Increased aerobic metabolism is essential for the beneficial effects of caloric restriction on yeast life span

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Abstract Calorie restriction is a dietary regimen capable of extending life span in a variety of multicellular organisms. A yeast model of calorie restriction has been developed in which limiting the concentration of glucose in the growth media of *Saccharomyces cerevisiae* leads to enhanced replicative and chronological longevity. Since *S. cerevisiae* are Crabtree-positive cells that present repression of aerobic catabolism when grown in high glucose concentrations, we investigated if this phenomenon participates in life span regulation in yeast. *S. cerevisiae* only exhibited an increase in chronological life span when incubated in limited concentrations of glucose. Limitation of galactose, raffinose or glycerol plus ethanol as substrates did not enhance life span. Furthermore, in *Kluyveromyces lactis*, a Crabtree-negative yeast, glucose limitation did not promote an enhancement of respiratory capacity nor a decrease in reactive oxygen species formation, as is characteristic of conditions of caloric restriction in *S. cerevisiae*. In addition, *K. lactis* did not present an increase in longevity when incubated in lower glucose concentrations. Altogether, our results indicate that release from repression of aerobic catabolism is essential for the beneficial effects of glucose limitation in the yeast calorie restriction model. Potential parallels between these changes in yeast and hormonal regulation of respiratory rates in animals are discussed.

Keywords Calorie restriction · Crabtree effect · Free radicals · Aging · Respiration

Introduction

Calorie restriction, or the reduction of caloric intake without lack of essential nutrients, is a dietary regimen capable of extending life span in a variety of laboratory animals ranging from *C. elegans* to mice and, probably, primates. The effects of this diet are widespread, and involve physiological, metabolic, hormonal, gene expression and morphological changes. It is not yet clear which of these observed changes are directly related to decreased incidence of age-related pathologies and increased life span (Weindruch and Walford 1988; Partridge and Gems 2002).

Yeast models of calorie restriction have been developed in order to study the results of this diet in a less complex organism. In *Saccharomyces cerevisiae*, growth in rich media with lower glucose concentrations significantly increases both replicative and chronological longevity (Jiang et al. 2000; Lin et al. 2000). Interestingly, the beneficial effects of glucose restriction in yeast are related to increases in respiratory rates that occur when glucose levels in the media are lower (Lin et al. 2002; Barros et al. 2004). These enhanced respiratory rates elevate intracellular

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NAD^+ levels, which may be involved in the regulation of life span by modulating the activity of Sir2 family proteins (Lin and Guarente 2003) and reducing mitochondrial reactive oxygen species release (Tahara et al. 2007).

S. cerevisiae are Crabtree-positive cells, yeast in which fermentation is the preferred metabolic pathway and aerobic metabolism is inhibited, despite the presence of oxygen, when glucose levels are high (De Deken 1966). Taking this into account, we questioned whether repression of aerobic catabolism was necessary for the efficacy of the yeast caloric restriction model. In order to investigate this possibility, we compared chronological life spans of S. cerevisiae in the presence of different substrates metabolized by the glycolytic pathway which lead to different levels of respiratory repression. We also compared the effects of glucose limitation in S. cerevisiae and glucose limitation in Kluyveromyces lactis, a Crabtree-negative yeast (Breunig et al. 2000).

Experimental procedures

1. S. cerevisiae and K. lactis: BY4741 S. cerevisiae (Brachmann et al. 1998) and CBS 2359 K. lactis were used for all experiments.
2. Culture media: Culture media used for all experiments were liquid yeast extract (1%) and peptone (2%) plus 0.2–3.0% glucose, galactose, raffinose or glycerol plus ethanol, as indicated.
3. Chronological life span determinations: Chronological life span was determined by colony forming ability and metabolic integrity, or the ability to accumulate calcein. *Colony-forming ability:* 100 cells from S. cerevisae or K. lactis in late stationary phase (96 h) cultured in liquid media (1% yeast extract, 2% peptone and 0.2–3.0% glucose, 0.2–3.0% galactose, 0.2–3.0% raffinose or 0.2–3.0% glycerol plus 0.2–3.0% ethanol, as indicated) were plated in 2% solid YPD media (1% yeast extract, 2% peptone, 2% glucose and 2% bacteriological agar). After ~36 h of growth at 30 °C, the number of colonies formed was counted (Tahara et al. 2007). *Metabolic integrity:* Yeast in late stationary phase were centrifuged at 1,000×g for 5 min at 30 °C and washed twice using ultrapure water. Cells were ressuspended ($2×10^6$) in 1 mL of 0.6 M sorbitol, 32.5 mM K-phosphate, 10 mM Tris–Cl and 1 mM EDTA (pH 7.5) and were incubated with 1 µg/mL calcein-AM for 20 min. Cytometry parameters used were: FS=17.6 (gain=5.0); SS=243 (gain=50.0); FL1=752 (gain=2.0); discriminant value=20.0.
4. Spheroplast generation: Spheroplasts were obtained through yeast cell wall digestion with 20 U zymolyase/g of cells, for ~45 min at 37 °C, under mild shaking, in

