, 2004) and yeast (Lee et al., 2002; Swan & Watson, 1997) *in vivo*. DPH aligns with the phospholipid acyl chains and becomes evenly distributed in the bilayer core (Van Blitterswijk, Van Hoeven & Van der Meer, 1981). DPH anisotropy thus reflects the overall fluidity of the membrane (Denich et al., 2003).

First, the reversibility of fluidity changes was assessed by measuring DPH anisotropy before and after the application to yeasts of lethal physical perturbations of different energy levels and sources. Second, for osmotic stresses, anisotropy was measured during treatments of increasing intensity for two yeast strains in order to compare cell death rates and the anisotropic changes induced by the treatments.

Materials and Methods

Yeast growth

Two *Saccharomyces cerevisiae* strains were used in this study, CBS1171, which was obtained from the Centraalbureau voor Schimmelcultures (CBS), (Utrecht, The Netherlands) and ATCC60218, which was obtained from the American Type Culture Collection (Rockville, MD). Both strains were grown aerobically in 250-ml conical flasks containing 100 ml of modified Malt Wickeram medium (10 g glucose, 3 g pancreatic peptone, 3 g yeast extract, 1.5 g NaH_2PO_4 in 1 liter water/glycerol solution at an osmotic pressure of 1.38 Mpa, pH adjusted to 5.8). Cultures of *S. cerevisiae* CBS1171 were performed at 25°C and cultures of *S. cerevisiae* ATCC60218 were performed at 30°C. Both culture types were held on a rotary shaker and allowed to grow to early stationary phase.

Preparation of Binary Water/Glycerol Solutions of Different Osmotic Pressures

The Norrish equation (Norrish, 1966) was used to determine the mass of solute to be added to 1,000 g of distilled water in order to adjust the osmotic pressure to the desired level (Π):

$$\Pi = (1 - X_s)e^{-KXs^2}$$

where X_s is the molar fraction of the solute and K is the Norrish coefficient of the solute used to increase the osmotic pressure.

The solute used in all these experiments was glycerol (Sigma-Aldrich, Saint Quentin Fallavier, France), for which K = 1.16 (Chirife & Ferro-Fontan, 1980). Glycerol was chosen as a solute for osmotic stresses because it is nontoxic for yeast and can be used to prepare solutions with a large range of osmotic pressures. For steady-state fluorescence analysis, spectrophotometry-grade glycerol (Sigma-Aldrich) was used and phosphate buffer (10 mM, pH 5.8) replaced distilled water.

The osmotic pressure of all solutions was checked with a dew-point osmometer (Decagon Devices Inc., Pullman, WA).

Osmotic, High-Pressure and Thermal Treatments

Osmotic treatments

Osmotic treatments were performed with the two strains of *S. cerevisiae*. Samples (50 ml) of culture were centrifuged (5 min, 2,200 x *g*) and washed twice in a binary water/glycerol mixture (1.38 MPa). The pellets were resuspended in 5 ml of the same medium. Cell suspensions, rehydration solutions and shock solutions were incubated for 10 min in a water bath placed in a thermostatically controlled room, to reach the appropriate temperature of 25°C. The temperature was checked using a thermocouple. When the temperature was homogeneous, an osmotic shock was induced by suddenly introducing 1 ml of cell suspension into

9 ml of a binary water/glycerol solution (final osmotic pressure 144.5, 95.4, 68.0, 49.1 or 30.7 MPa). The cells were maintained for 30 min under hyperosmotic conditions. For two osmotic pressure levels, 95.4 and 49.1 MPa, cells were also maintained for 5, 15 and 60 min under hyperosmotic conditions. Then, 1 ml of the cell suspension in the shock solution was suddenly introduced into 9 ml of distilled water for rehydration.

High-pressure treatment

A cell suspension of strain CBS1171 in phosphate buffer/glycerol solution at 1.38 MPa was divided into sterile polyethylene bags, which were then heat-sealed and placed in a high-pressure vessel (GEC, Alsthom, France). They were exposed to high pressure at 300 MPa and 25°C for 10 min.

Thermal treatment at 75°C

Samples (29 ml) of a phosphate buffer/glycerol solution at 1.38 MPa were incubated in a thermostatically controlled bath at 75°C until the temperature was stable. An aliquot (1 ml) of a cell suspension of strain CBS1171 in water/glycerol solution at 1.38 MPa was suddenly introduced into the former solution and maintained for 1 min in the thermostatically controlled bath. Then, the suspension was quickly cooled on ice until the temperature reached 25°C.

Thermal treatment at 50°C

Samples (99 ml) of a phosphate buffer/glycerol solution at 1.38 MPa were incubated in a bain-marie at 50°C until the temperature was stable. An aliquot (1 ml) of a cell suspension of strain CBS1171 (3×10^8 cells/ml) in water/glycerol solution at 1.38 MPa was suddenly introduced into the former solution and maintained for 60 min in the bain-marie. The suspension was then cooled to ambient temperature before analysis.

Measurement of Yeast Viability

After each physical treatment, cell viability was estimated in triplicate by the colony-forming units (CFU) method. The initial cell suspension was used as the control.

