Dihydrolipoyl dehydrogenase as a source of reactive oxygen species inhibited by caloric restriction and involved in *Saccharomyces cerevisiae* aging

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**Abstract** Replicative life span in *Saccharomyces cerevisiae* is increased by glucose (Glc) limitation [calorie restriction (CR)] and by augmented NAD<sup>+</sup>/H<sub>11545</sub>evisiae is increased by glucose (Glc) limitation [calorie restriction (CR)]. Indeed, deities are an important source of CR-preventable mitochondrial reactive oxygen species (ROS). Results indicate that matrix-soluble dihydrolipoyl-dehydrogenase is decreased oxygen consumption and increased mitochondrial H<sub>2</sub>O<sub>2</sub> release, reversed over time by CR. These null mutant strains also present decreased chronological longevity in a manner rescued by CR. Furthermore, we observed that changes in mitochondrial H<sub>2</sub>O<sub>2</sub> release alter cellular redox state, as attested by measurements of total, oxidized, and reduced glutathione. Surprisingly, our results indicate that matrix-soluble dihydrolipoyl-dehydrogenases are an important source of CR-preventable mitochondrial reactive oxygen species (ROS). Indeed, deletion of the *lpd1Δ*, *npt1Δ*, and *bna6Δ* mutants. Furthermore, pyruvate and α-ketoglutarate, substrates for dihydrolipoyl dehydrogenase-containing enzymes, promoted pronounced reactive oxygen release in permeabilized wild-type mitochondria. Altogether, these results substantiate the concept that mitochondrial ROS can be limited by caloric restriction and play an important role in *S. cerevisiae* senescence. Furthermore, these findings uncover dihydrolipoyl dehydrogenase as an important and novel source of ROS leading to life span limitation.—Tahara, E. B., Barros, M. H., Oliveira, G. A., Netto, L. E. S., Kowaltowski, A. J. Dihydrolipoyl dehydrogenase as a source of reactive oxygen species inhibited by caloric restriction and involved in *Saccharomyces cerevisiae* aging. *FASEB J.* 21, 274–283 (2007)

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McCay et al. (1) originally observed that rodents submitted to low-calorie diets [calorie restriction (CR)] had increased life spans compared to animals fed *ad libitum*. Their results were later reproduced in a wide range of multicellular organisms, including rotifers, arachnids, worms, fish, mice, rats, and primates (for reviews, see refs. 2, 3). Although increases in life span through CR certainly occur due to multiple alterations in metabolic regulation and gene expression, a common finding is that the generation of free radicals and other ROS by mitochondria from CR animals is decreased (4–6). Concomitantly, many groups have found that increases in levels of oxidative stress markers during aging are prevented by CR (7, 8). These findings support the idea that CR prevents mitochondrial ROS formation and the accumulation of oxidative cellular modifications that lead to cell damage during aging.

Mitochondria are the main source of ROS in most cells due to multiple one-electron transfer reactions. Within the electron transport chain, a small quantity of the electrons transported is sidetracked to oxygen at intermediate points such as Complexes I and III, generating superoxide radical anions, which are transformed into mitochondrial H<sub>2</sub>O<sub>2</sub> and other ROS (9–13). In addition to the electron transport chain, recent work has indicated that ROS may also be generated by matrix-soluble enzymes such as pyruvate and α-ketoglutarate dehydrogenases (14, 15). Each mitochondrial ROS source responds differently to substrates, changes in energy metabolism, and O<sub>2</sub> tensions (10). As a result, mitochondrial ROS generation varies widely with metabolic conditions and the effects of CR on redox state are still not fully understood (4, 11).

*Saccharomyces cerevisiae* has been used as a model to study the effects of CR, with the advantage of exhibiting short life spans and allowing simplified metabolic and

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genetic manipulation. Two forms of aging are typically measured in *S. cerevisiae*: replicative and chronological (16–18). Replicative life span measures the number of generations produced by a single mother cell. This measurement is the most common form of life span determination in yeast (19–21). On the other hand, chronological life span measures cell survival during the stationary growth phase, in which budding rates are low (18). The correlation between these forms of yeast life span and aging in multicellular animals has yet to be determined, but it has been suggested that chronological life span may resemble survival in nondividing cells, while replicative life span mimics aging in dividing tissues (18).