1.2 M sorbitol, 10 mM $MgCl_2$ and 50 mM Tris, pH 7.5. Spheroplasts were ressuspended in 75 mM K-phosphate buffer with 1.2 M sorbitol and 1 mM EDTA, pH 7.5, to a final concentration of 10 mg protein/mL (Tahara et al. 2007). Protein concentrations were determined using the Lowry method.

5. Cytochrome absorption spectra: Respiratory chain cytochrome spectra were assayed with mitochondria prepared by the method of Faye et al. (1974), except that zymolyase 20T instead of glusulase was used for the conversion of cells to spheroplasts. Mitochondria were extracted at a final protein concentration of 4 mg/mL with 1% deoxycholate to solubilize all cytochromes (Tzagoloff et al. 1975). Difference spectra of sodium dithionite-reduced versus potassium ferricyanide-oxidized extracts were recorded at room temperature.
6. NADH-cytochrome c reductase activity: NADH oxidation and cytochrome c reduction were estimated as described previously (Tzagoloff et al. 1975). Briefly, 20 µg of mitochondrial proteins, previously permeabilized with 0.1% of potassium deoxycholate, were incubated in 10 mM potassium-phosphate buffer, pH 7.5, containing 0.1 mM KCN and 0.08% cytochrome c. The rate of cytochrome c reduction was measured at 550 nm, at room temperature, after the addition of 1 mM NADH.
7. O_2 consumption: O_2 consumption was monitored over time in 800 µg/mL spheroplast suspensions in the presence of 2% ethanol and 1 mM malate/glutamate as substrates, using a computer-interfaced Clark-type electrode operating with continuous stirring, at 30 °C. Spheroplasts were permeabilized using 0.004–0.006% digitonin, as described by Tahara et al. 2007.
8. H_2O_2 production: H_2O_2 release from mitochondria was monitored for 10 min in 100 µg/mL spheroplast suspensions in the presence of 50 µM Amplex Red, 0.5 U/mL horse radish peroxidase, 2% ethanol and 1 mM malate/glutamate, using a fluorescence spectrophotometer operating at 563 nm excitation and 587 nm emission, with continuous stirring, at 30 °C. Spheroplasts were permeabilized using 0.002–0.003% digitonin, as described by Tahara et al. (2007). Measurements were calibrated by adding known quantities of H_2O_2, as described previously (Ferranti et al. 2003).

Results

Decreasing the concentration of glucose in the culture media results in an enhancement of replicative (Jiang et al. 2000; Lin et al. 2000) and chronological (Barros et al. 2004; Tahara et al. 2007; Smith et al. 2007) life span in S.

cerevisiae. Indeed, we found that *S. cerevisiae* cultured until the late stationary growth phase in media containing lower glucose concentrations (0.2–0.5%) generated significantly more colonies (representing more viable cells) than those cultured in higher glucose concentrations (1–3%, Fig. 1, upper left Panel). This higher generation of colonies in the late stationary phase represents an extended chronological life span (Tahara et al. 2007; Smith et al. 2007). In order to verify if this effect was dependent on the significant repression of aerobic catabolism promoted by growth in high concentrations of glucose, we compared glucose restriction to the effect of restricting galactose, raffinose, and glycerol plus ethanol.

