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Glucose as a Potent Inducer of Cell Death in Yeast

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Abstract: The aim of the present work was the study of the apoptotic mechanism induced by glucose in *Saccharomyces cerevisiae TPS1* deletion mutant. The *TPS1* gene encodes the Trehalose-6-phosphate synthase. When cells of this mutant were exposed to glucose, they lost progressively the capacity to proliferate but maintained the membrane integrity for much longer time, what was evaluated by using the fluorescent probes oxonol and propidium iodide. The overexpression of *pPDE2* recovered viability of the *tps1*Δ mutant to a high extent when compared to the wt. After glucose addition, the presence of apoptotic and/or necrotic phenotypes was analyzed by the detection of accumulated intracelullarly reactive oxygen species (ROS), assessed by 2′,7′-dichlorodihydrofluorescein diacetate and DihydroRhodamine 123, by the presence of DNA fragmentation evaluated by TUNEL assay, phosphatidylserine (PS) externalization visualized by the fluorescent Annexin-V binding and finally, by the release of cytochrome c to the cytosol, detected by western blot.

Altogether, the results obtained indicate that exposition of yeast cells with *TPS1* gene deleted to different glucose concentrations, from 5 to 100 mM glucose, results in growth arrest originated by apoptotic cell death, rather than necrotic death.

Keywords: *Saccharomyces cerevisiae,* glucose signaling, cAMP pathway, ROS production, apoptosis and necrosis.

INTRODUCTION

Programmed cell death (PDC) defines all modes of death under molecular control and carried out in a controlled manner. Apoptosis has been classified as PCD and the role of apoptosis in multi-cellular organisms has been documented extensively. The molecular hallmarks of apoptosis in cells of higher eukaryotes have also been discovered in cells of microbial microorganisms such as yeast. This has led to suggestions for a functional role of apoptosis in colonies or multicellular aggregates of single-celled microorganisms. The apoptotic markers in yeast can be triggered by a rather diverse array of unrelated compounds (Carmona-Gutierrez et al., 2010). Recently, evidence suggested that necrosis, first described as an accidental uncontrolled form of death, can be regulated in yeast and also occurs under normal physiological conditions like aging. A variety of agents capable of inducing yeast apoptosis at low doses could shift cell death to a rather necrotic phenotype when used at higher concentrations (Eisenberg et al. 2010).

Our previous results show that glucose, the most-preferred carbon substrate of many microorganisms, is a potent inducer of apoptosis in yeast cells in which downregulation of hexokinase activity is compromised. Yeast hexokinase is inhibited by trehalose-6-phosphate, the product of the Tps1 enzyme. Absence of the inhibitor causes rapid expression of classical apoptotic markers followed by cell death upon addition of glucose (unpublished results). Deletion of the *HXK2* gene, encoding the major yeast hexokinase, prevents the glucose-induced apoptotic cascade. Interestingly, this suggests a process in yeast very similar to the well-known involvement of hexokinase and mitochondrial

respiration in the control of apoptosis in mammalian cells. Our results also show that downregulation of the yeast Ras-cAMP-PKA pathway suppresses to some extent apoptosis and cell death in the *tps1*Δ. This suggests that hyperphosphorylation of sugar by hexokinase triggers apoptosis by overactivation of this signaling pathway. The Ras-cAMP-PKA pathway is well known to act as trigger of apoptosis and cell death in yeast (Gourlay and Ayscough, 2006)

The Tps1 control of hexokinase was discovered because of the inability of *tps1* mutants to grow on glucose and related rapidly-fermented sugars. Addition of glucose to *tps1*Δ causes hyperaccumulation of sugar phosphates and depletion of ATP and free phosphate (Neves et al. 1995). This metabolic deregulation has logically been thought off as the reason why *tps1* mutants arrest growth after addition of glucose but it poorly explains the extreme sensitivity to even low levels of glucose. Our results show that glucose addition to cells of a *tps1* mutant growing on galactose, not only causes growth arrest but rapid loss of viability. This loss of viability is associated with rapid and strong expression of classical apoptotic markers: cytochrome c release, dramatic formation of reactive oxygen species (ROS), phosphatidylserine exposure and chromatin fragmentation.

Yeast mutants with an overactive Ras-cAMP-PKA pathway are well known to rapidly loose viability under conditions of slow growth or growth arrest. This is associated with appearance of typical apoptotic markers. This finding has identified the Ras-cAMP-PKA pathway as a central controller of yeast apoptosis but the connection with glucose as a trigger of this pathway (Hlavatá et al. 2008) has never been made. In the absence of the GPCR system, glucose is able to trigger partial cAMP signaling through this glucose phosphorylation-dependent pathway. Although the mechanism involved is unclear it may be due to a partial stimulation of Ras activity being required for sensitization of adenylate cyclase to stimulation by Gpa2 (Colombo et al. 1998). The growth defect on glucose of the tps1 mutant can be suppressed by lowering the activity of the cAMP-PKA pathway. This was accomplished by overexpression of the high-affinity cAMP phosphodiesterase, encoded by PDE2, in the tps1 mutant. These results suggest that unbridled hexokinase activity triggers apoptosis by overstimulation of the cAMP-PKA pathway, possibly by enhancing Ras activity.