Cell Membrane Labeling and Anisotropic Measurements under Hyperosmotic Conditions

Measurements of DPH anisotropy inserted into yeast membranes at different osmotic pressures were performed with the two strains of *S. cerevisiae*. The probe DPH (Sigma-Aldrich) was prepared in tetrahydrofuran to produce a 5 mM stock solution. This probe is known to label the core of the phospholipid bilayer of membranes, where its quantum yield is greatly enhanced. An aliquot (1 ml) of cell suspension (10^8 cells/ml) in phosphate buffer (pH 5.8) was labeled with 9 µl of the DPH stock solution (final probe concentration 45 μM) for 10 min.

An aliquot (100 µl) of labeled cells was added to 2.9 ml of a phosphate buffer/glycerol solution prepared at a specific osmotic pressure in a spectroscopic cuvette (probe concentration 1.5 μM for 3×10^6 cells/ml). Steady-state anisotropy of DPH was measured in a Fluorolog-3 spectrometer (Jobin-Yvon/Horiba Group, Longjumeau, France), using T-Format fluorescence polarizers. The excitation and emission wavelengths were 360 ± 2 and 431 ± 5 nm, respectively. Steady-state fluorescence anisotropy was calculated as follows:

$$r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$$

where $G = I_{hv}/I_{hh}$ is a correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light.

Measurements were begun less than 5 min after the osmotic shock.

An aliquot (100 µl) of nonlabeled cells in 2.9 ml of the same phosphate buffer/glycerol solution was used as the control. For each glycerol concentration tested, the fluorescence intensities I_{vv}, I_{vh}, I_{hv} and I_{hh} of the control were <10% of the intensities of the assay and did not affect the r values.

To evaluate the effect of glycerol on the anisotropy measured, the intensity of DPH fluorescence was also measured directly in glycerol solutions as a function of the glycerol concentration (probe concentration 1.5 μM). Fluorescence intensity increased with increasing glycerol concentrations and reached 17% of the assay intensity (labeled cells in a glycerol suspension at the same concentration) for the highest glycerol concentration tested in this study. However, at each osmotic pressure tested, the cell suspension was filtered after the assay with a 1.2-µm-pore filter (VWR, Fontenay sous Bois, France). The fluorescence of the filtrate was measured; this was at least 10 times lower than the fluorescence of the whole assay and did not influence the values of r. Thus, the influence of glycerol on anisotropy was considered negligible.

Cell Membrane Labeling and Anisotropic Measurements after High-Pressure and Thermal Treatments

Cell suspensions of the yeast CBS1171 were centrifuged after treatment and suspended in a phosphate buffer/glycerol solution at 1.38 MPa (3×10^6 cells/ml). An aliquot (3 ml) of these cell suspensions was labeled with 3 µl of a 1.5 mM stock solution of DPH for 10 min, and the steady-

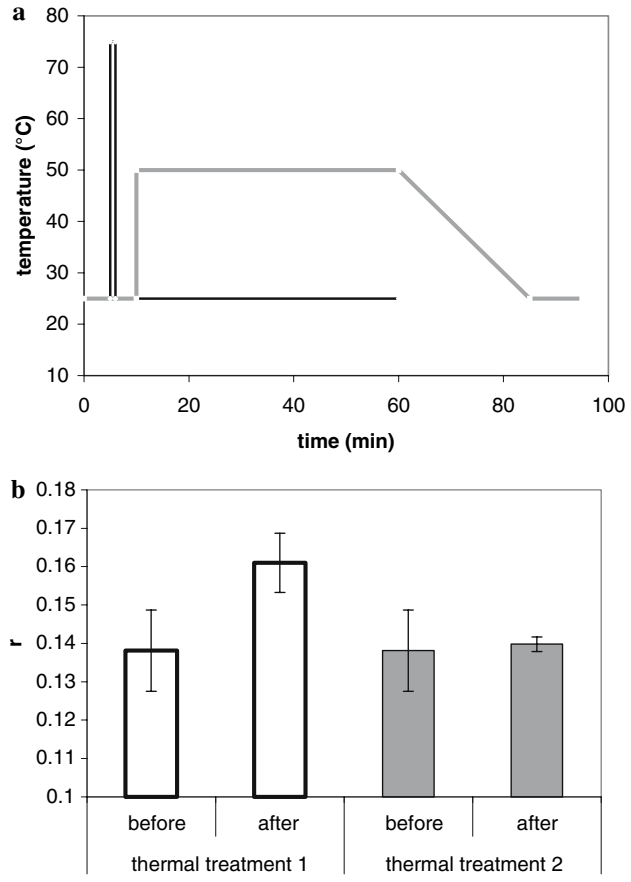

Fig. 1 Fluorescence anisotropy of DPH inserted into the membranes of the yeast strain CBS1171 before and after two heat treatments leading to 100% of cell destruction. Thermal treatments were performed at 75°C (thermal treatment 1, *black line*) and 50°C (thermal treatment 2, *gray line*) (**a**), and the anisotropy of DPH inserted into the cell membranes was assessed before and after each thermal treatment (**b**). Error bars correspond to confidence intervals at the 0.05 level

state fluorescence of DPH was measured as described above. A suspension of whole live cells was used as a control and tested in the same way. Each experiment was repeated three times.