Galactose is metabolized by the Leloir pathway to produce glucose 6 phosphate, which is then degraded by the glycolytic pathway. Although metabolism of this sugar leads to some degree of respiratory inhibition, the effect is significantly smaller than that observed with glucose (Gancedo 1998; Rodríguez and Gancedo 1999). Raffinose is a trisaccharide composed of galactose, fructose, and glucose, which also promotes less significant changes in aerobic catabolism than glucose, due to the lower concentrations of free glucose produced (Gancedo 1998). Interestingly, no beneficial effect of limiting galactose or raffinose was seen on *S. cerevisiae* chronological life span (Fig. 1). Furthermore,

limitation of glycerol plus ethanol, which are non-fermentable substrates, also did not enhance chronological life span, suggesting that repression of aerobic catabolism is essential for the beneficial effects of the yeast caloric restriction model. Indeed, limiting raffinose and glycerol/ethanol decreased cell survival.

In order to ascertain that high glucose concentrations were not exclusively decreasing replication and colony-forming ability, we also determined chronological life span by measuring cellular metabolic integrity, or the ability of the cells to accumulate fluorescent calcein when incubated with calcein-AM. Flow cytometry fluorescence histograms (Fig. 2) demonstrate that restriction of glucose, and to a lesser extent galactose, but not raffinose or glycerol plus ethanol, lead to significantly improved cell integrity, as indicated by peaks at higher fluorescence levels.

We have previously shown that a beneficial effect associated with glucose restriction leading to extended chronological life span is the prevention of oxidative stress. While antioxidant levels are largely unchanged (Lin et al. 2002), glucose restriction limits mitochondrial reactive oxygen species generation in mitochondria (Barros et al. 2004; Tahara et al. 2007). In order to compare the redox effects of fermentative versus respiratory substrates (promoting maximal and minimal respiratory repression, respectively), we

Fig. 1 Glucose, but not galactose, raffinose or glycerol/ethanol restriction increases chronological life span (colony-forming ability) in *S. cerevisiae*. One hundred late stationary cells cultured using glucose, galactose, raffinose or glycerol plus ethanol (as indicated) as substrates were plated in solid 2% YPD. Colonies were counted after 36 h of growth at 30 °C. *$p<0.05$ versus 3.0%
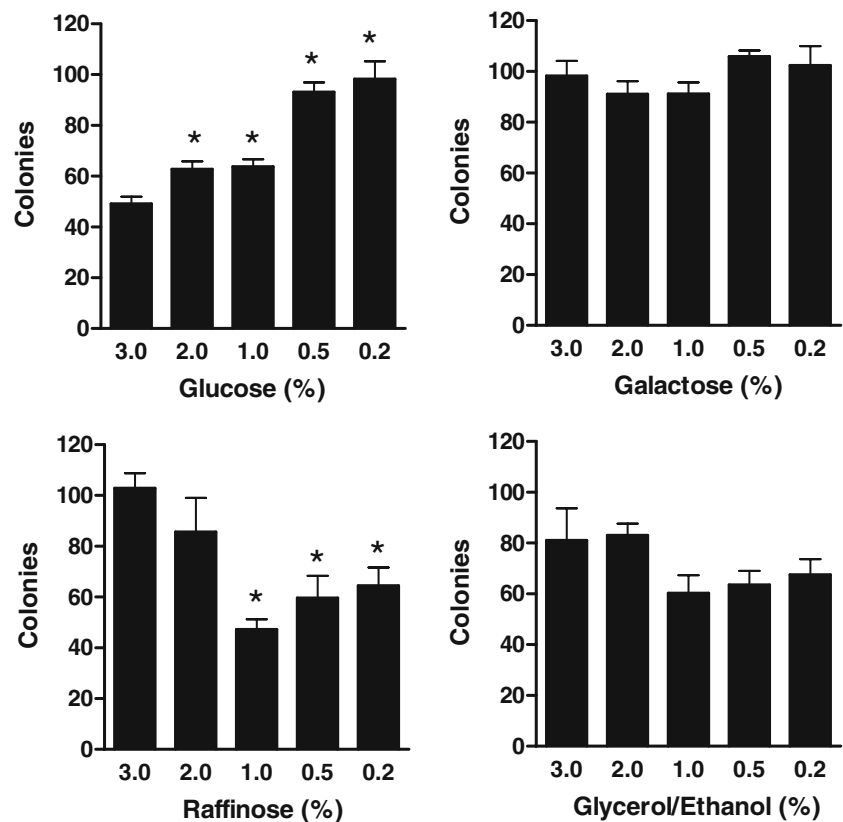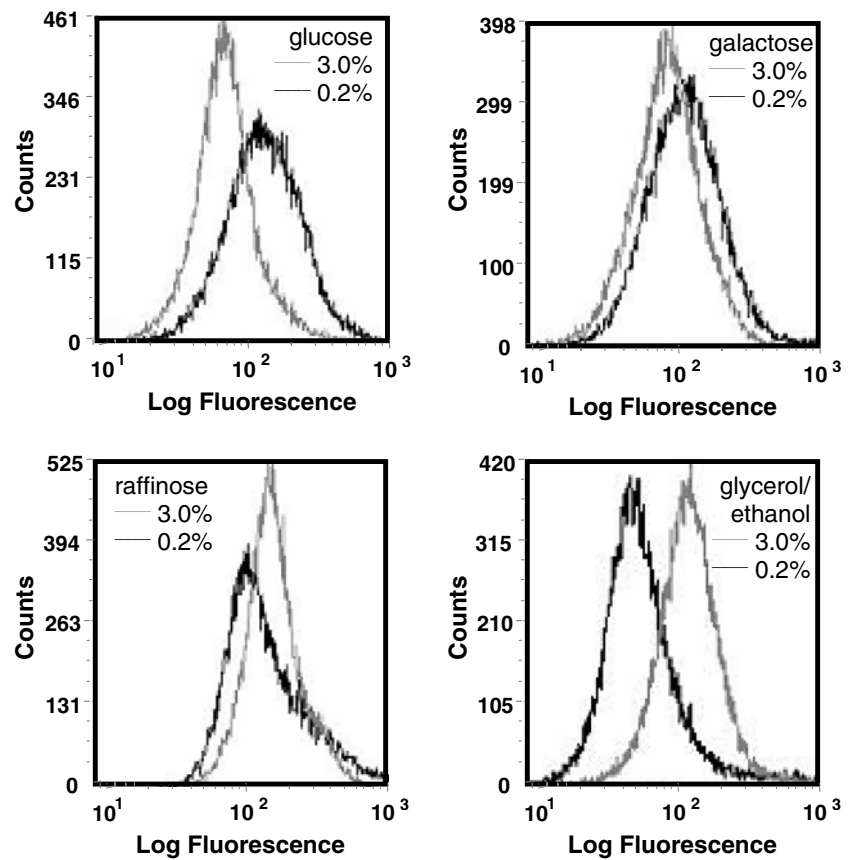
Fig. 2 Glucose, but not galactose, raffinose or glycerol/ethanol restriction increases chronological life span (metabolic integrity) in *S. cerevisiae*. Calcein retention of late stationary cells cultured in 0.2% (*black lines*) or 3.0% (*grey lines*) glucose, galactose, raffinose or glycerol plus ethanol was measured as described in "Experimental procedures"