Most studies in yeast have focused on genes that regulate replicative life span such as *SIR2*, which causes increased life span when overexpressed and decreased longevity when deleted (see ref. 22 for review). *SIR2* encodes Sir2p, a highly conserved NAD$^+$-dependent histone deacetylase involved in telomeric and rDNA silencing (19, 23), repressing the generation of toxic extrachromosomal ribosomal DNA circles (21). Lin *et al.* (20) and Jiang *et al.* (24) demonstrated that CR, promoted by decreasing the concentration of Glc or amino acids in growth media, extends *S. cerevisiae* replicative longevity, in a Sir2p-sensitive manner. Enhanced respiratory rates promoted by CR (21) result in higher NAD$^+/$/NADH ratios (25), which may activate Sir2p and augment replicative life span. On the other hand, Anderson *et al.* (26) have proposed that CR also up-regulates Pnc1p, an enzyme in the NAD$^+$ salvage pathway, reducing nicotinamide levels and consequently increasing Sir2p activity (22). Independently of the proposed mechanism, it seems clear that NAD metabolism plays a central role in the control of replicative life span by CR. In addition to increasing replicative life span, we found that CR-promoted respiratory increments in yeast enhance chronological longevity in a manner independent of Sir2p (27). Indeed, artificial increments in respiratory rates using mitochondrial uncouplers improve both replicative and chronological life span (27). Furthermore, survival in the stationary phase is decreased when cellular antioxidants such as superoxide dismutase (SOD) are absent (27–29), suggesting links between CR, mitochondrial respiratory rates, redox balance, and chronological longevity similar to those observed in multicellular animals. Unfortunately, further mechanisms regulating chronological longevity remain to be uncovered, since studies involving this form of life span are fewer than those relating to replicative longevity. However, the finding that CR and changes in respiratory rates lead to increments in both replicative and chronological life span indicates that mitochondrial metabolism is a central regulatory point for both forms of aging in yeast (30).

Here, we further investigate the link between respiration and yeast life span, focusing on redox balance. We found that CR enhances O$_2$ consumption and concomitantly prevents mitochondrial ROS formation and glutathione oxidation. Indeed, a strong inverse correlation between respiratory rates and ROS release was observed. We also found that decreased NAD$^+$ synthesis inhibits respiration, enhances mitochondrial ROS release, and decreases chronological life span. Surprisingly, our results suggest that the main CR-sensitive ROS source was not the electron transport chain but matrix dihydrodiployl dehydrogenases. This finding implicates a new mitochondrial ROS source in cellular life span limitation.

**MATERIALS AND METHODS**

**Culture media and yeast strains**

Yeast were cultured with continuous shaking at 220 rpm, 30°C, in liquid YPD (1% yeast extract, 2% peptone, and 2.0% or 0.5% Glc). Cells were inoculated (10$^7$/ml) and grown for 16 or 64 h to reach early and late stationary growth phases, respectively, as confirmed by growth curves (results not shown). Under these conditions, Glc levels in the culture media were undetectable by HPLC analysis after 24 h for both 2.0 and 0.5% Glc cultures. Strains used were wild-type BY4741 and BY4742 and single null mutants of BY4741: sir2Δ, pnc1Δ, npt1Δ, bna6Δ, and lpd1Δ obtained from the EUROFAN collection (http://web.uni-frankfurt.de/ftb15/mikro/euroscarf/index.html). Double mutants bna6Δlpd1Δ and npt1Δlpd1Δ were, respectively, generated by crossing null allele mutants of BNA6 and NPT1 with a *LPD1* mutant of opposite mating type. The resultant diploids were sporulated. After tetrad analysis, the double mutants were selected from true tetrads with 2:2 segregation for genetic resistance. Single and double mutations were confirmed by polymerase chain reaction (PCR) using the following primers located in the promoter region of the respective gene: BNA6F 5’-GGTACAGCTTGGTACAAAC, NPT1F 5’-GCCCTGCAAAGCTTATAAAG, LPD1F 5’-GGCAAGCTTCGATTGCTCTTGTGC, with the reversed primer present in the *kanMX* disruption cassette: kanB 5’-CTGCAAGGGAGCCG-.