Results

Membrane Anisotropy of Cells before and after Treatments: Steady States

Two heat treatments, one high-pressure and one hyperosmotic treatment, were performed on the yeast *S. cerevisiae* CBS1171.

Figure 1 schematically represents the heat stresses applied (a) and the corresponding anisotropy of the inserted DPH before and after each treatment (b). The first treatment consisted of a rapid thermal shock at 75°C with a 1-min maintenance period, followed by rapid cooling. The second treatment consisted of a rapid thermal shock at 50°C, with 1 h of maintenance. These treatments were chosen for their ability to kill yeasts because they both involve 100% inactivation. Times of treatments were the minimal times for which 100% of cells died. The anisotropy of DPH inserted into the membranes of living cells was used as the control and was equal to 0.137. After the first thermal treatment and the return to the initial temperature, anisotropy increased to 0.161, showing a strong modification of the yeast membrane structure. After the second heat treatment, in contrast, plasma membrane anisotropy did not change after the return to the initial temperature.

In the same way, Figure 2a schematically represents the high-pressure treatment, i.e., 300 MPa for 10 min, applied to the yeast, which involved 100% destruction, as did the heat shocks. The corresponding anisotropy, before and after treatment, is presented in Figure 2a and shows that the cell membrane recovered its initial anisotropy.

The DPH anisotropy of the labeled cells was also assessed before and after a strong hyperosmotic treatment at 144.5 MPa for 30 min, represented schematically in Figure 2b1, which involved a rate of cell death of 75%. Results showed that anisotropy was not modified by the treatment after the return to isotonic conditions (Fig. 2b2).

Based on these results, it can be inferred that permanent membrane rigidification can be induced with a lethal treatment, as in the drastic thermal treatment, but is not necessarily relevant to cell death, as shown by the mild heat, high-pressure and osmotic treatments.

Membrane Anisotropy of Cells during Osmotic Treatments: Transient State

To investigate the death mechanisms involved during physical stresses, osmotic perturbations were studied more thoroughly because such stress can lead to a large range of yeast viabilities and does not involve irreversible rigidification of the membrane. Osmotic shocks were performed at 30.7, 49.1, 68 and 95.4 MPa with the two strains of *S. cerevisiae;* and DPH anisotropy of the labeled cells was measured under hyperosmotic conditions. First, the results in Figure 3 show that the DPH anisotropy values were different with the two strains of *S. cerevisiae* tested. r values ranged between 0.137 and 0.195 when DPH was inserted into membranes of strain CBS1171, whereas they ranged between 0.220 and 0.250 in membranes of strain ATCC60218. Second, results show that osmotic pressure increase in the medium caused a significant increase in DPH anisotropy compared with the values measured under isotonic conditions at osmotic pressures higher than 30.7 MPa for CBS1171 and higher than 49.1 MPa for ATCC60218. In fact, for CBS1171, there was no signifi-

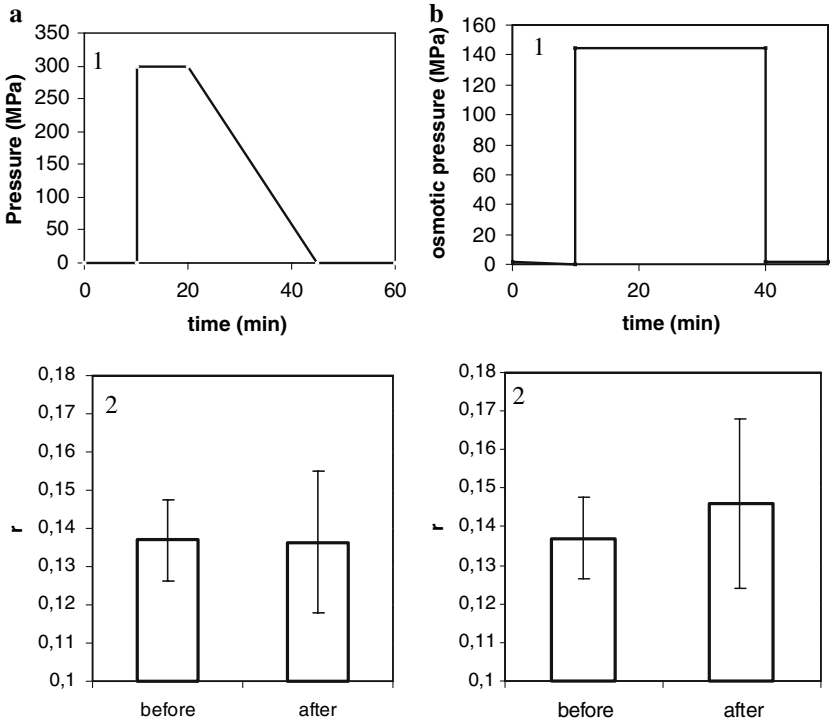

Fig. 2 Fluorescence anisotropy of DPH inserted into the membranes of the yeast CBS1171 before and after a high-pressure treatment at 300 MPa (**a1**) (100% of cell destruction) and an osmotic shock at 144.5 MPa (**b1**) (75% of cell destruction). Anisotropy of DPH inserted into the cell membranes was assessed before and after the treatment (**a2** and **b2**). Error bars correspond to confidence intervals at the 0.05 level