measured oxygen consumption and the release of H_2O_2 (a membrane-permeable reactive oxygen species) in digitonin-permeabilized spheroplasts (Tahara et al. 2007) from *S. cerevisiae* grown in 2.0% or 0.5% glucose or ethanol/glycerol

(Fig. 3). As expected, we found that yeasts grown in 2.0% glucose present lower respiratory rates than those grown in ethanol/glycerol. In addition, glycerol/ethanol-grown cells presented lower levels of both absolute (H_2O_2) and relative

Fig. 3 Glucose restriction or glycerol/ethanol decrease H_2O_2 release in *S. cerevisiae*. Spheroplasts obtained from early and late stationary phase yeast were incubated in 1.2 M sorbitol, 1 mM EDTA, 75 mM phosphate, 2% ethanol, 1 mM malate and 1 mM glutamate, pH 7.5 (KOH). Plasma membrane permeabilization was obtained using digitonin (0.002–0.006%), and O_2 consumption and H_2O_2 release rates were measured as described in "Experimental procedures". *$p<0.05$ versus 2.0% glucose; #$p<0.05$ versus 16 h
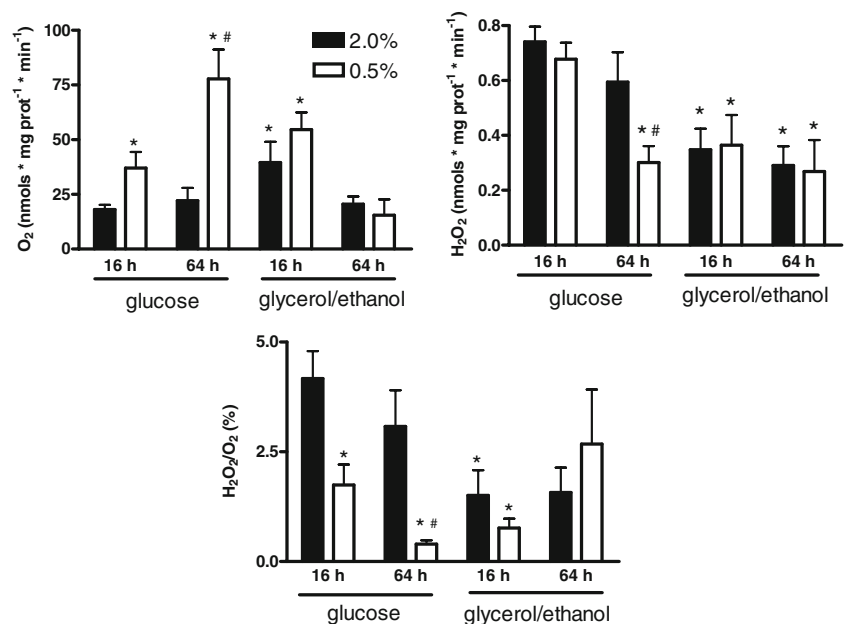
(H_2O_2/O_2) mitochondrial H_2O_2 production. Taken together, the results presented to this point strongly suggest that enhanced chronological life span and prevention of oxidative stress in yeasts grown in limited glucose are due to the loss of repression of aerobic catabolism promoted by high glucose growth conditions.

If loss of repression of aerobic catabolism is indeed necessary for the beneficial effects of calorie restriction in yeast, these effects should only be observed in Crabtree-positive yeasts. We thus investigated if a Crabtree-negative yeast, *K. lactis*, responded to glucose restriction similarly to *S. cerevisiae*. As expected, restriction of glucose in *S. cerevisiae* cultures leads to a clear increment in respiratory cytochromes content (Fig. 4, black lines), both in early and late stationary growth phases. On the other hand, *K. lactis* mitochondrial respiratory cytochromes did not show any

significant increment when cells were cultured under glucose restricted conditions. Consistently, the respiratory activity of *K. lactis*, reflected here as NADH-cytochrome c reductase activity, did not change when the two growth conditions were compared. This enzymatic activity is strongly stimulated in *S. cerevisiae* mitochondria isolated from cells cultured under glucose restriction (open bars), at both 16 and 64 h.

We then determined the functional effects of these respiratory capacity changes by measuring oxygen consumption and release of H_2O_2 in digitonin-permeabilized spheroplasts of these yeasts (Tahara et al. 2007). Figure 5 shows that *S. cerevisiae* grown in low glucose exhibit significantly higher respiratory rates after 16 and 64 h in culture, despite the fact that glucose was undetectable after only 16 h (Tahara et al. 2007). As discussed previously,

