Establishing a Low Redox Potential in Giant Yeast Colonies: Effects of Media and Rotation

Holly H. Birdsall1,2,3,4, Patricia L. Allen4, Jeffrey S. Hammond5, Margaret A. Gunter5, and Timothy G. Hammond3,6,7

1Department of Veterans Affairs Office of Research and Development, Washington, DC; 2Departments of Otorhinolaryngology, Immunology, and Psychiatry, Baylor College of Medicine, Houston, TX; 3Space Policy Institute, Elliott School of International Affairs, Washington, D.C.; 4Durham VA Medical Center, Research & Development Service, Durham, NC; 5Institute for Medical Research, Durham, NC; 6Nephrology Division, Department of Internal Medicine, Duke University School of Medicine, Durham, NC; 7Durham VA Medical Center, Medicine Services, Durham, NC

ABSTRACT

Giant yeast colonies develop a low redox potential, which mimics the electrophilic milieu of both the mitochondrial drug metabolizing compartment and the hypoxic core of many tumors. The major metabolic mediators of low redox potential include: ATP, glutathione, NAD+/NADH, and NADP+/NADPH. Ammonia signaling is the critical mechanism that induces stratification of the giant yeast colonies to allow a low redox potential. A comparison of two powerful investigative models for drug pathways using Saccharomyces cerevisiae have been compounded by the use of different growth media and stimuli to the system. Chemogenetic profiling, which uses a pool of yeast deletion mutants to determine survival changes, is heavily slanted to the use of rich media. Giant yeast colonies studies are heavily slanted to the use of poor media. The current study answers the question "what is the difference over time in

ACRONYMS

ATP  Adenosine 5'-triphosphate
DC-FDA  2',7'-dichlorodihydrofluorescein diacetate
Em  Emission
Ex  Excitation
mBCL  Monochlorobimane
NAD  Nicotinamide adenine dinucleotide (oxidized form)
NADH  Nicotinamide adenine dinucleotide (reduced form)
NADP  Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH  Nicotinamide adenine dinucleotide phosphate (reduced form)
PBS  Phosphate buffered saline
PI  Propidium iodide
ROS  Reactive oxygen species
SEM  Standard error of the mean
Sok2A  Suppressor of Kinase 2 = ammonia deficient strain
WT  Wild type
YE  Yeast extract (“poor media”)
YPD  Yeast peptone dextrose (“rich media”)
redox potential, and its major metabolic mediators, between giant yeast colonies grown on rich and poor media? Using gene deletion tools, we show that cell death in giant yeast colonies is ammonia-dependent. In poor nutrient, ammonia-depleted (Sok2 deletion mutants) giant yeast cultures, rotation can allow manipulation of reactive oxygen species, providing a model to compare high and low redox states without chemical administration. Mechanistically, these changes are not due to detectable NAD/NAPH or NADP/NADPH changes, but are related in changes in glutathione and ATP concentration.

INTRODUCTION

Several lines of evidence suggest that giant yeast colonies can make important contributions to our understanding of human physiology and pathology, specifically, differentiation and stratification of cell colonies (Cap et al., 2009), cell survival and aging (Ayer et al., 2014; Cap et al., 2009), and cancer pharmacogenomics (Acharya et al., 2010; Cap et al., 2012b). Yeast have broad genetic homology to human cells and are simple to genetically manipulate (Lee et al., 2014; Nislow et al., 2015). Yeast offer the ability to quantitatively determine the effects of gene deletion on phenotype, including cell survival (Lee et al., 2014; Nislow et al., 2015), and this allows identification of drug metabolism pathways (Lee et al., 2014).