**Spheroplast generation**

*S. cerevisiae* spheroplasts were obtained through yeast cell wall digestion (31) for 45 min at 37°C with 20 U zymolyase/g cells in 1.2 M sorbitol, 10 mM MgCl$_2$, and 50 mM Tris, pH 7.5, after 15 min pretreatment with 30 mM dithiotreitol at room temperature. The resultant spheroplasts were washed twice with 1.2 M sorbitol, 10 mM MgCl$_2$, and 50 mM Tris, pH 7.5, at 4°C and resuspended to a final concentration of 5 mg protein/ml in 75 mM phosphate buffer, pH 7.5 (KOH), with 1.2 M sorbitol and 1 mM EDTA. Protein was quantified using the Lowry method.

**Mitochondrial isolation and permeabilization**

Mitochondria from yeast strains grown in 2% Glc YPD were isolated as described elsewhere (27). One-hundred micrograms of the resulting mitochondria were incubated at room temperature in 2 ml reaction media (0.6 M sorbitol, 32.5 mM phosphate, 10 mM Tris, and 1 mM EDTA, pH 7.5, KOH) supplemented with 5 μg alamethicin for permeabilization. Samples were then washed and resuspended in the media described in Fig. 1.
**O₂ consumption assay**

O₂ consumption was monitored over time using a computer-interfaced Clark electrode operating at 30°C with continuous stirring. Spheroplasts were suspended at 800 µg/ml in 75 mM phosphate, 1.2 M sorbitol, and 1 mM EDTA, pH 7.5 (KOH) in the presence of 2% ethanol and 1 mM buffered malate/glutamate. Digitonin (0.004–0.006%) was added as necessary to promote plasma membrane permeabilization, maintaining mitochondrial integrity (31).

**H₂O₂ production assay**

H₂O₂ production was monitored by following resorufin fluorescence (27) in 100 µg/ml spheroplasts suspended in 75 mM phosphate, 1.2 M sorbitol, 1 mM EDTA, 50 µM Amplex Red, 0.5 U/ml horseradish peroxidase (HRP), 2% ethanol, and 1 mM malate/glutamate, pH 7.5 (KOH), using a Hitachi F-4500 fluorescence spectrophotometer operating at 563 nm excitation and 587 nm emission, with continuous stirring, at 30°C. Digitonin (0.002–0.003%) was added as necessary to promote spheroplast permeabilization (31). Permeabilized mitochondria were assayed at 50 µg/ml in media described in Results, supplemented with Amplex Red and HRP.

**Glutathione assays**

Oxidized glutathione (GSSG), reduced glutathione (GSH), and total glutathione were determined in the late stationary phase using a DTNB colorimetric assay, as described by Monteiro et al. (32). Values are expressed as glutathione content per gram cells.

**Resistance to H₂O₂**

Yeast were cultured in YPD containing 2.0 or 0.5% Glc for 16 h. Culture quantities were determined by measuring the absorbance at 600 nm. Cells were then plated on solid minimal media (0.67% yeast nitrogen based media supplemented with amino acids and 2.0% Glc) in the presence or absence of H₂O₂. Spots were compared and photographed after 36–40 h growth at 30°C.

**Chronological life span determinations**

Chronological life span can be defined as the measure of survival in the stationary phase (18). Survival was measured in two distinct manners: metabolic integrity determinations or the ability to metabolize the FUN 1 probe (Molecular Probes, 33), and reproductive integrity or the ability to form colonies when plated in solid media.