Fig. 4 Glucose limitation increases cytochome contents and NADH-cytochrome c reductase activity in *S. cerevisiae*, but not *K. lactis*. Cytochrome spectra (*upper panels*) and NADH-cytochrome c reductase activity (*lower panels*) were measured as described in "Experimental procedures" in *S. cerevisiae* or *K. lactis* cultured for 16 or 64 h, as shown. *Letters above the spectra* indicate the absorption peaks of specific cytochromes. *$p<0.05$ versus 2.0% glucose
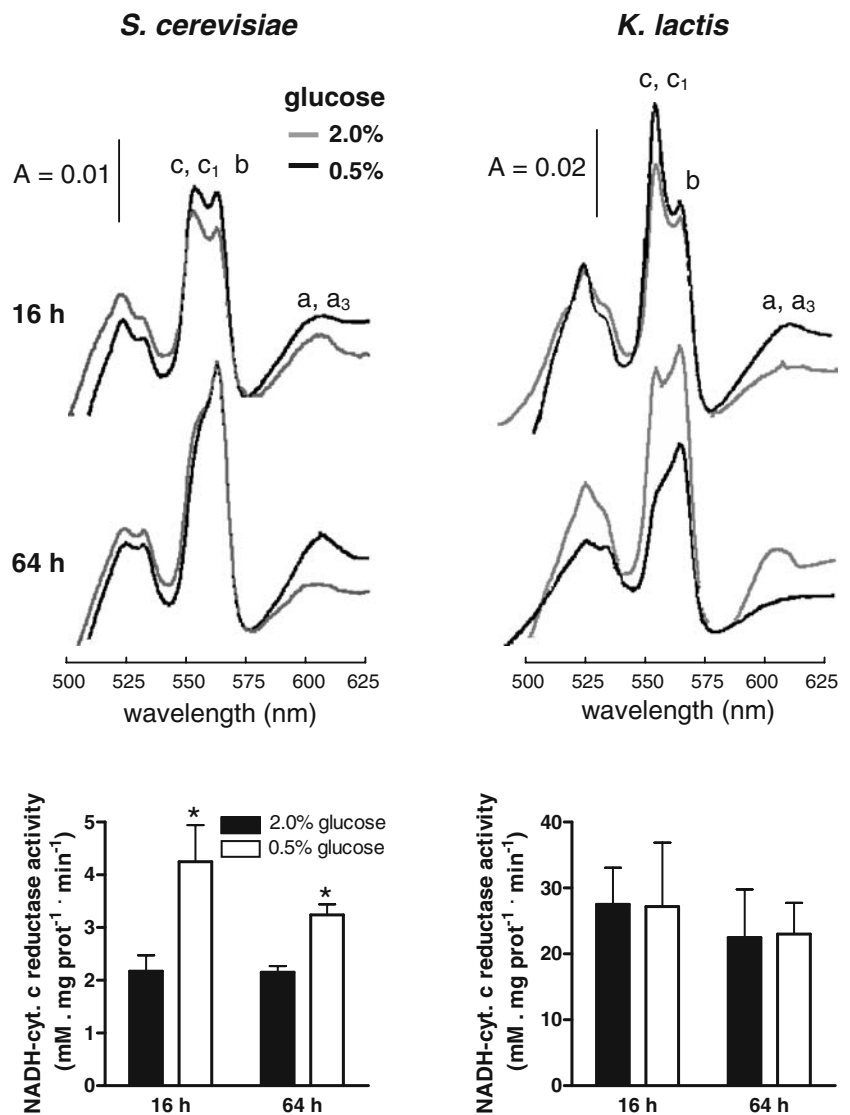
Fig. 5 Calorie restriction decreases H_2O_2 release in *S. cerevisiae*, but not *K. lactis*. Spheroplasts obtained from early and late stationary phase yeast were incubated in 1.2 M sorbitol, 1 mM EDTA, 75 mM phosphate, 2% ethanol, 1 mM malate and 1 mM glutamate, pH 7.5 (KOH). Plasma membrane permeabilization was obtained using