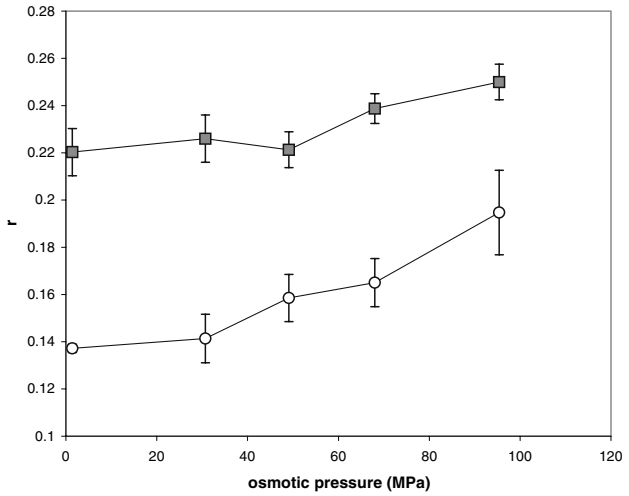

Fig. 3 Steady-state fluorescence anisotropy of DPH inserted into the membranes of *S. cerevisiae* CBS1171 (○) and ATCC60218 (■). Anisotropy was measured with cell suspensions in phosphate buffer/glycerol solutions at different osmotic pressures. Error bars correspond to confidence intervals at the 0.05 level

cant difference between the r values measured at 1.4 and 30.7 MPa. After an osmotic shock of 49.1 MPa, r increased from 0.140 to 0.160. Osmotic shocks at 68.0 and 95.4 MPa induced increases in r to 0.165 and 0.190, respectively. For ATCC60218, the r value was unchanged at 30.7 and 49.1 MPa in comparison with isotonic conditions and increased to 0.239 and 0.250 at 68.0 and 95.4 MPa, respectively. Furthermore, for each strain and each osmotic pressure tested, the initial anisotropy value was recovered after the treatment and rehydration (as shown in Fig. 2a for CBS1171), showing that the cell membranes were subjected to a transient change in fluidity for osmotic shocks higher than 30.7 MPa for strain CBS1171 or 49.1 MPa for strain ATCC60218, i.e., a decrease in fluidity induced by the osmotic stress. For each osmotic pressure tested in this study, the r value was constant for at least 30 min of maintenance under hyperosmotic conditions.

A Link between Transient Anisotropy Variations and Yeast Survival

To study the link between the transient variations in plasma membrane fluidity and the rate of cell death induced by different osmotic treatments, viability after hyperosmotic shocks at 30.7, 49.1, 68.0 and 95.4 MPa, 30 min of maintenance under hyperosmotic conditions and rehydration to 1.4 MPa was measured by CFU for the two strains of yeasts studied. Results are presented in Figure 4. For strain CBS1171, viability was not changed by an osmotic shock at 30.7 MPa. It decreased to 80.0% for osmotic shocks at 49.1 and 68.0 MPa and to 60.0% for osmotic shock at 95.4 MPa. The viability of strain ATCC60218 was not significantly changed by osmotic shocks to osmotic pressures equal to or lower than 68.0 MPa and decreased to 80.6% after osmotic shock at 95.4MPa.

Then, the difference between anisotropy measured under hyperosmotic conditions and that measured under isotonic

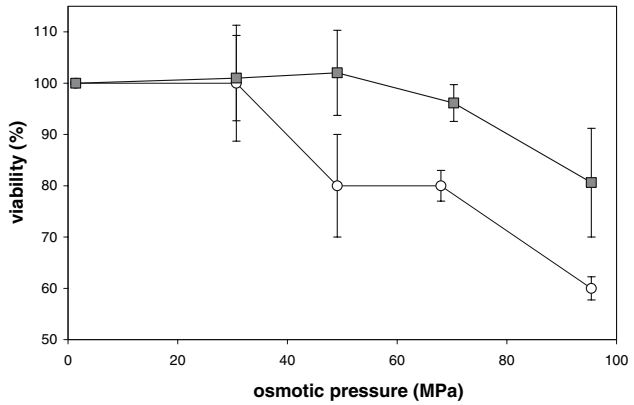

Fig. 4 Percentage viability measured by CFU after osmotic shocks in water/glycerol solutions and 30 min maintenance under hyperosmotic conditions as a function of osmotic pressure of the shock for CBS1171 (○) and ATCC60218 (■). Error bars correspond to confidence intervals at the 0.05 level