FUN 1 determinations were performed as described by Barros et al. (27). This probe marks metabolically active vs. inactive cells, which fluoresce at 580 and 535 nm, respectively, when excited at 470 nm; 2×10⁶ cells were added to 1.5 ml of reaction buffer consisting of 5 µM FUN 1, 2.0% Glc, and 10 mM HEPES, pH 7.5 (NaOH). Fluorescence differences were monitored until stable using a Hitachi F-4500 fluorescence spectrophotometer. FUN 1 metabolism occurs both in aerobic and anaerobic cells (33) and has been previously shown to correlate with colony-forming ability (34). It should be noted that FUN 1 fluorescence changes allow for qualitative, not quantitative, metabolic activity determinations.

Reproductive survival was quantitatively measured by plating 100 stationary phase cells (as determined by absorbance at 600 nm after being washed in distilled water) in individual solid YPD plates. Colonies were counted in each plate after 36 h growth at 30°C.

**RESULTS**

**CR decreases ROS release from mitochondria**

Lin et al. (21) demonstrated that increments in cellular oxygen consumption are necessary for replicative life span extension promoted by CR in yeast. In support for this finding, we observed that artificially enhancing mitochondrial respiration improves both replicative and chronological longevity (27). To study the respiratory effects of CR and directly relate them to possible changes in mitochondrial ROS release, we measured O₂ consumption and H₂O₂ generation in mitochondria within permeabilized S. cerevisiae spheroplasts, both in early (16 h) and late (64 h) stationary growth phases. In the early stationary phase (Fig. 1A), mitochondria within cells grown in 0.5% Glc (CR, empty bars) exhibit significantly higher respiratory rates when compared to 2.0% Glc (control, full bars), a result that confirms measurements conducted in intact cells (21) and isolated mitochondria (27). High Glc levels are well known to inhibit respiration in S. cerevisiae through Glc repression (35), which may account for the changes in respiratory rates observed in the early stationary growth phase. Interestingly, although Glc levels were undetectable after 24 h under both culture conditions (results not shown), O₂ consumption by mitochondria grown under control conditions decreased significantly when cells reached the late stationary phase, while CR cells maintained high O₂ consumption over time.

Parallel H₂O₂ release measurements indicated that cells grown in 2.0% Glc maintained similar H₂O₂ release rates over 3 days growth, whereas lower levels of H₂O₂ (Fig. 1B) and H₂O₂/O₂ (Fig. 1C) were detected in CR cells after a similar interval. These results indicate that the cumulative release of ROS from CR mitochondria over 64 h in culture is lower than that of control cells.

**Defective NAD⁺ synthesis or salvage results in CR-sensitive decrease in O₂ consumption**

CR increases NAD⁺/NADH ratios, a determinant effect in yeast replicative longevity linked to changes in O₂ consumption (20, 21, 25). To verify the importance of NAD⁺/NADH in mitochondrial respiratory and redox metabolism, we tested strains with altered NAD⁺ synthesis. PNC1 encodes a nicotinamidase for the NAD⁺ salvage pathway that, when absent, decreases intracellular...
that span extension mediated by CR (20, 26, 36). We found intracellular NAD levels in nicotinate. Deletions of this gene also promote reduced early stationary phase, as described in Materials and Methods. * were measured in parallel, in early and late stationary growth (2.0% Glc, full bars) or CR (0.5% Glc, empty bars) conditions.