The objective of this research was to identify cellular markers that would allow the characterization of the mode(s) of cell death induced by glucose in *tps1*Δ and also to study the correlation between glucose, Ras activation and apoptotis in the yeast. It will be also interesting to investigate if necrotic cell death is involved. Other proteins of the fungal apoptotic network already described as key proteins in regulating apoptotic-like cell death, will be studied as well by constructed new deletions mutants will be for this purpose.

MATERIALS AND METHODS

Strains and Growth Conditions The yeast strains used were all isogenic to W303-1A. The YEplac 195 expression vector contains the promoter and terminator sequences of the 5' and 3' regions of the PDE2 gene. Strains were grown on standard yeast extract/peptone (YP) media or yeast nitrogen base/ammonium sulphate media supplemented with 2% carbon source, as indicated (Sherman et al., 1983).

Yeast cells were maintained on YP agar plates containing galactose (2%, w/v), yeast extract (1%, w/v), bactopeptone (2%, w/v) or a selective synthetic minimal media appropriated for the auxotrophic marker and agar (1,5%, w/v). In experiments, cells were

subcultured in liquid YPGal and incubated at 30 ℃ and 200 r.p.m. until mid-exponential phase.

Drop Test and Clonogenic Assay Cells suspensions were diluted in 1 ml of water to an OD600 of 1. The suspension was sequentially diluted (10 times each dilution) until an OD600 of 0.001. For the drop test 5 µl of each dilution was spotted on YPGal and YPD solid media containing different concentrations of the carbon source (from 1, 2, 5, 10, 20, 50, 100 mM). For the clonogenic assay samples were taken after 0h, 2h, 4h, 6h, 8h and 24h of glucose addition (glucose final concentration of 5, 20, 50 and 100 mM). 100 µl of the most diluted suspension were plated in YPGal (three replicates) and viability was determined by c.f.u. counts. Plates were incubated at 30℃ for 2 to 3 days. The percentage of viable cells was calculated using the number of colony-forming units (c.f.u.) at zero time as reference (100%). The values are the average of three independent experiments.

Isolation of Released Cytochrome c (adapted from Tzagoloff *et al.*, 1975). After incubation with glucose for the desire time, cells were harvested by centrifugation at 2500 g and 4ºC for 10 min and washed once with 1.2 M sorbitol. The cells were weighted and resuspended in 5 ml of Digestion buffer (1,2 M Sorbitol; 60 mM Potassium Phosphate pH 7,5; 1 mM EDTA; 15 mM Mercaptoethanol; 1 mg Zymolyase 20T) per g of cells (wet weight) and the suspension was incubated for 40 min to 1 h at 30℃ to digest the cell wall. The spheroplasts were collected at 1000 g for 10 min with soft brake, gently washed two times with 1.2 M sorbitol and resuspended in 2.5 ml of ST-PMSF buffer (1,2 M Sorbitol; 20 mM Tris HCl pH 7,5; 1 mM EDTA; 0,2 M PMSF; 1 tablet Protease inhibitor/10 ml (Roche)) per g cell (wet weight). The suspension was transferred to a Wheaton dounce and stroked until the spheroplasts were broken. Homegenates were centrifuged at 2500 g for 10 min then the supernatant was collected and centrifuged at 12000 g for 15 min at 4℃ to sediment mitochondria. The post-mitochondrial supernatant (PMS) was transferred to new tube and the mitochondrial pellet was resuspended in 500 µl ST-PMSF buffer. Samples were centrifuged again at 12000 g for 15 min and the final mitochondrial pellet resuspended in 250 µl ST-PMSF buffer. Both fractions were stored at -20º until analysis by Western Blotting. The anti-cytochrome c antibody was custom made (Eurogentec, 1:5000 dilution (v/v) in 5% milk in TBST), and the anti Cox II antibody was from Mitosciences (MS419, dilution of 1:200 (v/v) in 5% milk). The secondary anti-rabbit antibody for cytochrome c and anti-mouse for Cox II, horseradish peroxidase-conjugated anti IgG, used and detected by enhanced chemiluminescence.