Giant yeast colony stratification is indelibly linked to gravity-driven convection, as ammonia production and gravity-driven convection mediate giant yeast colony differentiation (Cap et al., 2009). Growth and differentiation of giant yeast colonies is a month-long cycle of cell differentiation and production of signaling molecules (Cap et al., 2012a; Cap et al., 2012b; Hammond et al., 2015). Without ammonia the differentiation is interrupted, but provision of exogenous ammonia recapitulates the process (Cap et al., 2009). Yeast colonies growing on solid medium begin at 1-2 weeks into their development to produce volatile ammonia, and to alkalize their surroundings. Ammonia serves as a long-range signal between neighboring colonies and influences multiple aspects of colony biology—including metabolic reprogramming and differentiation (Cap et al., 2010). An ammonia signal emitted by aging colonies triggers metabolic changes that localize yeast death only in the colony center (Vachova and Palkova, 2005). The remaining population can exploit the released nutrients and survive (Vachova and Palkova, 2005). Colonies of the yeast Saccharomyces cerevisiae form sharply divided layers of sporulating and non-sporulating cells. Sporulation is initiated in the colony’s interior, and this region expands upward as the colony matures (Piccirillo et al., 2010; Vachova and Palkova, 2005). Hence, yeast colonies on agar do not have the characteristic exponential growth-to-stationary phase of liquid cultures. Rather, they differentiate in layers with divergent metabolism and functions (Piccirillo et al., 2010; Vachova and Palkova, 2005).

As there is greatly reduced convection in microgravity (Hammond and Hammond, 2001; Klaus et al., 2004), and since ammonia signaling to differentiate giant yeast colonies is gravity-driven convection (Klaus et al., 2004; Showman and de Pater, 2005), studies in true and simulated microgravity appear to have much to offer the understanding of giant yeast colony differentiation from aging to cell survival, as well as drug metabolism (Ayer et al., 2014; Cap et al., 2012a; Cap et al., 2012b; Lee et al., 2014; Nislow et al., 2015). The current study is the first to rotate giant yeast colonies to disperse ammonia, modulate ammonia-mediated colony differentiation, and quantitate the effects on the metabolic milieu.

Another important chiasm characterizes the differences between studies of giant yeast colonies and yeast applications to study drug metabolism. Specifically, giant yeast colony studies are almost universally performed on poor media (Cap et al., 2012a; Cap et al., 2009; Cap et al., 2012b), while yeast drug metabolism studies are almost universally performed in rich media (Lee et al., 2014; Nislow et al., 2015). Few, if any, studies directly address in a systematic quantitative manner the effects of media choice on middle metabolism—such as NAD:NADH or NADP:NADPH ratio, ATP (adenosine 5’-triphosphate), glutathione, and reactive oxygen species (ROS). The major components of the citric acid cycle electron transport chain of S. cerevisiae—also known as the tricarboxylic acid cycle or Krebs cycle—on the inner mitochondrial...
(which is the major determinant of reactive oxygen status) begin with NADH and end with ATP (Murray et al., 2011). Due to its low redox potential (E0 = 0.24 V) and high concentration (2-3 mM) in yeast (Ayer et al., 2014), glutathione is a ubiquitous, low molecular weight thiol that plays a major role in determining the cellular redox environment in most cellular compartments. NADPH is the immediate and predominant source of H+ for cellular antioxidant detoxifying systems in the cytosol, peroxisome, and mitochondrion (Ayer et al., 2014). NADPH is the major reducing agent to maintain pools of glutathione (Foyer and Noctor, 2005).

This study addresses exactly these issues—the effects of media choice on middle metabolism metrics, including the redox potential critical to drug metabolism (Acharya et al., 2010; Vander Heiden, 2011)—as well as using rotation as a stimulus to disperse ammonia (Klaus et al., 2004) to determine the effects of perturbing the giant yeast colony system and its redox status.

METHODS AND MATERIALS

Chemicals and Reagents

_Saccharomyces cerevisiae_ strain BY4743 (i.e., wild type (WT)), was used to match our earlier studies (Hammond et al., 2015). This clone, as well as Sok2 deletion, Msn4 deletion, Sfp1 deletion, and TRR1 eGFP yeast clones and the fluorescent dyes, monochlorobimane (mBCL) and 2′,7′-dichlorodihydrofluorescein diacetate (DC-FDA), were purchased from Life Technologies (Grand Island, NY). The ENLITEN® ATP Assay System for the quantitative detection of ATP and the NAD/NADH-Glo™ Assay kit and NADP/NADPH-Glo™ Assay kit were purchased from Promega (Madison, WI). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Giant Yeast Colony Cultures: Static and Rotating Cultures

