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Yeast growth in raffinose results in resistance to acetic-acid induced programmed cell death mostly due to the activation of the mitochondrial retrograde pathway

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ABSTRACT

In order to investigate whether and how a modification of mitochondrial metabolism can affect yeast sensitivity to programmed cell death (PCD) induced by acetic acid (AA-PCD), yeast cells were grown on raffinose, as a sole carbon source, which, differently from glucose, favours mitochondrial respiration. We found that, differently from glucose-grown cells, raffinose-grown cells were mostly resistant to AA-PCD and that this was due to the activation of mitochondrial retrograde (RTG) response, which increased with time, as revealed by the up-regulation of the peroxisomal isoform of citrate synthase and isocitrate dehydrogenase isoform 1, RTG pathway target genes. Accordingly, the deletion of *RTG2* and *RTG3*, a positive regulator and a transcription factor of the RTG pathway, resulted in AA-PCD, as shown by TUNEL assay. Neither deletion in raffinose-grown cells of *HAP4*, encoding the positive regulatory subunit of the Hap2,3,4,5 complex nor constitutive activation of the RTG pathway in glucose-grown cells due to deletion of *MKS1*, a negative regulator of RTG pathway, had effect on yeast AA-PCD. The RTG pathway was found to be activated in yeast cells containing mitochondria, in which membrane potential was measured, capable to consume oxygen in a manner stimulated by the uncoupler CCCP and inhibited by the respiratory chain inhibitor antimycin A. AA-PCD resistance in raffinose-grown cells occurs with a decrease in both ROS production and cytochrome *c* release as compared to glucose-grown cells *en route* to AA-PCD.

1. Introduction

Cell homeostasis depends on a complex signal transduction network regulating cell capability to adapt or succumb to environmental stress in which different cell components are involved; in this regard besides being cell powerhouse, mitochondria are central organelles in the integration of intracellular signalling pathways and play a pivotal role in the regulation of programmed cell death (PCD) [1–3]. Since signal transduction components and mechanisms are highly conserved among eukaryotes, the unicellular yeast *Saccharomyces cerevisiae* is a suitable model organism to study this issue. In fact, yeast undergoes a PCD process which shares a variety of features with mammalian apoptosis including oxidative stress and the release of pro-apoptotic proteins, including cytochrome *c* (cyt *c*), with attendant late mitochondrial dysfunction [4,5]. With a model system in which PCD of yeast grown on glucose is induced by acetic acid (AA-PCD), the time course of key events which take place *en route* to PCD, including ROS production, cyt *c* release and mitochondrial dysfunction, was described in some detail [5–7]. It was also shown that AA-PCD can also occur in cells lacking cyt *c* and/or the yeast metacaspase *YCA1* via a N-acetyl cysteine (NAC)-insensitive death pathway [8–10]. Although AA-PCD has already been investigated in detail, to date whether and how the cellular energy metabolism can somehow influence the occurrence of either AA-PCD pathways remains to be established.

Glucose is a fermentable carbon source responsible for the down-regulation of respiration [11,12], while raffinose is a poorly fermentable carbon source in which respiration is de-repressed [13]. Here we investigated yeast cell sensitivity to AA-PCD in yeast cells grown either in glucose (GLU-WT cells) or in raffinose (RAF-WT cells). We found that, when raffinose is used as a sole carbon source, differently from what occurs in glucose-grown cells, yeast cells become resistant to AA-PCD in a manner mostly dependent on the retrograde (RTG)-pathway activation. In this regard, that yeast dysfunctional mitochondria can communicate

Abbreviations: AA, acetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cyt *c*, cytochrome *c*; DCF, dichlorophluoresceine; GLU, glucose-grown; H_2DCH-DA, 2,7-dihydrodichlorofluorescein diacetate; NAC, N-acetyl cysteine; PCD, programmed cell death; RAF, raffinose-grown; RTG, retrograde; TMRM, tetramethylrhodamine methyl ester; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling; WT, wild type

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with the nucleus via the RTG signalling pathway has already been ascertained in the Butow's laboratory: the expression of a broad array of nuclear target genes was first demonstrated to be largely increased in cells grown on raffinose with impaired mitochondria including those lacking mitochondrial DNA (ρ°) [14,15]. Yeast RTG signalling pathway has been characterized in details [16,17] and its activation was shown to extend replicative life span in yeast [18,19], possibly providing a defense mechanism by which cells can respond to mitochondrial stress leading to mitochondria impairment [20–22]. Surprisingly enough we first found that RTG pathway is active in yeast cells in which mitochondria are still coupled.