Evaluation of S-nitrosylation of GAPDH After o.n. incubation at 30℃, 100 mM glucose or 2 mM DETA/NO donor were added to the proper samples and the culture was further incubated for 2 h. Then cells were harvested by vacuum filtration, washed with ice-cold PBS 1x, collected in an eppendorf and were immediately frozen in liquid nitrogen. The pellets were thawed in ice and resuspended in 500 µl of lysis buffer (1% Triton X-100; 20 mM NaCl; 50 mM Tris-HCl pH 7,4; 2 mM EDTA; 10% Glycerol; Protease inhibitor; 1 mM PMSF). One volume of glass beads was added to break the cells by vortexing, seven times for 1 min each with intervals of 1 min on ice. The homogenate was centrifuged at 2000 g for 5 min at 4℃. The supernatant centrifuged again at 20000 g for 30 min at 4℃ and protein concentrations were quantified. For the immunoprecipitation of nitrosilated proteins, 3 µl of the antibody rabbit anti-S-nitrosocysteine (Sigma-Aldrich) were added to 500 µg of protein lysate in a total volume of 500 µl. The suspensions were incubated for 4h at 4℃ with rotation (as a negative control, 500 µl of lysate were incubated without antibody). The samples were then added to 20 µl of washed protein G plus/protein A-agarose beads and gently rocked with the beads-Ab complex o.n. at 4℃. The agarose beads were collected by centrifugation at 1000 g for 1 min at 4℃ and washed twice with 1 ml of each ice-cold lysis buffer and wash buffer. The elution of the beads was performed in 20 µl of sample buffer and boiled for 5 min. The beads were collected by centrifugation at 20000 g for 1 min and the supernatant analysed by Western Blot. The monoclonal anti-GAPDH mouse antibody (MAB374, Chemicon) has a dilution of 1:200 (v/v). Horseradish peroxidase-conjugated anti-mouse IgG secondary antibody was used and detected by enhanced chemiluminescence.

Measuring Protein Concentration The protein concentration of the samples was determined by the Thermo Scientific Pierce 660 nm Protein Assay. The standard curve was designed by different known concentrations of a BSA (Bovine Serum Albumine) solutions and water was used as blank. 300 µl of Pierce® reagent was added to 20 µl of sample (two replicates) and absorbance was measured at 660 nm using Spectra Max Plus 384 (Molecular Devices, Brussels) after 5 min incubation in a 96 well cuvette.

Blotting Technique Protein samples with the dye were boiled for 5 min and pulled down before applying on gel. Protein samples were resolved by pre-prepared SDS-PAGE (NuPAGE® 4-12% Bis-Tris gel 1.0 mm x 10 wells, Invitrogen) with 1xSDS Running buffer (NuPage® 20x, Invitrogen) at 150V (Power PAC 300) for 1.5 h and blotted on nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, UK) in a blotting buffer (20% methanol; 1x MOPS running buffer) at 300 mA for 1.5 h, placed inside a western blot chamber (BIO-RAD Mini Protean®3Cell). The membranes were washed once with TBS and blocked for 2 h with 5% milk in TBST buffer at r.t. Then they were incubated o.n. with the primary antibody in milk TBST solution at 4℃ with gentle shaking. Then membranes were washed three times (10 min) with TBST buffer (1 x TBS; 0.05% v/v Tween 20) and incubated for 1 h with secondary antibody (1:5000 dilution (v/v) in 5% milk solution) at r.t. with gentle shaking. Once more, the membranes were washed three times (10 min) with TBST buffer. The detection was achieved by rapidly incubating the membranes with SuperSignal® WestPico Chemiluminescent (Pierce Chemical, Rockford), 1:1 peroxide and luminal solution. Subsequently visualization was performed using Las-4000 mini Luminescent Image Analyzer apparatus (Fuji Film) and the software Las-4000 image reader. The data obtained was then analyzed through the program AIDA (advanced image data analyzer) version 4.22.034.

Evaluation of Reactive Oxygen Species Accumulation Two different ROS types formation were visualized by adding cell permeant appropriate dyes: DCDHF–DA and DHR 123. Hydrogen peroxidase formation was detected by using Dihydrorhodamine 123 (DHR 123, Invitrogen). Different glucose concentrations and 2 µl/ml of DHR 123 or 1 µl/ml of H_2DCF-DA were added and the cultures (OD_{600} of 1) were further incubated at 30℃ for the desired time. ROS accumulation was visualized through Axioplan 2 imaging from Zeiss, using the filter set 10 (excitation BP 450-490 nm, emission BP 515-565 nm). Superoxide anion formation was detected by using 2'7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Invitrogen).

Phosphatydylserine Externalization. Different concentrations of glucose were added to the cultures according to the experiment and the cells were incubated at 30℃ for the desired time. Then the samples were transferred to a pre-weighted falcon and centrifuged at 2500 g for 10 min. The supernatant was discard and the pellets were washed with sorbitol 1,2 M. The supernatants were discarded, the falcons dried and the cells weighted. The cells were resuspended in 5 ml of Lyticase buffer (1,2 M Sorbitol; 35 mM Potassium buffer pH 6,8; 0,5 mM $MgCl_2$; Lyticase 120 U/ml) per g of cells and the suspension was incubated for 40 min to 1 h at 30℃. The spheroplasts were collected by centrifugation at 1000 g for 10 min with soft break, gently washed with 50 ml of sorbitol 1.2 M and centrifuged once more at 1500 g for 5 min. The supernatants were discarded with a tip and washed with 500 µl Annexin buffer (1,2 M Sorbitol; 10 mM HEPES/NaOH pH 7,4; 140 mM NaCl; 5 mM $CaCl_2$). The pellets were then resuspended in 250 µl Annexin buffer and and 38 µl of this suspension were incubated with 2 µl Annexin-V-FLUOS and 2 µl PI for 20 min in the dark(staining kit from Roche). The cells were centrifuged at 1500 g for 2 min and washed with Annexin buffer, ressuspended and 5 µl were applied to a slide. Externalization of phosphatydylserine residues was visualized through Axioplan 2 imaging from Zeiss (AxioVisio 4.6). Annexin-V-FLUOS excitation and emission wavelengths are 488 nm 518 nm, respectively. Propidium iodide excitation and emission wavelengths range are 488 to 540 nm and 617 nm, respectively.

