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Regular Articles

Respiratory stress in mitochondrial electron transport chain complex mutants of *Candida albicans* activates Snf1 kinase response

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ABSTRACT

We have previously established that mitochondrial Complex I (CI) mutants of *Candida albicans* display reduced oxygen consumption, decreased ATP production, and increased reactive oxidant species (ROS) during cell growth. Using the Seahorse XF96 analyzer, the energetic phenotypes of Electron Transport Chain (ETC) complex mutants are further characterized in the current study. The underlying regulation of energetic changes in these mutants is determined in glucose and non-glucose conditions when compared to wild type (WT) cells. In parental cells, the rate of oxygen consumption remains constant for 2.5 h following the addition of glucose, oligomycin, and 2-DG, but glycolysis is highly active upon the addition of glucose. In comparison, over the same time period, electron transport complex mutants (CI, CIII and CIV) have heightened activities in both oxygen consumption and glycolysis upon glucose uptake. We refer to the response in these mutants as an "explosive respiration," which we believe is caused by low energy levels and increased production of reactive oxygen species (ROS). Accompanying this phenotype in mutants is a hyperphosphorylation of Snf1p which in *Saccharomyces cerevisiae* serves as an energetic stress response protein kinase for maintaining energy homeostasis. Compared to wild type cells, a 2.9- to 4.4-fold hyperphosphorylation of Snf1p is observed in all ETC mutants in the presence of glucose. However, the explosive respiration and hyperphosphorylation of Snf1 can be partially reduced by the replacement of glucose with either glycerol or oleic acid in a mutant-specific manner. Furthermore, Inhibitors of glutathione synthesis (BSO) or anti-oxidants (mito-TEMPO) likewise confirmed an increase of Sfn1 phosphorylation in WT or mutant due to increased levels of ROS. Our data establish the role of the *C. albicans* Snf1 as a surveyor of cell energy and ROS levels. We interpret the "explosive respiration" as a failed attempt by ETC mutants to restore energy and ROS homeostasis via Snf1 activation. An inherently high OCR baseline in WT *C. albicans* with a background level of Snf1 activation is a prerequisite for success in quickly fermenting glucose.

1. Materials and methods

1.1. Strains, media and chemicals

C. albicans SN250 are wild type (WT) strains used in this study. Three mitochondrial ETC CI mutants (*ndh51Δ* (JM02), *nuo1Δ* and *nuo2Δ*) as well as an apparent regulator of CI (*goa1Δ*) and their reconstituted strains (GOA32 (Goa1R), JM03 (Ndh51R), Nuo1R and Nuo2R) were described previously (Bambach et al., 2009; She et al., 2015; McDonough et al., 2002). Other than the CI mutants, two additional ETC mutants are included in order to discriminate any CI-specific effect. Those mutants are *mt6918Δ* (CIII) and *mt230Δ* (CIV). Both mutants were constructed by Dr. William Fonzi (unpublished data). Both mutants have respiration defects due to an insufficient assembly of ETC CIII and CIV, respectively.

All strains were stored at −80 °C. Cultures were initially grown on YPD agar (2% glucose, 2% peptone and 1% yeast extract) and then cultured overnight in YPD broth, unless otherwise indicated in experiments with Seahorse assays described below. For other experiments, YPD was always used to grow cells to exponential phase for experiments. When 2% glucose is replaced with 2% glycerol or 2% oleic acid, the YP based media are referred to as YPG or YPO, respectively.

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A commonly used oxidation-sensitive fluorescent dye for detecting cellular H_2O_2 (dihydrofluorescein diacetate, DCFDA) was obtained from Sigma-Aldrich, Inc. For inhibitor studies, buthionine sulfoximide (BSO), carbonel cyanide 3-chlorophenylhydrazone (CCCP) and triethyltin bromide (TET) were obtained from Sigma-Aldrich, Inc. mito-TEMPO (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl triphenylphosphonium chloride was purchased from Cayman Chemicals.