digitonin (0.002–0.006%), and O_2 consumption and H_2O_2 release rates were measured as described in "Experimental procedures". $*p < 0.05$ versus 2.0% glucose; $^#p < 0.05$ versus 16 h
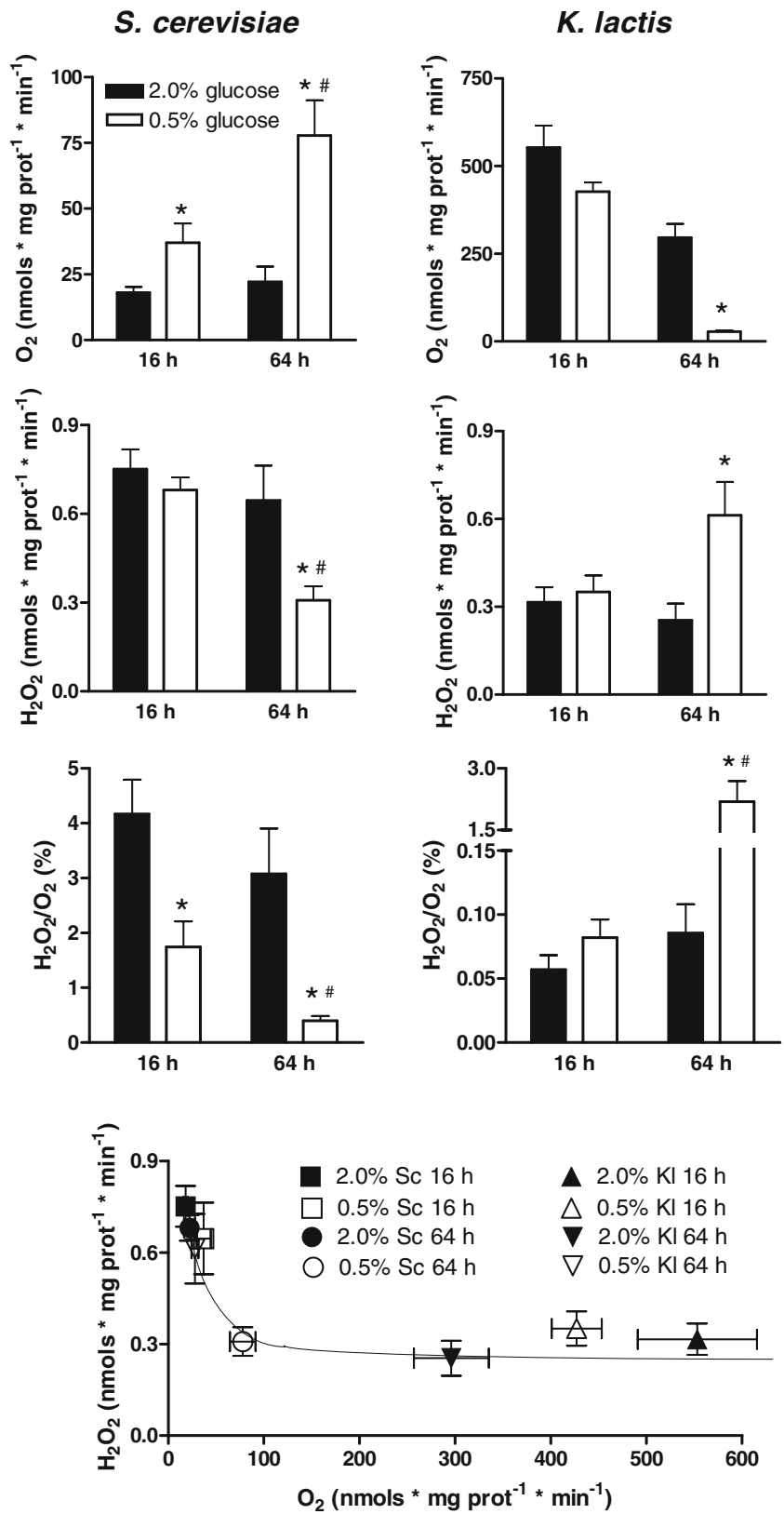
higher respiratory rates in *S. cerevisiae* are accompanied by a decrease in both absolute (H_2O_2) and relative (H_2O_2/O_2) mitochondrial H_2O_2 production, most noticeably after 64 hours in culture. However, in *K. lactis* the results were strikingly different. Respiratory rates were not enhanced by low glucose concentrations, and, instead, showed marked inhibition after longer culture times, accompanied by an increment in H_2O_2 release rates. These results clearly indicate that the beneficial effects of caloric restriction on *S. cerevisiae* redox state do not occur in the Crabtree-negative *K. lactis*.