TUNEL Assay The detection of DNA fragmentation was made by Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay. Glucose was

added in different concentrations and the cultures were further incubated for 4h. Cell fixation was done by adding 268 µl of formaldehyde 37% (w/v) and samples were incubated for an extra hour. Cellular suspensions were centrifuged at 2500 g for 5 min, supernatants discarded and pellets washed with 5 ml of 0.1 M potassium phosphate buffer pH 7.5 containing 1.2 M sorbitol. Then the pellets were resuspended in the same buffer with 1.5 µl 2-mercaptoethanol and 1 mg zymolyase 20T per ml and incubated for 40 min at 30℃. The cells were collected after centrifugation at 1000 g for 5 min with low break and washed once with PBS containing 1.2 M sorbitol and resuspended in 1 ml of the same buffer. 30 µl of sample were transferred to a polylisine treated glass slide (Poly-L-Lysine 10 min, washed with water and air dried) and incubated at r.t. for 10 min. After washed three times with 30 µl PBS 1x, 30 µl of sodium citrate were added and samples were incubated on ice for 2 min, washed twice with PBS 1x and incubated again for 1 h at 37℃ with 30 µl of reaction mix (450 µl Label solution with 50 µl Enzyme solution, In Situ Cell Death Detection kit, Roche). Visualization was achieved by fluorescent microscopy (filter set 40 (BP360}51, BP485/17, BP560/18) from Zeiss, excitation filter BP 450-490, beam splitter FT510 and emission filter LP520) after having the samples washed three times with PBS 1x.

As a positive control wt cells were treated with DNAse I in tris HCl pH 7.5, 10 mM $MgCl_2$ and BSA. The negative control was obtained by incubating wt cells with label solution without terminal transferase.

Cell Membrane Integrity Monitoring With the dye Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3); FlouroPureTM,Molecular Probes, USA); a quantitative evaluation of live and dead cells in a population after glucose addition was possible in a flowcytometry study performed using FACScan flow cytometer (FACSCalibur cytometer, Becton Dickinson) equipped with a 488 nm argon lazer at low flow rate and simultaneous scattering and fluorescence signal measurements. An optimized sheath fluid was used (BD FACSFlowTM, BD Biosciences) and the setting were setup: Threshold on FSC at 500 V and FSC and SSC detector voltages were E00 and 300 V, respectively. Fluorescence detectors were adjusted to 600 V and a FL1 filter was applied (530/30 nm).

Samples were taken at defined intervals of time reported in the figures. Cell suspensions diluted in milliQ water until a good flow rate is reached (approximately 100 cells per second) were incubated with Oxonol (1,6 µg/ml of final concentration) in polystyrene round bottom tube (BD Falcon™, BD Biosciences, USA). Samples were then resuspended in a vortex and measured after being incubated for 15 min in the dark at r.t. and further vortex-mixed. Data was obtained and analyzed using the program BD Cell Quest™ Pro software version 4.0.2. A dot plot FSC-H vs FL-1H was represented and percentage of dead cells of each region was calculated after the clouds of cells were gated.

Determination of Ras2 Activity Cells were harvested by centrifugation at 2500 g for 10 minutes and washed with MilliQ water. The cells were weighted and 500 µL of lysis buffer (25mM HEPES; 150mM sodium chloride; 1% Non-Idet P-40; 0.25% sodium deoxycholate; 1mM EDTA; 1mM sodium vanadate; 10% glycerol; 25mM sodium fluoride; 10mM magnesium chloride; 1 tablet of protease inhibitor (Roche)) was added per 400 mg of cells. The cells were broken by 0.3 g of glass beads and 2 times running in Fast prep instrument (Precellys 24) at 25 s each. The cells were centrifuged at 7000 g for 5 min and the supernatant was collected. Then, 500 µL of lysis buffer was added and the protein was quantified. Afterwards, 300 µg of protein were taken and 20 µL of RasBindingDomain (RBD)-glutathione-beads (Ras activation assay Biochem Kit™, Cytoskeleton, Inc.) were added and the total volume was adjusted to 500 µL with lysis buffer, followed by incubation with rotation for 1 h at 4ºC. Afterwards, the samples were centrifuged at 1000 g for 1 min and carefully washed with wash buffer (1x PBS; 0.1% Triton; 2 mM magnesium chloride; 1 mM EDTA; 1 mM DDT) for 3 times. After removing the supernatant just leaving approximately 20 µL, the protein loading buffer was added and the results were obtained by Western Blotting. For the detection of Ras2, Anti-Ras (Santa Cruz Biotechnology, Inc.) with 1:250 v/v dilution was used as primary antibody and a secondary anti-goat antibody (Santa Cruz Biotechnology, Inc.).