1.2. Growth under alternative carbons and ATP generation

5-mL overnight cultures of *C. albicans* WT and ETC mutants were grown in a 15 mL Falcon tubes (16 h, 200 rpm, 30 °C) in YPD broth. Batch cultures were prepared by transferring 5×10^6 in 10 mL pre-warmed YPD broth in a 50 mL baffled flask. Shake cultures were grown for a total of 9 h at 30 °C (200 rpm). At 1 h intervals, a 100 µL aliquot of each strain was sampled from each batch culture, and the optical density at 600 nM was measured in a clean 96-well plate by Spectramax 190 (Molecular Devices). At 9 h, the batch cultures of each strain were harvested, washed and divided into 3 portions, which were re-suspended in 3 mL of YPD, YPG, or YPO broth, respectively in 15 mL Falcon tubes for an additional 18 h. A 100 µL aliquot of each strain was sampled at 1 h intervals for optical density measurements.

The ATP Colorimetric/Fluorometric Assay Kit (Biovision catalog#K354-100) was used to determine cellular ATP levels. For the ATP assay test, all strains (2×10^6) were incubated at 30 °C in 10 mL YPD broth overnight at 150 rpm. 5 mL of culture was used to measure ATP (YPD). The remaining 5 mL of culture was collected, washed with PBS buffer and transferred into YPG media for 1 h at 30 °C, 150 rpm before ATP measurement (YPG). To better determine cellular ATP levels, sonication was used to disrupt cells on ice (15 s on, 45 s off, 70% pulse for a total of 30 min). After disruption, 500 µL of each sample was transferred to 10 kDa spin columns (Millipore Corporation) and centrifuged at 10,000*g* for 30 min at 4 °C. The samples were kept in dry ice or stored at −80 °C for assays. Black costar 96-wells micro-plate was used to measure fluorescence according to instructions (excitation 535 nm, emission 587 nm) at 37 °C.

1.3. Determination of energy phenotypes using XF96 Seahorse assays

The Seahorse XF analyzer (XF^e 96) was used to measure the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) as previously described in yeast (Simpkins et al., 2016; He et al., 2013). Changes in rate were recorded in real-time over a 2.5 h time course. Exponentially grown yeast cells of all strains (OD ≈ 0.5) in YPD were washed twice with PBS (pH, 7.0), adjusted to 10^6 cells/well in 175 µL of XF assay medium as recommended by the manufacturer, and then distributed into Poly-D Lysine (50 mg/ml) XF 96-well pre-coated microplates (Seahorse Bioscience). Six replicates of each strain or treated condition were used per experiment. Seahorse basal medium (Seahorse Bioscience cat. 102353) was freshly made and supplemented with 2 mM L-glutamine (Sigma). For non-glucose conditions, cells of each strain at OD ≈ 0.5 were collected, washed with PBS, and suspended in YPG (glycerol) or YPO (oleic acid) broth for 1 h before washing. Cells were centrifuged and suspended in the Seahorse assay medium as described above and seeded into assay plates. All cell preparations in this assay medium (lacking glucose) were maintained in 96-well plates for 1 h at 30 °C to permit cell adhesion to the microtiter plates before analysis.

The two energy pathways (indicated by OCR and ECAR) of each strain were measured by the Seahorse XF 96 as follows: baseline levels of both pathways were measured three times (5 min intervals) during the first 18 min at 30 °C. In this phase, mitochondrial respiration is driven by 2 mM L-glutamine in the Seahorse assay medium without glucose. Then, 10 mM glucose, 1.0 µM oligomycin (or 100 µM TET), and 50 mM 2-deoxyglucose (2-DG) were sequentially injected automatically into assay wells. For each treatment, rates of OCR and ECAR were determined for six time points at ~40 min intervals. Oligomycin is a mitochondrial ETC CV inhibitor that blocks the classical respiration chain (CRC) and stimulates ECAR in mammalian cells (Grandjean et al., 2016). 2-Deoxyglucose was used to inhibit glucose metabolism since it is a competitive inhibitor of the glucose hexokinase.