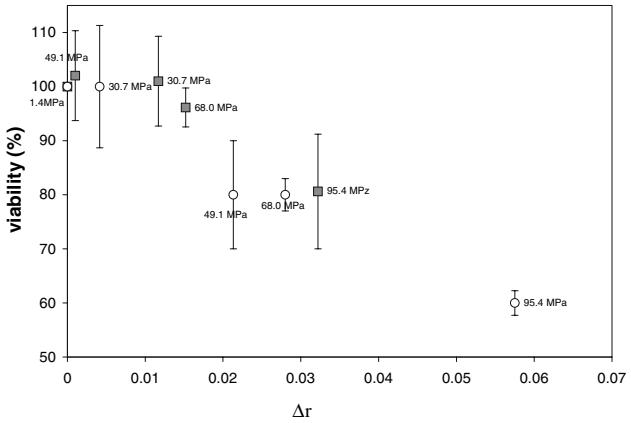

Fig. 5 Percentage viability measured by CFU after osmotic shocks in water/glycerol solutions and 30 min maintenance under hyperosmotic conditions at the same osmotic pressure as a function of Δr (changes in anisotropy induced by osmotic perturbation). Error bars correspond to confidence intervals at the 0.05 level

conditions (Δr) was calculated for each strain and each osmotic pressure tested and compared to the rate of cell death caused by the stress. Viability is represented *vs.* Δr for the two strains in Figure 5. Our results show that Δr correlates with cell mortality for the two strains. In fact, regarding the results obtained with strain CBS1171, the results shown in Figures 3 and 4 demonstrate that anisotropy was not affected by osmotic shock at 30.7 MPa and that this shock did not cause cell death. However, with higher osmotic pressure shifts, r increased by + 0.02, +0.028 and +0.050 at 49.1, 68.0 and 95.4 MPa, respectively, and cell viability decreased proportionally. For strain ATCC60218, Figure 3 shows that anisotropy remained constant for osmotic pressures ranging 1.4–49.1 Mpa, and such treatments did not provoke any cell death (Fig. 4). For osmotic shifts to 68.0 and 95.4 MPa, increases in r of +0.015 and +0.032 were recorded and viability decreased to 96.1% and 80.6%, respectively. Thus, for the two yeast strains an increase in r higher than 0.015 was correlated with an increase in cell death. A Δr between +0.02 and +0.03 induced around 20% of cell death and an increase in r by +0.057 provoked 40% of cell death.

Yeast Viability Is a Function of Exposure Time under Hyperosmotic Conditions

Yeast viability after hyperosmotic shocks at 49.1 and 95.4 MPa for strain CBS1171 and at 95.4 MPa for strain ATCC60218 was assessed after various periods of osmotic stress. These treatments were chosen because they provoke cell death and are associated with a reversible variation of membrane fluidity. Figure 6 shows viability, expressed as a percentage of the control value, measured after 5, 15, 30 and 60 min of exposure to 49.1 or 95.4 MPa. For strain CBS1171, at 49.1 MPa (Δr = +0.020), viability decreased

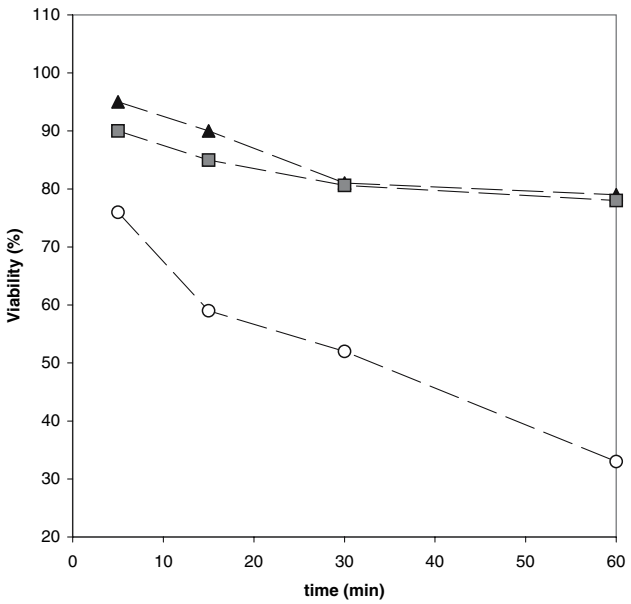

Fig. 6 Effect of maintenance time under hyperosmotic conditions on the viability of yeast after hyperosmotic shocks at 49.1 MPa for strain CBS1171 (▲) and at 95.4 MPa for strain CBS1171 (○) and for strain ATCC60218 (■) in water/glycerol solutions, measured by CFU

to 80% after both 30 and 60 min of exposure. At 95.1 MPa (Δr = +0.05), viability decreased to 75% during the first 5 min of exposure, reaching 50% and 35% after 30 and 60 min of exposure, respectively. Thus, the greater the osmotic shock, the faster the decrease in viability. For strain ATCC60218, an osmotic shock at 95.4 MPA (Δr = +0.032) led to a decrease in viability of 20% during the first 30 min of exposure, as for an osmotic shock to 49.1 MPa (Δr = +0.020) for strain CBS1171.