RESULTS

Deletion of *TPS1* Causes Growth Defect in Glucose Containing Medium Exposure of *S. cerevisiae tps1*Δ to glucose at different concentrations (1 - 100 mM) resulted in growth affectation with only just 1 mM and growth absence when glucose concentrations exceeded 2 mM. Nevertheless, *tps1*Δ growth was restored when they had the *PDE2* gene overexpressed, although not completely. wt showed no alterations (Fig. 1). Growth curves obtained in liquid medium supported the observations obtained with the spot assay (not shown).

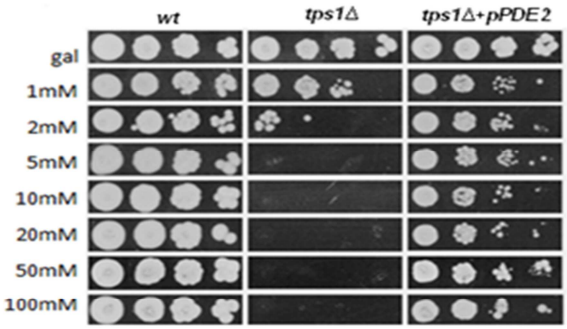

Figure 1 - Growth of wt, *tps1*Δ and *tps1*Δ+p*PDE2* on 100 mM galactose and several glucose concentrations (from 1 mM to 100 mM) in YPagar medium.

***Tps1*Δ Mutant Presents a Glucose Induced Loss of Viability** The percentage of survival, estimated by c.f.u. counts, decreased with increasing glycose concentrations. After 7h, 5h, 4h and 3h of glucose addition to final concentrations of 5 mM, 20 mM, 50 mM and 100 mM, respectively, only 50% of the *tps1*Δ cells. The *tps1*Δ+p*PDE2* cells, in every condition, showed viability higher than 75% of the cells able to form colonies after 24h of glucose addition, while tps1Δ cells showed a percentage of viable cells lower than 7%. Also at 24h after glucose addition, the number of wt c.f.u. dropped to values between 83% and 60%. Probably the low values can be explained by the presence of cell aggregates, therefore forming a reduced number of colonies (Fig. 2). Conversely, the integrity of

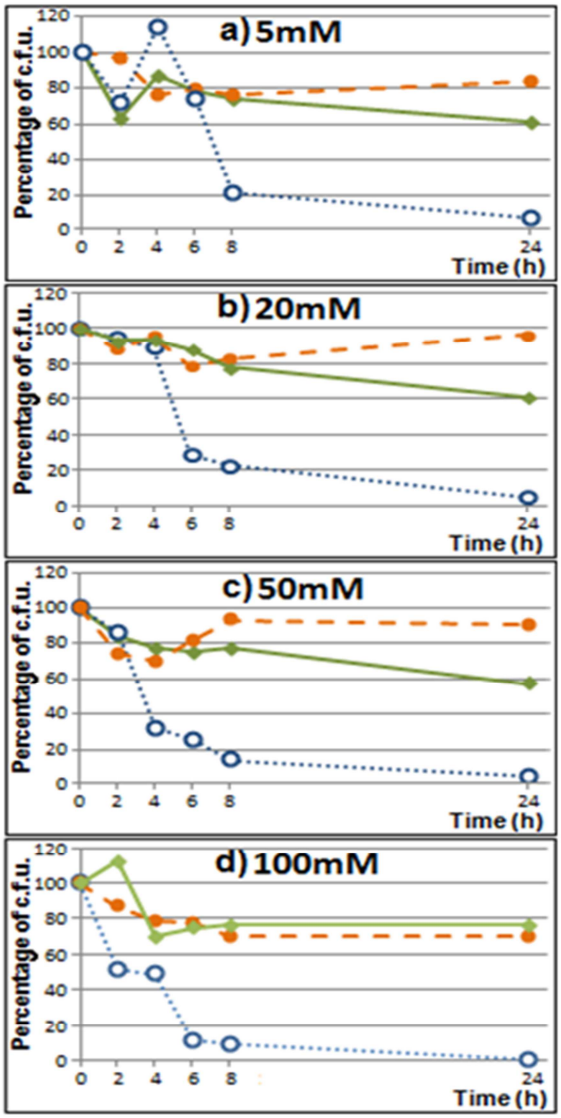

Figure 2 - Clonogenic assay for wt (full line), *tps1*Δ (dotted line), *tps1*Δ+p*PDE2* (dashed line). Glucose was added, at time zero, to obtain different final concentrations: 5 mM (a), 20 mM (b), 50 mM (c) and 100 mM (d). Samples were collected at the time indicated. Viability was estimated by c.f.u. counts. Are presented the most representative results from at least three independent experiments.

*tps1*Δ cell membrane was maintained up to 8h after glucose addition, until when was kept above 80% and dropping to values of around 45%, 24h after glucose addition. No significant reduction of cell membrane integrity occurred (merely about 10%) in wt cells exposed to glucose up to 24 h, when evaluated using Oxonol. The *tps1*Δ+p*PDE2* cells showed a decrease in viability, which was recovered after 8h of glucose addition (Fig. 3).

