Extracellular pH and high concentration of potassium regulate the primary necrosis in the yeast *Saccharomyces cerevisiae*

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**Abstract**
Extracellular pH and concentration of K+ as well as their gradient across the plasma membrane have a significant impact on the physiology of the yeast cell, but their role in cell death has not been thoroughly investigated. Here we observed that increasing extracellular pH, as well as supplementing with K+ ions had a mitigating effect on cell death in yeast occurring under several conditions. The first is sugar induced cell death (SICD), and the second is death caused by several specific gene deletions, which have been recently identified in a systematic screen. It was shown that in both cases, primary necrosis is suppressed at neutral pH. SICD was also inhibited by the protonophore dinitrophenol (DNP) and 150 mM extracellular K+, with the latter condition also benefiting survival of cell dying due to gene mutations. In the case of SICD, these effects could not be mitigated by perturbing known pH-dependent signaling pathways, and thus are likely to be realized via direct effects on the plasma membrane potential. Thus, (a)—we show that stabilization of external pH at a neutral level can suppress different types of primary necrosis, and (b)—we suggest that changes to the cellular membrane potential can play a central role in yeast cell death caused by different factors.

**Keywords** Yeast · Necrosis · Sugar-induced cell death · Extracellular pH · Membrane potential

**Introduction**
Fungi and yeast are capable of growing over a wide range of extracellular pH, and the extracellular pH has a significant effect on the physiology of the fungal cell. It is known that extracellular pH, among other things, regulates gene expression (Serrano et al. 2002; Penalva et al. 2008), ionic homeostasis (Ke et al. 2013), and controls the cell cycle (Hayashi et al. 1998).

Cell death that has some resemblance to apoptosis in yeast cells was first reported in 1997 (Madeo et al. 1997). Necrosis was considered to be the result of a fatal exposure of the cell to an excessive amount of an apoptosis-inducing agent (Ludovico et al. 2001; Liang and Zhou 2007). External and internal triggers of cell death in yeast have been reviewed in (Falcone and Mazzoni 2016; Karginov et al. 2021).

It has been shown that glucose, in the absence of other nutrients, induces apoptosis in stationary yeast (Granot et al. 2003). This phenomenon was termed glucose (sugar)-induced cell death (GICD or SICD). Later it has been shown that SICD is independent of yeast metacaspase and dependent on reactive oxygen species (ROS) production (Hoeberichts et al. 2010), and that stationary yeast lacking the Trk2 transporter are the most susceptible to SICD (Dušková et al. 2021). We recently reported on the necrotic nature of SICD in exponentially growing yeast (Valiakhmetov et al. 2019).

While SICD is an acute treatment which causes death of a considerable share of the cell population, primary necrosis has also recently been reported in the context of dividing cells harboring various deletions and down-regulating mutations in a large number of genes (Alexandrov et al. 2021). This type of cell death, termed rapid division-associated necrosis, is chronic, does not seem to be related to ROS and is likely to be a case of primary necrosis.
In this work, we present data on the effect of extracellular pH and changes in membrane potential on SICD, as well as on increased cell death during division caused by several gene deletions.

**Materials and methods**

**Culture growth**

Strains used in this study are listed in Supplementary Table 1. Culture growth for SICD assay was done as in (Valiakhmetov et al. 2019). For experiments on necrosis during cell division in mutants, cells were grown in YPD medium (or YPD medium with pH stabilized at 7) to logarithmic phase (OD_{600} = 0.3–0.6). YPD buffering was performed by titration with 2 M NaOH to pH 7, after which the medium was supplemented with 20 mM phosphate buffer, pH 7. Growth for testing the effects of KCl was performed by growing cells in YPD that was supplemented with 150 mM of KCl.

**Incubation with glucose**

Incubation with glucose for SICD assay was done as in (Valiakhmetov et al. 2019).

**Staining the cells and flow cytometry**

For SICD assay, we stained dead cells with FITC-dextran (FD), m.w. 4 kDa (Sigma, USA) and counted them as described in (Valiakhmetov et al. 2019). For experiments on necrosis during cell division in mutants, cells were stained with propidium iodide (PI) (Sigma, USA) by direct addition to the cultivation medium (final concentration—2 μg mL⁻¹, incubation time—1 h) and measured on a Beckman Cytoflex S flow cytometer using the 488 nm laser for excitation and a 525/40 nm filter for the emission.