Interestingly, respiratory rates and H_2O_2 release presented a strong inverse correlation in both cell types, regardless of culture phases (Fig. 5, lower panel). This suggests that respiratory rates are a major determinant of yeast oxidant generation. Since we have previously shown that redox state limits chronological longevity in *S. cerevisiae* (Barros et al. 2004; Tahara et al. 2007), our results support the notion that enhancement of aerobic metabolism is an important step in chronological life span extension promoted by glucose limitation.

In this sense, we investigated next if *K. lactis* responded to glucose restriction with enhanced chronological longevity. We found that *K. lactis* does not present an increase in life span, as measured by colony-forming ability or calcein retention (Fig. 6), when incubated in decreasing concentrations of glucose. In *K. lactis*, no colony formation and low calcein retention were observed at the late stationary growth phase in media containing low glucose levels, despite the finding that cell replication occurred normally, as indicated by cell growth in the

Fig. 6 *K. lactis* does not display enhanced chronological life span with glucose restriction. In the leftmost panel, colony-forming ability was measured by plating 100 late stationary cells cultured in 0.2–3.0% liquid glucose on solid media. Colonies were counted after 36 h of growth at 30°C. *$p < 0.05$ versus 3.0% glucose. In the rightmost panel, metabolic integrity was determined by measuring calcein retention in cells cultured in 0.2% (*black lines*) or 3.0% (*grey lines*) glucose, as described in "Experimental procedures"

logarithmic phase (results not shown). Thus, *K. lactis* clearly does not present an increase in chronological life span under conditions of low glucose that favor life span extension in *S. cerevisiae*.