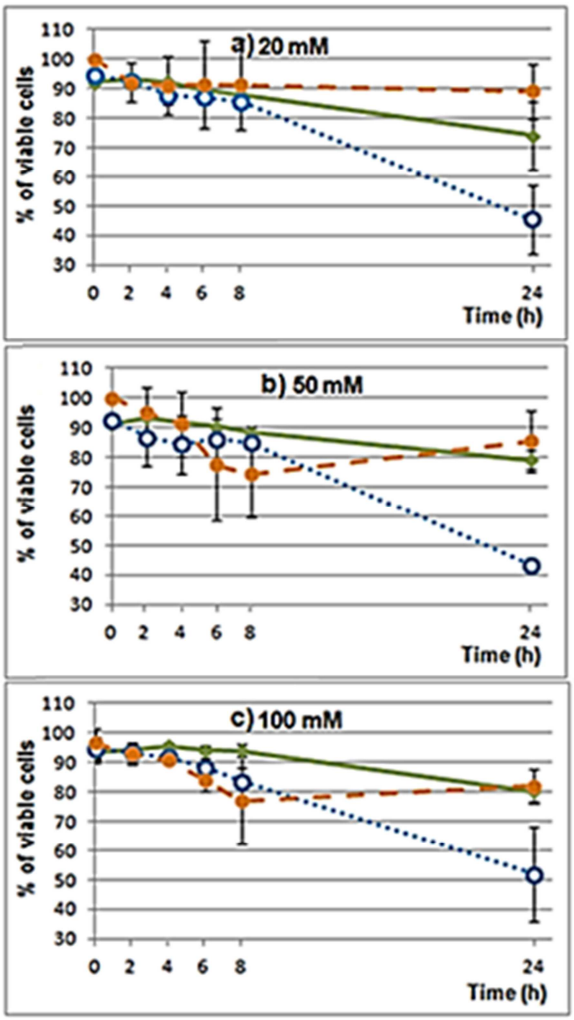

Figure 3 – Cell membrane integrity of wt (full line), *tps1*Δ (dotted line), *tps1*Δ+p*PDE2* (dashed line) during exposure to glucose. Glucose was added, at time zero, to several final concentrations: 20 mM (a), 50 mM (b) and 100 mM (c). Samples were collected at the times indicated. Viability was estimated by depolarized cells: evaluation of loss of plasma membrane integrity assessed with Oxonol. Are presented the mean values from at least three independent experiments.

***Tps1*Δ Mutant Presents Apoptotic Markers after Glucose Addition** Samples of cellsuspension treated with glucose were assayed for different apoptotic markers, namely ROS accumulation, PS externalization, DNA fragmentation and cytochrome c release. Intracellular accumulation of ROS leads to cell damage; in addition, ROS also act as cell death regulators and its presence has been linked with the phenomenon of apoptosis in yeast cells (Carmona-Gutierrez et al. 2010). With the help of DHR123, it was observed that after 2 h of glucose exposition, ROS were intracellular accumulated in the majority of the *tps1*Δ cells (Fig. 4Ac); conversely, wt cells evidenced no fluorescent (Fig. 4Aa). The exposure of PS at the outer surface of the cytoplasmic membrane occurs at the early stages of apoptosis (Martin et al., 1995) when membrane integrity is still retained. Therefore, using simultaneous staining with Annexin-V and the membrane impermeant fluorochrome PI, those early apoptotic cells only stain green, indicating the presence of phosphatidylserine at the outer surface of the plasmamembrane (annexin V (+), PI (-), Fig. 4Ba). Yeast cells in more advanced apoptotic stages, or necrotic cells, stain green and red due to the inability of the cell membrane to exclude PI (Annexin V (-), PI (+), Fig. 4Be). wt cells did not exhibit any fluorescence. Treatment of *tps1*Δ with 50 and 100 mM glucose resulted in a TUNEL-positive (Fig. 4c), indicating the occurrence of DNA strand breaks. Cytochrome c analysis revealed that it is exclusively released into cytoplasm in *tps1*Δ cells subjected to 20 mM and 50 mM glucose concentrations (**Error! Reference source not found.** 4D), in contrast to wt and *tps1*Δ+p*PDE2* cells (not shown). Apparently, with 20 mM of glucose, cytochrome c release in *tps1*Δ is only detected after 3h of glucose addition and is maintained up to 5h, while with 50 mM it is detected between 1h and 4h after glucose addition.