1.4. Immunoblot assays of the Snf1p kinase pathway in C. albicans

The activation of Snf1 complex is measured by phosphorylation of Snf1 as described previously (Orlova et al., 2008). In brief, protein extracts from WT and mutants were obtained from cell cultures in YPD (OD ≈ 0.5), an additional 1 h growth in YPG medium, in the presence of the inhibitors such as 0.6 mM BSO (ROS inducer), 25- or 50-µM CCCP (mitochondrial uncoupler), and 10 µM mito-TEMPO (mitochondrial targeted antioxidant) respectively after exponential growth in YPD. BSO is an inhibitor of glutathione biosynthesis that creates oxidative stress to promote an apoptotic process on mammalian cells (Takahashi et al., 2010).

1.4.1. Protein extraction

All cultures were immediately submerged in a boiling water bath for 3 min, and then cells were pelleted by centrifugation at 3000*g* for 5 min. Each culture pellet was incubated with 150 µL of 0.2 M NaOH-TE buffer for 5 min at room temperature (RT). After alkaline treatment, each aliquot in 30 µL of extracted protein collected by centrifugation at 10,000*g* for 1 min was then mixed with a volume of SDS-PAGE sample buffer (80 mM Tris-HCl, pH 6.8, 10 mM EDTA, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 5% β-mercaptoethanol). The volume of the loading buffer used was 50 µL for cell density as $OD_{600} = 1.0$. All samples were boiled for 5 min, air-cooled, and cleared by centrifugation for 5 min at 10,000*g*. The cleared supernatants were loaded onto 10% SDS-PAGE gels at 5 µL/lane.

1.4.2. Western blotting

Proteins were transferred to Immobilon-P PVDF membranes (Millipore) using a field strength of 250 mA for 90 min at 4 °C. The membranes were blocked in 1 × TBS-T (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween-20), supplemented with 5% BSA for 1 h at RT with gentle shaking. Blots were washed in 1 × TBS-T three times at RT (each for 5 min). To detect phosphorylated Snf1 (Snf1p), blots were incubated with a primary antibody phospho-Thr172-AMPK (Cell Signaling Technology; Cat. No. 2531), diluted 1:1000 in 1× TBS-T. After a 12 h incubation in sealed plastic bags at 4 °C, the blots were then washed with 1 × TBS-T for 3 times at RT, and incubated in a plastic tray at RT for 1 h with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.) diluted 1:10,000 in 1× TBS-T. All blots were washed in 1× TBS-T as described above and phosphorylation measured using a chemiluminescence kit (ECL Plus, Amersham Biosciences). The level of phosphorylated Snf1 protein in each strain, with or without inhibitors, was standardized by comparing phosphorylation of Snf1 with α tubulin blots (internal controls). The primary antibody α tubulin was purchased from Cell Signaling Technology and blotted at 1:1000 at 4 °C for 12 h after the same blot had been stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) at 37 °C for 15 min. Total Snf1 was also measured in each loading sample with an AMPKα antibody (Cell Signaling Technology; Cat. No. 2532) (1: 1000) in 1× TBS-T and standardized with α tubulin.

1.5. Cytosol ROS measurements

Given the complex nature of ROS communication among cell organelles (Dios et al., 2010), a fluorescent dye DCFDA was used to measure the total cytosolic ROS in each tested mutant and WT as described previously (She et al., 2015). In brief, each yeast culture was collected from overnight growth in YPD and washed once with PBS (pH 7.2). One set of cells (5×10^6) in 1-mL of PBS was stained with 20 µM DCFDA in PBS (pH 7.2) at 30 °C for 15 min, and washed with PBS once