Figure 1 summarizes the matrix of experiments performed. A 100 µl stationary culture inoculum was placed in 5 ml of fresh YPD or YE media, and grown overnight in a shaking incubator at 30°C. Round, 10 cm diameter agar plates were poured with YPD agar ((1% yeast extract, 2% peptone, 2% dextrose (D-glucose) (“rich media”), and 20 g agar/L) or YE agar (1% yeast extract, 3% glycerol, 1% ethanol, 2% agar, and 10 mM CaCl2) (“poor media”). For the imaging studies in Figure 2, propidium iodide (PI) was included in the agar (16 µg/ml final). Giant yeast colonies were initiated by placing 10 µl
spots of an overnight liquid culture on the agar plates, with six spots per plate. After drying and sealing the lid with Parafilm M® (Bemis, Oshkosh, WI), the plates remained at room temperature and were static or rotating for 7, 14, 21, or 28 days prior to harvest. Rotation was achieved by attaching the Petri dishes to the base of a four head, rotating cell culture system (Synthecon Inc., Houston, TX). The plates turned at 12 rpm around their long axis. Fresh liquid cultures in YE or YPD broth were inoculated 18 hours before the assay, and all samples were harvested and assayed simultaneously. There were a minimum of six replicates and three independent experiments for data presented here, but results mostly reflect data from 18 replicates and five independent experiments.

Assays for Cell Death, Glutathione, and Reactive Oxygen Species

Our choices of yeast redox potential and glutathione probes have been previously validated (Hammond et al., 2015). On the day of assay, giant yeast colonies were removed from the agar plate with the side of a piece of a plastic drinking straw (approximately 2 cm long), placed in 1 ml (1 or 7 day cultures) or 2 ml (day 14, 21, 28 day cultures) of phosphate buffered saline (PBS), and suspended by vortex. 50 µl aliquots were assayed in clear, flat-bottom 96 well plates, as indicated below in the section describing the plate reader, and the plate was bottom-read on a spectrofluorometric plate reader. To compensate for the varying amounts of yeast in the wells, the quantity of protein present was estimated by measuring the absorbance at 620 nm. A relative concentration of yeast was calculated on the basis of a standard curve prepared with serial 1:3 dilutions of yeast. The harvest volumes of PBS had been previously determined, so as to estimate where the samples would fall on the linear range of the OD 620 absorbance curve (Asaduzzaman, 2002). A standard curve of OD 620 nm dilutions of yeast was constructed for each experiment (Asaduzzaman, 2002). Any values off the linear portion of the curve were diluted to fall in the linear range, prior to further analysis. Multiple wells containing PBS buffer alone, unstained yeast, and dye alone were included in all experiments. This allowed determination of background fluorescence, and background was subtracted from sample measurements to yield actual fluorescence measurements. The relative fluorescence or luminescence of each sample was divided by the yeast concentration—determined by OD 620 nm—to correct fluorescence values for differences in number of yeast, recognizing that 3e+7 cells/ml=OD 1.0 (Day et al., 2004). Cell death was assayed as PI fluorescence by flow cytometry. Flow cytometry was performed in the Duke Human Vaccine Institute Research Flow Cytometry Shared Resource Facility, under the direction of Dr. Gregory D. Sempowski (Durham, NC), using a Becton Dickinson (San Jose, CA) LSRII cell analyzer flow cytometer with excitation (Ex)/emission (Em) of 532/590-610 nm. Measurements were collected on at least 10,000 cells per sample. Data analysis was performed using Flo-Jo software (Ashland, OR).

Bioluminescent Assays for ATP, NAD/NADH, and NADP/NADPH

The ENLITEN® ATP Assay System (Promega, Madison, WI) was used for the quantitative detection of ATP. When ATP is the limiting component in the luciferase reaction, the intensity of the emitted light is proportional to ATP concentration. Individual NAD⁺ and NADH levels, and NADP⁺ and NADPH levels, were measured using the Promega (Madison, WI) NAD/NADH-Glo® Assay kit and NADP/NADPH-Glo® Assay kit, respectively. Using a 96 well plate format, standard curves were included on each plate assayed. Preliminary experiments allowed sample dilution to ensure assays were within the linear dynamic range. Bioluminescent assays were top-read in white opaque plates using a spacer, according to the manufacturer’s recommendations.