Table 1 Estimation of the energy brought to bear on one cell by the four physical perturbations studied

	High-pressure treatment	Osmotic pressure treatment	Thermal treatment 1	Thermal treatment 2
Energy brought by cell (J)	$4.16 \pm 0.8 \times 10^{-9}$	$5.65 \pm 0.4 \times 10^{-9}$	$1.16 \pm 0.01 \times 10^{-8}$	$5.80 \pm 0.01 \times 10^{-9}$

Thermal treatments were considered as adiabatic, and calculations were performed on a model spherical yeast cell with a 5-μm diameter and the physical characteristics of water. Temperature precision was estimated at ± 0.2°C. The high-pressure treatment was considered to be isothermal

Discussion

In this study, the membrane fluidity of the yeast *S. cerevisiae* strain CBS1171 was measured before and after three kinds of lethal physical stresses: heat shock, high-pressure treatment and osmotic shock. From the results (Figs. 1 and 2), only the strongest heat shock at 75°C induced an irreversible change in membrane fluidity. In this case, the DPH anisotropy of the dead cells was lower than that of the membranes of living cells. For all the other treatments, membrane fluidity recovered its initial value after the return to initial conditions. These results indicate that cell death is associated with a permanent modification to the membrane in cases of strong physical stress but with a transient and reversible modification in cases of mild physical perturbations. To understand the occurrence of previous reversible or irreversible membrane changes measured by DPH anisotropy, the energy applied in each physical treatment investigated was estimated.

Energy Brought by Physical Treatments Is Linked to the Reversibility of Membrane Anisotropy

Damage to the yeast membrane following the different perturbations was compared by evaluating the corresponding energy brought to one cell. These energies were calculated for high-pressure and thermal treatments without distinguishing work and heat and considering that the energy supplied to the cell was constant during the treatment time. For each treatment, the initial conditions were defined as follows: temperature = 25°C, pressure = atmospheric, osmotic pressure = 1.4 MPa. Yeast was considered to be a water sphere of 5 μm diameter (Perrier-Cornet, Hayert & Gervais, 1999).

For the heat treatment, equation 1 was used to calculate the corresponding energy.

$$E_1 = m\,Cp_{water}\Delta T \quad (1)$$

where Cp_{water} is the heat capacity of water, expressed in $J \cdot kg^{-1}\ K^{-1}$; m is the mass of one cell, expressed in kilograms; and ΔT is the change in temperature induced by the heat shock, expressed in Kelvin.

For the high-pressure treatment, considered as isothermic, the energy induced by the volume contraction of the cell was estimated using equation 2:

$$E_2 = |P|\Delta V \quad (2)$$

where P is the hydrostatic pressure, expressed Pascal, and ΔV is the change in cell volume, expressed in meters cubed.

For the hydrostatic pressure treatment at 300 MPa, the final cell volume was estimated to be 75% of the initial volume after 10 min, corresponding to the average volume compression measured by Perrier-Cornet et al. (1999).

For the osmotic treatment (144.5 MPa), the instantaneous energetic shift was estimated using Boyle-van't Hoff's law:

$$E_3 = \Delta\pi(Vi - b) \quad (3)$$

where $\Delta\pi$ is the osmotic shift (Pascal), Vi is the initial volume of the cell (meters cubed) and b is the nonosmotic volume of the yeast cell (40% of Vi, according to Gervais & Beney [2001]).

The results, expressed in joules per cell, are presented in Table 1. Comparison of these energy values shows that the energy brought to the cell by heat treatment of 75°C is two times higher than that brought by thermal stress at 50°C, 2.8 times higher than that brought by the high-pressure treatment and two times higher than that brought by the osmotic treatment. Thus, the permanent membrane modifications observed as a consequence of this thermal stress could be related to the high energy levels brought to cells and correspond to irreversible molecular structural changes.

Nature of Reversible and Irreversible Membrane Phenomena

According to several authors (Despa et al., 2005; Lepock, 2005), protein denaturation and aggregation are the main responses to a severe heat shock. Despa et al. (2005) reported that high temperatures (60–80°C) might lead to the formation of intramembranous protein aggregates in human cells. Therefore, in the case of a heat shock at 75°C, which is a highly energetic lethal stress with regard to the energy brought to one cell (*see* Table 1), proteins may have been strongly affected. When a protein unfolds, hydrophobic regions that were located in the interior become exposed. The possible translocation of DPH from the membrane lipidic core to proteins adsorbed to the cell surface has already been shown, and protein anisotropy values were

found to be considerably higher than any lipidic anisotropy value (Mely-Goubert & Freedman, 1980). Thus, our results could be interpreted as an increase in DPH accessibility to these hydrophobic areas, which were exposed by the treatment. This hypothesis is reinforced by the strong increase in DPH fluorescence intensity observed after a thermal treatment at 75°C (*data not shown*), which may point out a modification of the probe microenvironment. The permanent increase in DPH anisotropy could also be related to the direct influence of protein unfolding on the decrease in the mobility of membrane lipids. These hypotheses need to be investigated.

After other lethal physical treatments, i.e., mild thermal, osmotic and high-pressure treatments, DPH anisotropy recovered its initial value (*see* Figs. 1 and 2). It can be inferred that, during such stresses, any structural transition occurring in membrane lipids or proteins is reversible. In fact, according to Shimada et al. (1993), high hydrostatic pressure treatments of *S. cerevisiae* at 100–400 MPa only slightly damaged membranes and cell walls. The cell's outer shape, when treated with pressures of up to 300 MPa, is almost unaffected, as visualized by transmission electron microscopy. A few studies have described the plasmalemma changes in *S. cerevisiae* that occur after heat stress at 50°C. Only time-dependent phenomena have been described, including occasional membrane fusion with the tonoplast after heat stress at 52°C for 5 min (Webster & Watson, 1993) and the loss of membrane protein activity at 51°C (Felix et al., 1999). By analogy and considering the energy levels involved (Table 1), we deduce that the damage caused by a drastic osmotic shock (5.65×10^{-9} J/cell) is closer to that caused by high pressure (4.16×10^{-9} J/cell) or mild heat shock at 50°C (5.80×10^{-9} J/cell). Reversible membrane modifications could be lipid phase transitions and/or reversible protein denaturations. To confirm these assumptions, measurements of the DPH fluorescence lifetime would be helpful to improve our knowledge of the membrane state after physical treatments.