**1,2,3,-Dihydrorhodamine staining assay**

Determination of ROS by 1,2,3,-Dihydrorhodamine (DHR) staining in cells during SICD was done as in (Valiakhmetov et al. 2019).

**CFU assay**

0.1 mL of cells after incubation for 1 h with 100 mM glucose in water or in 50 mM HEPES pH 7 was plated on YPD-agar plates after the appropriate dilution. CFU numbers were counted after 48 h of growth at 28 °C. Cells incubated in water were used as a control.

**Dinitrophenol treatment**

Cells were prepared and loaded with DHR as described above. Dinitrophenol (DNP) (dissolved in water) at various concentrations and 2.5 μl of 2 M glucose were simultaneously added to 0.05 ml of the cell suspension. After 1 h of incubation at 30 °C, ROS and SICD were determined as described above.

**Extracellular pH measurement**

0.75 ml of 2 M glucose was added to 15 ml of cell suspension in kept at a constant 30 °C temperature with magnetic stirring, and the pH value was measured using a combined glass electrode on a Hanna-211 pH meter. To study the effect of the ΔμH⁺ modifiers, DNP or KCl was added so that the final concentration was 0.5 mM and 150 mM, respectively. To check the effect of the buffer, the cells were re-suspended in 50 mM HEPES pH 7.0.

**Determination of membrane potential by DiOC2(3) staining assay**

We used the SEY 6210 rho° strain to exclude the contribution of the mitochondrial membrane potential. The strain was obtained by treating S. cerevisiae SEY6210 cells with 0.1 mg mL⁻¹ ethidium bromide overnight. Thereafter, cells were plated on YPD plates and colonies whose cells did not show growth after 48 h of incubation in YP medium + 1% ethanol were used. Cells were prepared as described under Culture growth section (above). 0.05 mL of cells was added to 1 mL of MilliQ water or 1 mL of 50 mM HEPES pH 7.0. 0.1 mL of this cell suspension was plated in a 96-well black plate (Corning Costar, USA). 1 μL of 3 mM DiOC2(3) (ThermoFisher, USA) in DMSO was added to give a final dye concentration of 30 μM. The plate was incubated for 5 min at 30 °C to distribute the dye. To initiate the process, 5 μL of 2 M glucose was added to the wells. Fluorescence was recorded on a FilterMax F5 plate reader (Molecular Devices, USA). We used wavelength 485 nm for excitation, and emission was recorded at 625 nm. Readings were taken every minute with 30 s shaking before each measurement.

**Results and discussion**

**Stabilizing extracellular pH at 7 reduces SICD**

Yeasts respond to changes in extracellular pH in a complex manner (Arino 2010; Serra-Cardona et al. 2015). Basically, this response affects the functioning of the main signaling
pathways. However, until now, there were no data on the effect of extracellular pH on the process of yeast cell death. ROS play an important role in cell death processes. To assess the role of ROS, scavengers, such as glutathione, N-acetyl cysteine, and ascorbate, are widely used. However, ascorbic acid should be in neutralized form at pH 7 to avoid toxic effects on cells. As shown earlier, 10 mM ascorbate almost completely suppresses SICD (Valiakhmetov et al. 2019). Ascorbic acid (weak acid) neutralized with NaOH (strong base) to pH 7 is a buffer. The initial impetus for the present study was a neglect of the dual nature of neutralized ascorbate—which is a scavenger of ROS on the one hand, and a pH-buffering agent with pH 7.0 on the other hand. The incubation of *S. cerevisiae* SEY6210 with glucose in 50 mM HEPES buffer pH 7.0 led to a complete suppression of SICD, which is a case of primary necrosis. Suppression of ROS generation and SICD development has a clear pH relationship (Fig. 1). As the external pH decreased, the number of cells with ROS and SICD increased. This observation seems to link necrosis with the degree of the extracellular acidification. A similar effect was observed in the induction of apoptosis by valproic acid in the yeast *Schizosaccharomyces pombe* (Mutoh et al. 2011). To validate data obtained with dyes, we checked cell viability by CFU test—a crucial indicator of cell viability. The CFU test showed that after incubation of SEY6210 cells with glucose at pH 7.0, cell survival was 100%, and after incubation of cells in unbuffered media, it decreased by 18 ± 3%.