Plate Reader

Luminescent assays were read within 10 minutes of adding the detection reagent, as specified in the manufacturer’s instructions. mBCL and DC-FDA were read after four hours, as previously determined (Hammond et al., 2015). Fluorescence was measured in a Molecular Devices Spectramax M5e® spectrofluorometer using Ex and Em pairs of 405/525 nm and 485/530 nm. Kinetic measurements were taken
every 10 minutes for 8 hours at 30°C as the dyes entered the cells and were activated by cleavage and/or target binding. On each plate, endpoint assay of absorbance at 620 nm was recorded as a surrogate for protein concentration. Controls, including PBS buffer alone, unstained yeast, and dye alone, were included in every experiment and subtracted to arrive at net fluorescence.

**Colony Imaging**

Colony imaging was performed on a GE Healthcare Life Sciences IN Cell Analyzer 2200 Imaging System. Ex and Em filters were 488/525+20 nm and 525/617+20 nm.

**Statistics**

Data is presented as geometric mean ± standard deviation with a minimum of six (but typically 18) replicates pooled from at least three (typically five) independent experiments. Analysis of variance and post-hoc comparison using Tukey’s test was performed using Statistica 6.1 (StatSoft Inc., Tulsa, OK), using correlation matrix product moment and partial correlations.

**RESULTS**

This study documents the effects of rich YPD versus poor YE media, and rotation versus static culture conditions on cell death, as well as the content of ROS, glutathione, ATP, NADP, NADH, NAD, and NADH ratios in giant yeast colonies. The experimental design is outlined in Figure 1. Sok2 deletion clones were used to evaluate the effect of blocking ammonia production. Sfp1 deletion and Msn4 deletion clones were used to evaluate the role of stress responses previously shown to be involved in the response to microgravity (Coleman et al., 2008a).

Yeast cultured on solid agar for extended time periods form giant colonies. At 14 days, imaging shows the colony is stratifying with cells in the center, beginning to undergo apoptosis and take up PI (Figure 2, panel B). By day 28, there is a prominent core of non-viable cells, while cells at the edge of the colony continue to thrive (Figure 2, panel C).

The fraction of non-viable cells can be quantified by flow cytometry of the harvest colony (Figure 2). In rich YPD media, the colony remains largely viable for up to 21 days; but at 28 days, approximately a fourth of the colony is non-viable (Figure 3, lower left panel). If the cells are grown on YPD agar under rotating conditions, the fraction of non-viable cells at 28 days is almost two-fold higher than static controls (p=0.002) (Figure 3, lower panels).

The fraction of non-viable cells is also increased in cultures rotated on YE agar for 28 days compared to static YE agar controls (p=0.0006) (Figure 3, upper panels). Furthermore,

Figure 2. Effect of Culture Cell Death. Yeast were spotted on YPD agar impregnated with propidium iodide (PI) and imaged by fluorescence microscopy at 7 days (Panel A), 14 days (Panel B), and 28 days (Panel C). Green fluorescence is due to expression of eGFP linked to TRR1. Red fluorescence reflects cell death due to uptake of PI.
Figure 3. Effect of Media and Rotation and Media on Cell Death. Graphs show cell death in colonies harvested after 1, 7, 14, 21, and 28 days of growth on YE or YPD agar under static or rotating conditions, or in liquid YE or YPD media for one day. Values shown are the average percent of PI-positive cells as assessed by flow cytometry; error bars indicate +/- standard error of the mean (SEM) of six replicates from three separate experiments. Asterisks indicate time points where the Sok2 deletion clone (red line, squares) had significantly more cell death ($p<0.0001$), than either wild type (WT) (blue line, open circle), the Sfp1 deletion clone (purple lines), or the Msn4 deletion clone (green lines).

when cultured on YE agar, the colonies begin to lose viability earlier (compare day 21 values in the upper panels of Figure 3). This accelerated cell death starts even earlier in Sok2 deleted yeast that are unable to produce ammonia. Cell death is significantly higher ($p<0.0001$) at days 7, 14, and 21 in Sok2 deleted colonies compared to WT, Msn4 deleted, or Sfp1 deleted colonies (Figure 3, upper panels). Sok2 deleted colonies are also significantly less viable under rotation conditions at days 7 and 14, but this difference is lost at days 21 and 28 as rotation-associated cell death increases in the WT and other deletion clones.