Transient Membrane Anisotropic Changes and Cell Death during Hyperosmotic Stress

Membrane anisotropy was investigated during the application of several hyperosmotic perturbations for two different *S. cerevisiae* strains. Membrane fluidity, measured for each osmotic pressure tested, was different for the two strains studied; and whatever the osmotic pressure, the membrane of strain CBS1171 was more fluid (Fig. 3). It may be due to the different composition of lipids and proteins in the membranes because of the genetic differences between the two strains. Moreover, the growth temperature was higher for strain ATCC60218 and may have induced an increase in the degree of saturation in membrane lipids (Hazel, 1995) and thus a decrease in membrane fluidity at 25°C. The results presented in Figure 3 also indicate that osmotic shocks provoke a rigidification of the membrane for osmotic pressures higher than 49.1 MPa for strain CBS1171 and higher than 68.0 MPa for strain ATCC60218. Few studies report measurement of membrane fluidity during the course of a stress. This increase in DPH anisotropy as a response to an increase in osmotic pressure has only been observed in phospholipid vesicles (Yamazaki, Ohnishi & Ito, 1989) and yeasts (Laroche et al., 2001). Furthermore, it has been correlated with the occurrence of a liquid-to-gel phase transition in the membrane lipids of *S. cerevisiae* (Laroche et al., 2001, 2005).

Thus, phase transition may occur progressively with increasing osmotic pressures above 30.7 MPa for strain CBS1171 and above 49.1 MPa for strain ATCC60218, resulting in a progressive variation in DPH anisotropy. Also, lipid phase transition could take place over a large range of osmotic pressure levels because the anisotropy did not reach a plateau for the osmotic pressure levels tested in this study. This could mean that the membrane enters into a phase separation state above 30.7 MPa for CBS1171 and above 49.1 MPa for ATCC60218, with an increasing proportion of the lipids in the gel state as the osmotic pressure increases.

Besides, survival of the two yeast strains was assessed after osmotic treatments with the same magnitude and subsequent rehydration to 1.38 MPa. Results, presented in Figure 4, show that the survival rates measured after the same osmotic treatment for the two strains were different. Particularly, treatments were lethal for osmotic pressures higher than 30.7 MPa for CBS1171 and higher than 68.0 MPa for ATCC60218. This corresponds precisely to the osmotic treatments that involve transient fluidity variations of plasma membranes. Moreover, for these lethal treatments, viability was correlated to the magnitude of the membrane fluidity variation Δr (*see* Fig. 5). Thus, the more the plasma membrane was forced and maintained in a phase separation state, the more cells died, regardless of the initial fluidity of the membrane.

The link between the membrane phase transition and mortality during dehydration has been well established in the past. Van Steveninck & Ledeboer (1974) proposed that a gel-to-liquid-crystal phase transition occurs when *S. cerevisiae* is rehydrated at temperatures below 38–40°C and consequently provokes imbibitional damage. Laroche & Gervais (2003) studied the rate of cell death of *S. cerevisiae* induced by osmotic shock at different temperatures and concluded that changes in osmotic pressure when crossing a phase transition of the plasma membrane would result in membrane injury and cell death. Our results include a new observation of increasing cell death associated with the maintenance of the membrane in phase separation, with an increasing proportion of the lipids in the gel state.

Maintenance of the Membrane in a Transient State during Mild Perturbations Is Associated with Time-Dependent Cell Death

Cellular death depended on the time of maintenance under hyperosmotic conditions for the two yeast strains studied. Figure 6 shows that, for lethal osmotic pressure levels, viability decreased with an increase in the holding time under hyperosmotic conditions. Moreover, the higher the fluidity variation involved by the treatment, the faster the reduction in viability.

This time-dependent rate of cell death was also observed relative to the time of maintenance at 50°C after a heat shock by Guyot, Ferret & Gervais (2005) and relative to the time of maintenance under high pressure at 200 MPa by Hayert, Perrier-Cornet & Gervais 1997) for CBS1171. The results of these studies are reported in Figure 7 for a comparison with our results for cell death as a function of the time of maintenance under hyperosmotic conditions at 95.4 MPa. For the high-pressure and osmotic pressure treatments, the viability decrease was maximal during the first 15 min of treatment, whereas for the thermal treatment at 50°C, viability decreased dramatically after 15 min of treatment. Therefore, cell death appears to be a time-dependent event for these three physical treatments.