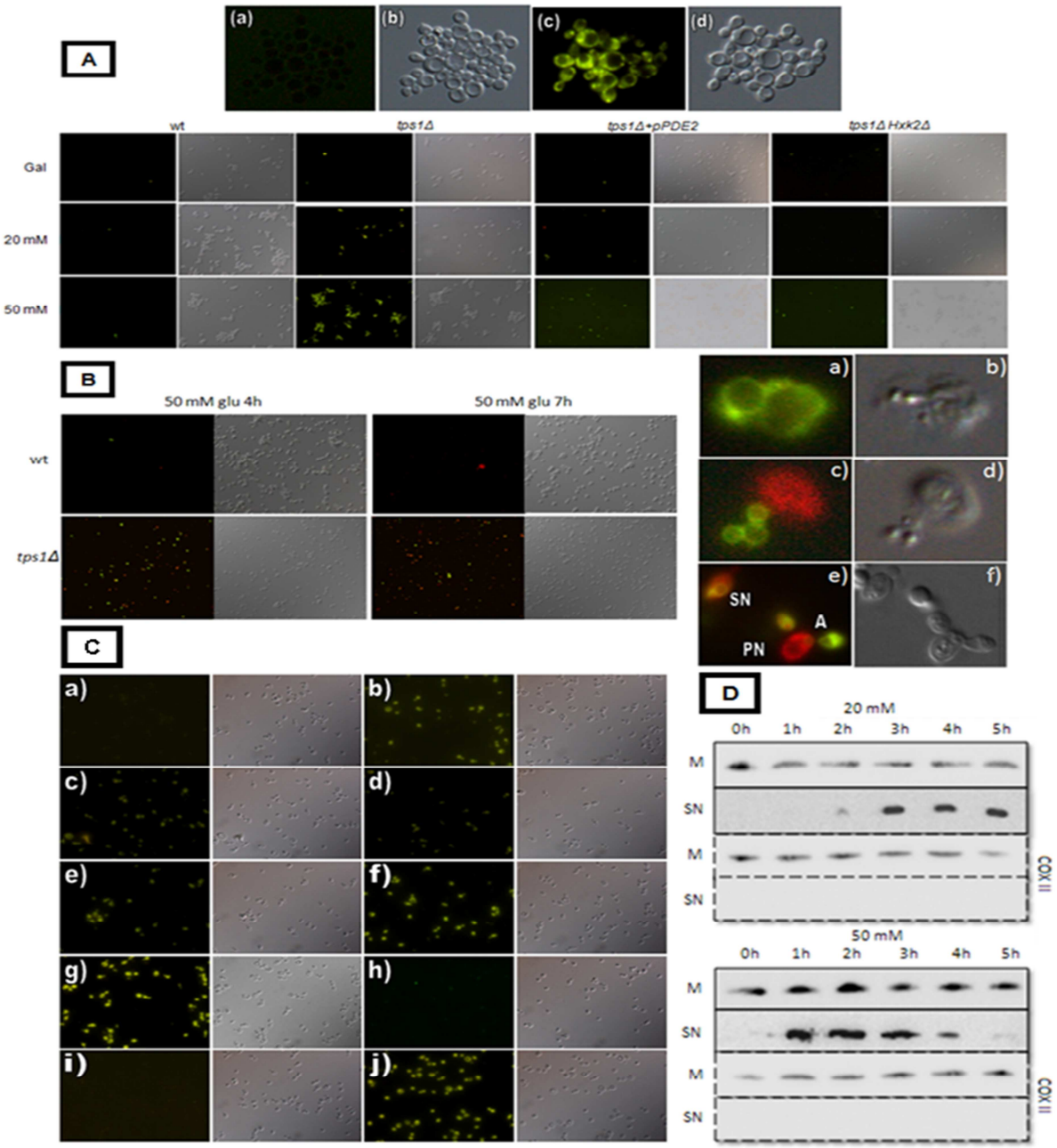

Figure 4 – A: Visualization of ROS production in wt, *tps1*Δ, *tps1*Δ*+pPDE2* and *tps1*Δ *Hxk2*Δ mutants, assessed by DHR123 after glucose addition to two final concentration. These samples were visualized at 2h after glucose addition. Wt showed always no level of ROS (a, b) as well as the *tps1*Δ*+pPDE2* and *tps1*Δ *Hxk2*Δ mutants. Meanwhile the *tps1*Δ showed an unequivocal increase in ROS levels with nearly all cells exhibiting ROS accumulation (c, d). A specific field was selected to better observe the ROS accumulation after 2h in 50 mM glucose. Fluorescent micrographs were observed with filter set FITC (a, c); DIC image of the same cells (b, d). B: Externalization of PS with Annexin V – FLOUS (green) and necrotic cells with PI (red), at the time and concentration indicated. A specific field was selected to evidence the membrane fluorescence of *tps1*Δ after 4h in 50 mM glucose. Apoptotic cell (A), primary necrotic cell (PN) and secondary necrotic cells (SN). Fluorescent micrographs were observed with filter set FITC (a, c, e); DIC image of the same cells (b, d, f). C: TUNEL assay, 4h after glucose addition to final different concentrations: 1 mM (a), 2 mM (b), 5 mM (c), 10 mM (d), 20 mM (e), 50 mM (f), 100 mM (g), wt (h), negative control (i), positive control (j). Fluorescent micrographs; DIC image of the same cells (right panels). D: Immunodetection of cytochrome c (full line) and Cox II (dashed line) at times indicated in mitochondria (M) and in postmitochondrial supernatant (SN) of *tps1*Δ with two glucose concentrations. The gel was loaded with 20 µg and 100 µg of protein from M and SN samples, respectively.