Since the extracellular pH influences the functioning of yeast cells in a variety of ways (Serra-Cardona et al. 2015), we examined the involvement of some of the signaling pathways and individual genes in the suppression of the necrosis by the neutral pH. We used commercially available knockout mutants derived from the BY4741 parent strain (Supplemental Table 1). The parental strain BY4741 was tested for the development of SICD under our conditions. Mitigation of ROS formation and SICD development in strain BY4741 (and its derivatives) upon incubation with 100 mM glucose in the presence of 50 mM HEPES pH 7 were in good agreement with the data obtained earlier for strain SEY6210 (Table 1).

In *S. cerevisiae*, the Rim101 pathway senses external alkalization and alteration in plasma membrane lipid asymmetry through a complex consisted of Rim8, Rim9 and Rim21 at the plasma membrane. It is known that Rim21p, acts as a sensor of extracellular pH (Obara et al. 2012; Nishino et al. 2015). It was logical to assume that deletion of the rim21 gene could lead to the restoration of SICD in a neutral environment. However, the Δrim21 strain showed the same suppression of SICD by neutral pH as the parental strain BY4741 (Table 1). We conclude that the Rim101/PacC signaling pathway is not involved in SICD.

The cAMP/protein kinase A (PKA) pathway is one of the major glucose-signaling pathways of budding yeast. Activation of the PKA pathway causes sensitivity to alkaline pH (Casado et al. 2011). Conversely, deactivation of this system increases resistance to alkaline stress. However, activation of the PKA pathway by deleting the *ira2* gene (upstream member of cAMP/PKA pathway) did not affect the development of SICD nor its suppression at neutral pH (Table 1). Thus, the development of SICD and its suppression by neutral pH turned out to be insensitive to the cAMP level.

**Table 1** Percentage of inhibition of ROS generation and development of SICD after incubation of cells with 100 mM glucose in 50 mM HEPES buffer pH 7. Mean±SD from 3 independent experiments

| Strain       | SICD  | ROS  |
|--------------|-------|------|
| SEY 6210     | 97±1.9| 85±4.3|
| SEY 6210 rho⁰| 89±7.6| 93±5.9|
| BY4741       | 96±0.96| 96±2 |
| Δafol       | 97±0.6| 93±2.1|
| Δend3       | 95±1.7| 94±2.4|
| Δira2       | 97±0.5| 96±1.5|
| Δrim21      | 97±0.8| 98±0.6|
| Δscp1       | 93±1.9| 88±11.6|
| Δsla1       | 91±2.2| 88±7.5|
| Δyca1       | 97±0.4| 95±1.2|
| Δyno1       | 97±0.8| 95±1.9|

![Fig. 1](image-url) Dependence of ROS generation and SICD development in SEY6210 strain on extracellular pH (Mean±SD from 3 independent experiments). ROS—percentage of the cells with ROS as estimated by DHR staining; SICD—percentage of the cells with SICD as estimated by FD staining. Buffer used for pH 7—50 mM HEPES; pH 6 and 5.1—20 mM MES; pH 4.2—20 mM glycine-glycine; pH 3.5 and starting point pH 3.3—water acidified by HCl.
SICD occurs in the presence of increased amounts of ROS. Two major sources of ROS are known in the yeast cell—the mitochondrial respiratory chain and NADPH oxidase (Rinnerthaler et al. 2012) of the ER. We found no data on regulation of ROS production by the ambient pH. However, if such regulation existed, it would explain the fact that SICD was suppressed by neutral pH. To test this assumption, we used two deletion mutants: Δafo1 (Heeren et al. 2009) with respiratory deficiency and Δyno1 in which there is no NADPH oxidase (Rinnerthaler et al. 2012). Surprisingly, both strains continued to exhibit generation of ROS when cells were incubated with glucose in an unbuffered medium. Incubation with glucose at pH 7 resulted in the suppression of the number of cells with ROS (and with SICD) by more than 90%. The data obtained indicate that the generation of ROS by the respiratory chain of mitochondria or NADPH oxidase of ER is not regulated by the extracellular pH.

Caspases are a family of protease enzymes playing essential roles in programmed cell death. Inhibition of caspases promotes alternative cell death pathways (Vandenabeele et al. 2006). It has also been shown that inhibition of caspases which occurs at acidic pH leads to a change in cell death from apoptosis to necrosis (Lan et al. 2007). Only one metacaspase, Yca1, is present in yeast (Madeo et al. 2002). We reasoned as follows. If metacaspase (by analogy with metazoan caspases), after inhibition in an acidic medium, leads to the switching of apoptosis to necrosis, then in Δyca1 strain, we either should not observe SICD in an acidic medium, or SICD will also be observed at neutral pH. However, the Δyca1 mutant did not show any of the expected responses (Table 1). Therefore, yca1 is not involved in SICD. This is in agreement with published data that only about half of the cell death scenarios are caspase-dependent (Madeo et al. 2009).