It can be inferred that the r value was transiently modified during each stress studied. A rigidification of the lipidic part of the membrane has been observed previously with increasing hydrostatic pressures (Chong & Cossins, 1983; Kato & Hayashi, 1999; Molina-Hoppner et al., 2004). A variety of pressure-induced phase transformations can occur in model biomembrane systems, particularly different kinds of gel phases (Winter & Dzwolak, 2005). In the case of heat stress, the transient state of the plasma membrane during the stress is different as the DPH anisotropy diminishes with increasing temperatures (Lentz, Barenholz & Thompson, 1976; Los & Murata, 2004), corresponding to an increase in fluidity.

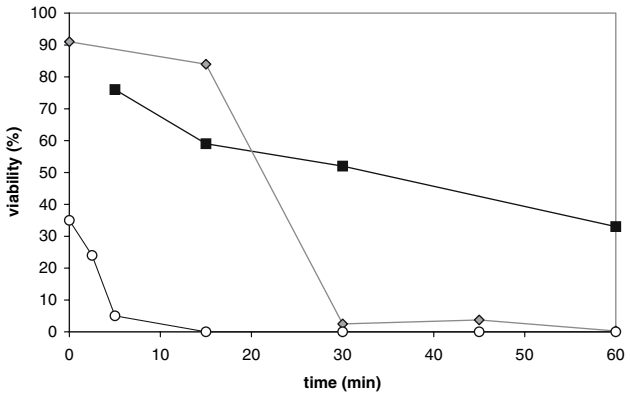

Fig. 7 Effect of maintenance time under supraphysiological thermodynamic conditions on the viability of the yeast *S. cerevisiae* CBS1171 at 95.4 MPa after a hyperosmotic shock (■), at 50°C after a heat shock (♦) (Guyot et al., 2005) and at 200 MPa after a high-pressure treatment (○) (Hayert et al., 1997) (CFU method)

Thus, whatever the physical stress applied to the cell, the maintenance of the membrane in a state differing from the optimal state induces time-dependent cell death. Moreover, this modified membrane state was different for the three stresses studied. A temperature increase induces an increase in membrane fluidity, whereas increases in hydrostatic or osmotic pressure induce a rigidification of the lipidic part of the membrane.

Hypothesis on the Mechanisms of Cell Death Due to Osmotic Perturbations

Cell death during the application of a hyperosmotic stress of sufficient intensity is time-dependent and associated with the maintenance of the plasma membrane in a transient state, namely a phase separation state, at high osmotic pressures.

On the one hand, phase transition may affect transmembrane permeability and the resistance of the membrane to shear forces (Crowe et al., 1992; Garcia-Manyes, Oncins & Sanz, 2005; Sparr & Wennerstrom, 2001). Thus, as a first assumption, cell death can be attributed to the time-dependent leakage of cell components through a modified membrane. This hypothesis has already been proposed by Bischof et al. (1995) concerning cell death at supraphysiological temperatures. In such a case, the membrane is maintained in a hyperfluid state. In the case of hyperosmotic shock, not only is the membrane in a transient state (lipid phase transition) but the cells are also contracted. It is well known that cells shrink in response to osmotic stress. Adya et al. (2006) have associated the surface topology of *S. cerevisiae* (irregular shape, surface roughness) and volume loss to the cell's resistance to both thermal and osmotic stress. Slaninova et al. (2000) reported the observation of deep invaginations in the plasma membrane and bulges in osmotically stressed yeasts. Such conditions of shrinkage associated with lipid phase separation, with an increasing proportion of lipids in the gel state, could lead to plasma membrane permeabilization and a time-dependent leakage of cellular components.

On the other hand, phase transition may also affect membrane proteins. Protein function is influenced by the fluidity of the surrounding lipids (Epand, 1998; Meder et al., 2006). Phase separation can induce protein diffusion to more fluid phases, leading to their aggregation and the modification of membrane resistance (Crowe, Crowe & Jackson, 1983). An increasing proportion of the phospholipids in the gel state would thus increasingly affect more membrane proteins. A second hypothesis could thus

involve the occurrence of a metabolic disequilibrium, caused by the malfunctioning of proteins. Furthermore, proteins are directly affected by physical perturbations. The displacement of their folding–unfolding equilibrium or their irreversible denaturation could also induce time-dependent cell death.

Conclusion

By measuring membrane fluidity during and after different physical perturbations, we have shown that causes of cell death related to membrane changes are different according to the level of energy brought to the cell by a physical treatment. In the case of a high-energy stress, permanent denaturation of membrane macromolecules is concomitant with a rapid occurrence of the whole cell population death. In the case of a mild-energy stress, a transient plasma membrane fluidity variation leads to a time-dependent cell death. The rate of cell death increases more slowly in the former case.

Considering the energy level brought to bear on the cells, the application of osmotic gradients could be compared with mild heating and high-pressure treatments. By measuring membrane fluidity during osmotic stresses of increasing intensity, the mechanism of cell death induced by osmotic perturbations could be approached and demonstrated with two different yeast strains. In fact, the rate of cell death after a given exposure time to an acute osmotic stress is proportional to the fluidity variation undergone by the plasma membrane independently of the strain used. Thus, it seems that the maintenance of the membrane in a transient state of lipid phase separation leads to time-dependent cell death. An increase in membrane permeability resulting from either phospholipid phase transition or reversible protein denaturation is now under investigation.

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