2. Materials and methods

2.1. Yeast strains, growth conditions and acetic acid treatment

The *S. cerevisiae* strains used in this study are listed in Table 1. Cells were grown at 30 °C in YPD or YPR (1% yeast extract, 2% bactopeptone, and 2% glucose or raffinose, respectively). Acetic acid treatment was carried out as described [23]. Briefly, cells were grown at 26 °C up to exponential phase (OD_{600} = 0.7–0.8) in YPD or YPR, resuspended (10^7 cells/ml) in the same medium adjusted to pH 3.00 with HCl, containing or not containing (control) 80 mM acetic acid and incubated for different times at 26 °C. Cell viability was determined by measuring colony forming units (cfu) after 2 days of growth on YPD plates at 30 °C.

2.2. TUNEL assay and intracellular ROS detection

DNA fragmentation was detected by TUNEL assay. Acetic acid-treated and control cells ($2x10^7$) were harvested at 150 min. Briefly, cells were fixed in 3.7 % formaldehyde solution in PBS, digested with 750 μg/ml zymoliase 20 T and incubated in permeabilization solution (0.1 % Triton-X100, 0.1 % sodium citrate) for 2 min on ice, and then with 30 μl TUNEL reaction mixture (In Situ Cell Death Detection kit, Fluorescein, Roche) for 1 hour at 37 °C. After incubation cells were washed, resuspended in PBS and observed using a Leica TCS SP5 confocal microscope. To detect intracellular H_2O_2 10 μg/ml 2,7-dihydrodichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes) dissolved in ethanol was added to cells either 30 min before or during cell treatment with or without acetic acid. $2x10^7$ acetic acid-treated or control cells were harvested at 15 min and oxidation to the fluorophore dichlorofluorescein (DCF) was detected by confocal fluorescence microscopy analysis.

2.3. Real-time polymerase chain reaction (PCR)

The mRNA levels of peroxisomal citrate synthase and isocitrate dehydrogenase isoform 1 encoding gene (*CIT2* and *IDH1*, respectively) were determined in exponentially growing cells (OD_{600} = 0.7) and in acetic acid-treated or control cells. 20 ml of cell suspension were centrifuged at 3000 ×*g*. Cell pellets were either stored at −80 °C or immediately used to extract total RNA with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) through mechanical disruption of cell walls with glass beads by Tissue Lyser (Qiagen). 1 μg RNA ($OD_{260}/OD_{280} \geq 1.9$) reverse transcription was immediately performed using QuantiTect Reverse Transcription Kit (Qiagen) and cDNA directly used for real-time PCR analysis or stored at −80 °C. Real-time PCR was carried out by QuantiTect SYBR Green PCR Kit (Qiagen) on an ABI Prism 7000 system using the following primer pairs: for *CIT2*: (F) 5′-CGGTTATGGTCATGCTGTGCT-3′ and (R) 5′-GGTCCATGGCAAACTTACGCT-3′; for *IDH1*: (F) TCGACAATGCCTCCATGCA and (R) AAAGCAGCGCCAATGTTGC; for *ACT1*: (F) 5′-CTTTGGCTCCATC TTCCATG-3′ and (R) 5′-CACCAATCCAGACGGAGTACTT-3′. The amount of *CIT2* and *IDH1* mRNA normalized with *ACT1* mRNA was calculated in arbitrary units (a.u.) using the standard curve method.