It is known that actin dynamics (indirectly through the mitochondrial membrane potential) affects the generation of ROS (Gourlay and Ayscough 2005). We tested the sensitivity of SICD to pH 7.0 in three knock-out mutants—Δsla1, Δscp1 and Δend3, involved in regulation of actin dynamics (Gourlay et al. 2003). All three mutants showed no SICD at pH 7.0. As shown in Table 1, pH 7.0 suppresses SICD in all the strains tested. Such results are to be expected, since End3p, Scp1p and Sla1p alter the ROS level indirectly by modulating the membrane potential of mitochondria, which, as can be seen from experiments with Δafo1, are not involved in the development of SICD.

Since we did not observe the sensitivity of SICD to neutral pH to depend on the functioning of several tested cellular systems, we hypothesized that ΔμH⁺ might be involved in this effect. During incubation with glucose, S. cerevisiae cells decrease the pH of the medium to pH 3.7 after 5 min (Fig. 2). This creates a large ΔpH on the plasma membrane, which is absent when cells are incubated in 50 mM HEPES pH 7. Hence, dissipation of ΔpH by the protonophore should lead to the suppression of SICD. Indeed, DNP suppresses SICD by more than 80% already at a concentration of 0.5 mM (Supplemental Fig. 1). This is in agreement with published data that only about half of the cell death scenarios are caspase-dependent (Madeo et al. 2009).

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membrane. As can be seen from Fig. 3, the incubation of cells in an aqueous solution of glucose leads to a significant increase in ΔΨ. Incubation of cells with glucose in a buffer with pH 7.0 or in the presence of 150 mM KCl completely suppresses this increase of ΔΨ. It is important to note that the acidification of the medium in the presence of 150 mM KCl even slightly exceeded acidification during incubation with glucose only (Fig. 2) probably due to K⁺/H⁺ antiport. A decrease in SICD in the presence of extracellular K⁺ against a background of high ΔpH indicates the predominant role of the ΔΨ in the regulation of necrosis. It has previously been suggested that plasma membrane hyperpolarization may cause SICD in stationary yeast (Hoeberichts et al. 2010). And it was recently shown that exogenous K⁺ activate the Trk2 K⁺ uptake system of stationary yeast, which leads to membrane depolarization and subsequent survival of SICD (Dušková et al. 2021). Thus, we can conclude that ROS generation and subsequent development of SICD depend on ΔμH⁺, with ΔΨ playing a larger role than ΔpH. Whether a hyperpolarized membrane can generate ROS on its own, or some abnormal cellular process is triggered via this hyperpolarization, is currently unclear. However, since we found a direct correlation between the SICD (which is ROS-mediated) and the magnitude of ΔΨ on the plasma membrane, we do not exclude the possibility of ROS production by some membrane-bound complexes at a high ΔΨ value.

Effect of pH and KCl on division-associated necrosis caused by gene deletions

To test how pH stabilization and addition of KCl affected other types of yeast cell death, we also tested its effects on several mutants that exhibit increased rates of cell death during division. These genes were identified in the course of a recent genome-wide screen which used the dye Phloxine B to search for mutants with increased numbers of dead cells in colonies (Alexandrov et al. 2021).

Our data show that at least 5 of the 12 tested mutants exhibited noticeable reduction of cell death under conditions of stabilized pH 7.0 (Fig. 4A), while addition of KCl (which should lead to dissipation of the ΔΨ) to the growth medium had a more universal effect, reducing the death rate of 11 out of the 12 tested mutants, and with effects of larger magnitude (Fig. 4B).

Summarizing the obtained data, we can state that both SICD and rapid division-associated necrosis in some mutants are suppressed (1) upon dissipation of ΔpH at neutral pH (or treatment with DNP for SICD) and (2) upon dissipation of ΔΨ in the presence of 150 mM extracellular KCl. Thus, we assume that ΔμH⁺ on the plasma membrane
regulates the development of various types of primary necrosis in the yeast *S. cerevisiae*.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02708-6.

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