To better understand the factors that influence cell viability under the different culture conditions, we measured ROS, glutathione, ATP,
NAD, NADH, NADP, and NADPH. ROS, as reported by DC-FDA fluorescence, did not differ significantly across time in WT yeast cultured on either YPD, or YE agar (Figure 4, upper panels). ROS also did not differ between rotated and static WT colonies (Figure 4, upper panels). In the ammonia-deficient Sok2 deleted colonies, the ROS levels were much higher at day 28 than earlier time points for both YE and YPD agar (Figure 4, lower panels). Furthermore, rotation was associated with a striking increase in ROS in the Sok2 deleted yeast grown on YE agar for 21 days (p=0.0001) (Figure 4, lower right panel). Yeast deleted for Sfp1 or Msn4 showed no significant change in ROS across time, in differing media, or in response to rotation (not shown).

Figure 4. Effect of Media and Rotation on Reactive Oxygen Species. Graphs show the amount of ROS—measured by DC-FDA fluorescence—in giant yeast colonies cultured on YE or YPD agar for 1, 7, 14, 21, or 28 days. Rotated samples are shown with filled red squares and a solid line; static samples are shown with open circles and a blue line. Results from one day liquid cultures are shown with an X. Values shown are the net fluorescence in relative units, normalized to the amount of yeast as determined by absorbance at 620 nm; error bars indicate +/- SEM of 18 WT replicates, or 6 Sok2 deletion replicates taken from three separate experiments. Double-headed arrow indicates the time point at which rotated samples were significantly different from static samples (p=0.0001 for Sok2 deletion clones on YE media at 21 days).
Glutathione, as reported by mBCL fluorescence, did not change in the WT cultures over the 28 day period on either YE or YPD, under rotating or static conditions (Figure 5, upper panels). Glutathione levels in the Sok2 deleted clones showed some changes with time, but were largely unaffected by rotation or media (Figure 5, lower panels). Overall, levels of glutathione in Sok2 deleted clones were higher than WT colonies at virtually all time points and conditions. Yeast deleted for Sfp1 or Msn4 showed no significant change in glutathione across time, in differing media, or in response to rotation (not shown).

ATP, as reported by a bioluminescent assay, tended to decrease with time in the colonies grown on YPD, but remained high in the colonies cultures on YE. Yeast deleted for Sfp1 or Msn4 showed no significant change in ATP across time, in differing media, or in response to rotation (not shown).

The ratio of NAD:NADH or NADP:NADPH, as reported by a bioluminescent assay, were not significantly or consistently different than the various clones cultured on YE or YPD under rotating or static conditions (not shown).

Figure 5. Effect of Media and Rotation on Glutathione. Graphs show the amount of glutathione—measured by monochlorobimane (mBCL) fluorescence—in giant yeast colonies cultured on YE or YPD agar for 1, 7, 14, 21, or 28 days. Rotated samples are shown with filled red squares and a solid line; static samples are shown with open circles and a blue line. Results from one day liquid cultures are shown with an X. Values shown are the net fluorescence in relative units, normalized to the amount of yeast as determined by absorbance at 620 nm; error bars indicate +/- SEM of 18 WT replicates, or 6 Sok2 deletion replicates taken from three separate experiments.
Figure 6. Effect of Rotation and Media on ATP. Graphs show the amount of ATP—measured by luminescence assay—in giant yeast colonies cultured on YE or YPD agar for 1, 7, 14, 21, or 28 days. Rotated samples are shown with filled red squares and a solid line; static samples are shown with open circles and a blue line. Results from one day liquid cultures are shown with an X. Values shown are the net luminescence in relative units, normalized to the amount of yeast as determined by absorbance at 620 nm; error bars indicate +/- SEM of 18 WT replicates, or 6 Sok2 deletion replicates taken from three separate experiments.

DISCUSSION

Giant yeast colonies grown on agar form and develop along a defined pathway of steps. The yeast colony grows until it exhausts its nutrients, and then ammonia production is triggered (Cap et al., 2009; Palkova et al., 1997). The ammonia forms alkali in the colony and supporting agar, and the colony center differentiates into basal ammonia–secreting, non-replicative L-cells and upper and peripheral non-ammonia-secreting replicative U-cells. Layer dominance is briefly reversed, as the replicative U-cells divide, the agar and colony acidify, and then status reverts to
ammonia-driven basal cell death in a high alkali colony and agar status, with upper and peripheral viable U-cell layers (Cap et al., 2009; Cap et al., 2010; Cap et al., 2012b).