Table 1
Strains of *Saccharomyces cerevisiae* used in this study.

Strain (name)	Genotype	Reference/source
W303-1B (WT)	MATα ade2 leu2 his3 trp1 ura3 ρ^+	X.J. Chen's lab*
W303-1B (ρ°)	MATα ade2 leu2 his3 trp1 ura3 ρ°	This study
Δrtg2	W303-1B rtg2Δ::LEU2	This study
Δrtg3	W303-1B rtg3Δ::LEU2	This study
Δhap4	W303-1B hap4Δ::kanMX4	This study
Δhap4Δrtg2	W303-1B rtg2Δ::LEU2 hap4Δ::kanMX4	This study
Δmks1	W303-1B mks1Δ::kanMX4	This study

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2.4. Mitochondrial membrane potential and O_2 consumption assay

Mitochondrial membrane potential was monitored essentially as in [24]. Briefly, cells (OD_{600} = 0.6) exponentially growing in YPD or YPR were incubated with 500 nM tetramethylrhodamine methyl ester (TMRM) (λ_{ex} 540/λ_{em}590) (Life Technologies) for 45 min at 26 °C in the dark and then resuspended (10^7 cells/ml) in the same medium adjusted to pH 3.00 with HCl, containing 500 nM TMRM. An aliquot was also incubated with 20 μM CCCP for 15 min to collapse the membrane potential. We confirmed that 20 μM CCCP was not toxic, but can inhibit the growth of WT cells on a non-fermentable carbon source. Cells (about $5x10^5$ cells/ml) were collected and resuspended in phosphate buffer saline (PBS) and analyzed with a FACS Calibur (Becton Dickinson) flowcytometer. Monoparametric detection of fluorescence was performed using FL-2 and data were analyzed using WinMDI 2.9 software. Background fluorescence without dye was analyzed in both glucose and raffinose and subtracted.

O_2 consumption was continuously measured at 25 °C in a thermostatically controlled chamber equipped with a Clark oxygen electrode (Oxygraf, Hansatech Instruments). In a typical experiment cell suspension ($1 x 10^7$ cells/ml) in YPD or YPR medium (final volume equal to 1 ml) was used. The oxygen uptake rate was measured as a tangent at the initial part of the progress curve and expressed as nmol O_2 min^{-1} cell $number^{-1}$. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and antimycin A (Sigma Aldrich) were dissolved in dimethyl sulphoxide and ethanol, respectively; control was made that the solvents have no effect on the electrode sensitivity.

2.5. Protein extraction and cell fractionation

Trichloroacetic acid (TCA) precipitation of total yeast cell proteins was carried out as described by Dilova and Powers [25], with some modifications. 5 ml of cell culture grown to exponential phase (OD_{600} = 0.7) were harvested by centrifugation for 5 min at 3900 rpm (Sepatech omnifuge 2.0 RS, Heraeus). Cells were lysed in 0.225 M NaOH/1 % 2-mercaptoethanol, 10 mM NaF, 1 mM Na_3VO_4 and 2 mM phenylmethylsulfonyl fluoride and proteins were precipitated with 6.1 % TCA. The protein pellet was then washed with 1 M Tris-HCl (pH 6.8) and resuspended in SDS PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 100 mM DTT, 0.002% bromophenol blue). Cytosolic and mitochondrial fractions were isolated from 200 to 400 ml of acetic acid-treated or control cell culture as described in [26]. Protein concentration was determined using the Bradford assay [27].

2.6. Immunoblotting

To detect Rtg3p phosphorylation, equivalent amounts of total cellular protein extracts were loaded on 7.5 % SDS-PAGE gels and transferred to polyvinylidenefluoride (PVDF) membranes (Millipore, Immobilon-P 0.4 μm) by semidry transfer units TE 70 (Amersham Biosciences). Membranes were probed using polyclonal anti-Rtg3p antibody and monoclonal anti-phosphoglycerate kinase (anti-Pgk1p) antibody (Molecular Probes). Immunodetection was performed with