Discussion

Calorie restriction by limiting glucose concentrations in the growth media has been widely shown to extend life span in *S. cerevisiae*. Most studies in this sense have been conducted measuring replicative life span, which consists in determining the number of generations a mother cell produces after removal from the growth media (Sinclair et al. 1998). Under these conditions, a variety of differing experiments (although not all, see Kaeberlein et al. 2005) suggest that the beneficial effects of glucose restriction are mediated by changes from fermentative to aerobic metabolism. These experiments include studies indicating that deletion or inhibition of respiratory chain components decreases life span and the response to glucose restriction (Lin et al. 2002), while activation of mitochondrial respiration by overexpressing Hap4 (Lin et al. 2002) or treatment with mitochondrial uncoupling agents (Barros et al. 2004) extends replicative life span.

We have previously shown that *S. cerevisiae* chronological life span, or the survival of cells in stationary phase (Fabrizio and Longo 2003), is intimately related to respiratory rates and mitochondrial levels of reactive oxygen species (Barros et al. 2004; Tahara et al. 2007). Interventions such as glucose limitation and mitochondrial uncoupling, which increase respiratory rates, decrease H_2O_2 release and augment chronological life span. Altogether, these results strongly suggest that enhanced life span in the yeast caloric restriction model depends on a phenomenon typical of *S. cerevisiae* and yeasts specifically adapted for fermentation: repression of aerobic catabolism upon incubation in the presence of high glucose concentrations.

This hypothesis is directly tested in this manuscript. Initially, we verified if limiting other carbohydrates as growth substrates promoted the same beneficial effects as glucose in *S. cerevisiae*, and found, using two distinct techniques to measure cell survival in the stationary phase, that improved chronological longevity was exclusive to the limitation of glucose (Figs. 1 and 2). Our results are in agreement with those of Smith et al. (2007), who did not see an increment in *S. cerevisiae* replicative capacity after long cultures in substrates other than glucose. In addition to life span extension, we show that *S. cerevisiae* cultured in the presence of lower glucose levels present a decrease in mitochondrial reactive oxygen species release associated with higher respiratory rates. On the other hand, cultures using respiratory substrates glycerol/ethanol present low
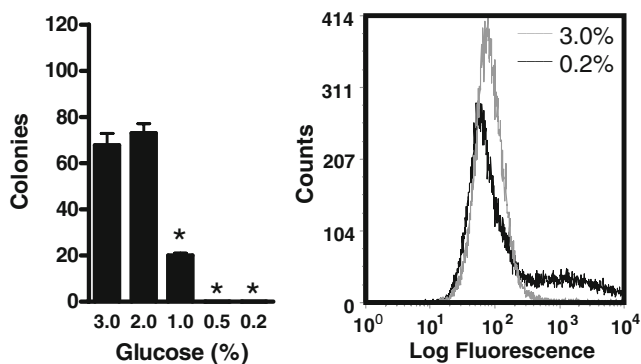
reactive oxygen release independently of the concentration of the substrate in the culture media (Fig. 3).

We also found that a model Crabtree-negative yeast, *K. lactis*, exhibited no beneficial decreases in reactive oxygen release (Fig. 5) or increments in life span (Fig. 6) when incubated in low glucose concentrations. Thus, our results using either alternative substrates or Crabtree-negative yeasts establish that repression of aerobic catabolism is the cause of lower life spans observed in high glucose cultures of *S. cerevisiae*.

It could be argued that the dependence on repression of aerobic catabolism for the effectiveness of the calorie restriction model in yeast underplays its importance as a model system to study this diet, since no such phenomenon is observed in animals. However, repression of respiration in *S. cerevisiae* resembles effects found in response to hormone signaling regulated by diet in more complex organisms. For example, calorie restriction in rats leads to enhanced mitochondrial respiratory rates, decreased proton-motive force and prevention of reactive oxygen species production, in a manner reversed by treatment with insulin (Lambert and Merry 2004). Indeed, many animal models including Klotho and dwarf mice (Bartke et al. 2001; Kurosu et al. 2005) suggest a close link between reduced insulin secretion levels, metabolic efficiency, respiratory rates and enhanced life span. In this sense, the *S. cerevisiae* model of caloric restriction holds many parallels with the mammalian effects of this dietary regimen.

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