In ground-based static cultures maintained for multiple days, yeast cells form giant multicellular colonies with characteristic organized morphologies (Cap et al., 2012a; Cap et al., 2009; Cap et al., 2012b). Around day nine, cells at the base of the colony begin to apoptose and also begin to secrete ammonia. Ammonia signalling induces cells at the top and leading edges of the colony to reprogram their metabolic pathways and divide rapidly, thereby allowing the colony to continue to expand. Interestingly, the differentiation of giant yeast colonies is also, at least in part, dependent on the promoters Msn4 (Cap et al., 2012a), which we have previously implicated in the response of yeast to suspension culture, mimicking some of the conditions of microgravity (Coleman et al., 2008a). The shear stress promoter in yeast, Sfp1, also provides a robust control for non-specific effects.

Hence, two very different modulations of yeast colonies, the response to real and simulated microgravity, and ammonia-dependent differentiation of giant yeast colonies may both be Msn4- and Sfp1-dependent. This is the primary mechanistic aim of the current studies. Are cell death, ROS, ammonia (Sok2), or shear stress promoters (Msn4 and Sfp1) dependent? Are these changes reflected in glutathione levels or middle metabolism, starting with the NAD system and ending in ATP?

The importance of these studies is to determine if we can modulate reactive oxygen status by physical (rotation) rather than pharmacological means to provide a drug-free system to compare the effects of reactive oxygen status on drug metabolism pathways. The answer is a resounding affirmative, as we observed large, highly reproducible changes in ROS, with rotation on day 21 on a Sok2 deleted yeast strain on poor media.

The previous evidence for a role of ammonia comes both from measurements of released ammonia, and provision of ammonia to modulate the timing of the colony differentiation (Cap et al., 2012a). However, there is scant, if any, data on yeast with the ammonia pathway genetically disrupted, such as deletion of the primary ammonia-producing gene Sok2. Using Sok2 deletion yeast strains, this paper demonstrates the central role of ammonia as a mechanism mediating cell death, middle metabolism changes (specifically ATP), and redox status, partially mediated by changes in glutathione.

Giant yeast colonies have practical utility to understand drug metabolism, as they have a very low reactive oxygen status that mimics the redox potential found in the core of tumors and the drug metabolizing membranes of mitochondria (Cap et al., 2012a). As the progression of giant yeast colony differentiation is triggered by exhaustion of nutrients, most studies have facilitated progression by using poor media (Cap et al., 2009). Sophisticated studies of drug metabolism pathways in the yeast deletion series have identified 46 key pathways. However, those studies have almost universally been carried out in rich liquid media (Lee et al., 2014).

The current study demonstrates that rich media ablates the ammonia-dependence of cell death. Rich media had little effect on giant yeast colony ROS status or glutathione—thought to mediate ROS status—leaving these parameters at the extremely low levels desirable for studies related to tumor biology and drug metabolism (Acharya et al., 2010; Jorgenson et al., 2013; Kobayashi and Suda, 2012).

Our lab has extensively studied the responses of yeast to real (flight-based) and simulated microgravity (Coleman et al., 2008a; Coleman et al., 2008b; Coleman et al., 2007; Hammond et al., 1999; Johanson et al., 2002; Nislow et al., 2015). We have found that yeast colonies spotted on agar respond to microgravity through changes in mitochondrial and ribosomal gene pathways. This genetic modulation of yeast colonies in real and simulated microgravity is dependent, at least in part, on both ammonia (Sok2) and the shear stress promoter Msn4, but not Sfp1.

Hence, using genetic deletion tools, we conclude that cell death in giant yeast colonies is ammonia-dependent. In poor nutrient media using ammonia-depleted (Sok2 deletion mutants) giant yeast cultures, rotation allows manipulation of ROS, providing a model to compare high and low redox states without chemical administration. These changes are not due to detectable
NAD/NAPH or NADP/NADPH changes, but are related to changes in ATP concentration.

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