S-nitrosilation of GAPDH is induced by glucose in *Tps1*Δ It was already demonstrated that NO and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are crucial mediators of yeast apoptosis (Almeida *et al.,* 2007). Considering the positive control DETA/NO treated cells the maximum reachable level and the negative control *tps1*Δ cells incubated in galactose the basal level, these results gave us some questionable values, since the difference between the basal and the obtained value for glucose incubated *tps1*Δ cells (Fig. 5) just differ about half unit. Additionally, upon comparison of *tps1*Δ cells incubated with glucose with the ones incubated with DETA/NO, there was still a difference of one unit, meaning that a higher value can be reached.

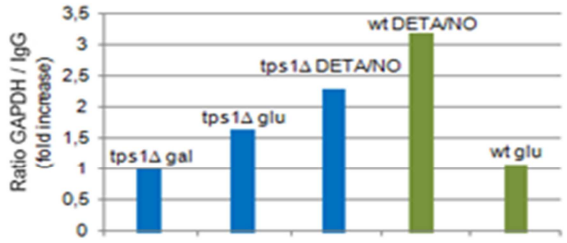

Figure 5 - GAPDH is S-nitrosilated during glucose-induced apoptosis. Band intensities were normalized to the intensity of IgG bands. Data express the GAPDH/IgG fold change in comparison to control (lane 1). Immunoprecipitation of S-nitrosilated GAPDH with an anti-CSNO antibody from cellular extracts of untreated, either glucose (100 mM) and DETA/NO-treated cell of wt (lanes 4 and 5) and *tps1*Δ (lanes 1 to 3).

***Tps1*Δ Presents RAS Overactivity after Glucose Addition** In order to verify if there is a correlation between Ras activation, glucose and apoptosis in *S. cerevisiae*, the activity of Ras2 proteins, which plays a key role in the cAMP-PKA pathway, was quantified. We could demonstrate that the Ras activity was enhanced in *tps1*Δ cells exposed to glucose. The Ras activation in the *tps1*Δ increased 8,5 times with 50 mM glucose. By the other hand the values of Ras activity in *tps1*Δ+p*PDE2* cells was conserved around the wt value in all conditions tested (**Error! Reference source not found.** 6).

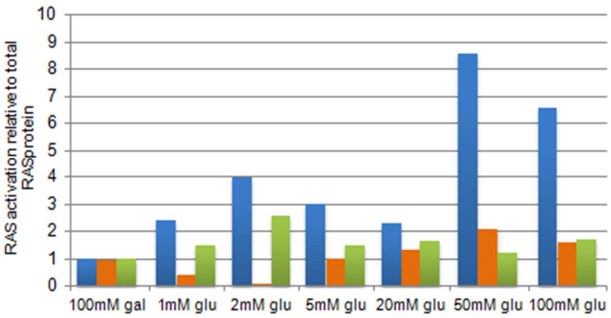

Figure 6 - Ras2 is activated during glucose-induced apoptosis. Band intensities were normalized to the intensity of pure protein bands. Data express the Ras activity fold change in comparison to control. Immunodetection of Ras activity from cellular extracts of untreated (100 mM galactose) and glucose-treated cell of *tps1*Δ (left bars) and *tps1*Δ+p*PDE2* (central bars) and wt (right bars) with the final concentrations mentioned.

Is of interest to study as well other proteins belonging to the fungal apoptotic network already described as key proteins in regulating apoptotic cell death. The serine protease NMA111, NUC1, the caspase-like ESP1 and MAC1 genes were deleted in the *tps1*Δ background. A spot assay was performed showing that the growth of the double mutants was not recovered after glucose addition, presenting the same *tps1*Δ growth arrest (not shown).

DISCUSSION

In the present study, it is shown that, in *tps1*Δ mutant, exposure to 20 to 100 mM glucose induces a programmed cell death pathway. The mode of programmed cell death after glucose addition was further characterized by the examination of cell death markers that are typical of apoptosis. The evidence obtained indicates that glucose can trigger an apoptotic phenotype in *tps1*Δ. The following concentration-dependent changes were observed after treatment with glucose: ROS accumulation, exposure of PS at the outer

surface of the yeast cytoplasmic membrane, formation of DNA strand breaks. And cytochrome c release. The present results suggest that at any condition tested occurred necrotic process. This interpretation is based on the fact that no reduction of apoptotic markers was detected. Observation indicates that, in our model, ROS accumulation precedes both PS externalizatrion and DNA fragmentation. The yeast *S*. *cerevisiae* has been used to study the mechanisms underlying apoptosis in higher eukaryotes. In the present work, we have shown that the apoptotic phenotype in *S*. *cerevisiae* can also be induced by acetic acid, pointing to the possibility that this mode of cell death may be more generalized in yeasts and extended to other stress agents. It is known that ROS play a central role in the regulation of apoptosis at various levels (Madeo *et al*., 1999). Further studies are in progress in order to evaluate ROS production in *S*. *cerevisiae* under the conditions where programmed cell death induced by acetic acid was detected. This would show if commitment of *S*. *cerevisiae* to a programmed cell death process in response to acetic acid is also mediated through a ROS dependent apoptotic pathway.

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