Figure 1. CR enhances O2 consumption (A) and prevents H2O2 release (B, C). O2 consumption and H2O2 release by spheroplasts prepared from WT cells grown under control (2.0% Glc, full bars) or CR (0.5% Glc, empty bars) conditions were measured in parallel, in early and late stationary growth phases, as described in Materials and Methods. *P < 0.05 vs. early stationary phase; #P < 0.05 vs. 2.0% Glc.

lular NAD+ and, consequently, replicative life span (26). NPT1 encodes nicotinate phosphoribosyl transferase, necessary for de novo NAD+ synthesis from nicotinate. Deletions of this gene also promote reduced intracellular NAD levels in S. cerevisiae and prevent life span extension mediated by CR (20, 26, 36). We found that npt1Δ and pnc1Δ strains in the early stationary phase presented diminished respiratory rates relative to WT strains grown in 2.0% Glc (Fig. 2A, full bars). Confirming that this decreased respiration is related to the lack of NAD+ synthesis, similar results were observed in cells devoid of Bna6p, an enzyme responsible for NAD+ synthesis from tryptophan-derived quinolinic acid (36). On the other hand, sir2 mutants behaved similarly to WT strains, indicating that the respiratory effect is related to defects in NAD+ synthesis and salvage but not use.

In the late stationary phase (Fig. 2B), respiratory rates of pnc1Δ, npt1Δ, and bna6Δ cells were more similar to WT strains than in the early phase, although still significantly lower in npt1Δ and bna6Δ strains. Interestingly, oxygen consumption by pnc1Δ, npt1Δ, and bna6Δ cells was considerably increased by CR (empty bars), resulting in complete respiratory rate recovery in the late stationary phase. Presumably, the alternative NAD+-generating pathway is up-regulated over time, in a manner stimulated by CR.

ROS release and O2 consumption are inversely correlated

In all cells and growth conditions studied, lower O2 consumption promoted by lack of NAD or high Glc growth conditions was accompanied by higher H2O2 release (Fig. 2G–F). Indeed, a strong inverse correlation between respiratory rates and H2O2 release was observed in early (Fig. 2G, r2=0.75, P=0.02) and late stationary phase cells (Fig. 2H, r2=0.79, P<0.001). In the early stationary phase (Fig. 2E, G), WT and sir2Δ cells grown in 0.5% Glc had the lowest H2O2/O2 relationships. WT and sir2Δ cells grown in 2.0% Glc and pnc1Δ, npt1Δ, and bna6Δ cells grown in 0.5% Glc formed an intermediate group. Finally, pnc1Δ, npt1Δ, and bna6Δ cells grown in 2.0% Glc presented the highest H2O2/O2 relationships. In the late stationary phase (Fig. 2F, H), a clear separation between CR and control growth conditions was observed, demonstrating that CR cells present very significant increments in respiration concomitantly to decreased ROS formation. As a result, cumulative ROS formation is lowest in CR cells with no lack of NAD, intermediate in control cells with unaffected NAD+ synthesis or CR cells with defective NAD+ synthesis/salvage, and highest in cells with defective NAD+ synthesis/salvage incubated in 2.0% Glc.

CR prevents cellular oxidative stress

Further evidence supporting the concept that CR and respiratory increments prevent oxidative stress was provided by glutathione measurements. We found that CR decreased oxidized glutathione (GSSG) levels (Fig. 3A) and improved GSSG/GSH ratios (Fig. 3C) in all strains. npt1Δ and bna6Δ cells presented significantly higher amounts of GSSG and total glutathione levels (Fig. 3B), a result typical of chronic oxidative stress, which stimulates glutathione synthesis (37). Thus, increased mitochondrial H2O2 release levels measured in Fig. 2 correlate closely with changes in intracellular redox potential.

The ability to grow in the presence of exogenously added H2O2, a reflection of levels of major peroxide-removing systems, was also tested for the different cell
types and growth conditions (Fig. 4). CR did not significantly alter the ability to grow in media supplemented with H$_2$O$_2$, a result supported by the finding that CR does not alter the expression of antioxidants in WT cells (21). The npt1/H9004 cells, but not the other mutants tested, presented a marked decrease in resistance to 0.6 mM exogenous H$_2$O$_2$. Even at higher H$_2$O$_2$ concentrations (0.9 mM), all other cell types and growth conditions presented similar sensitivity, indicating that other mutations and CR do not change cellular resistance to H$_2$O$_2$. 

**CR improves chronological longevity**

To verify if the changes in ROS release observed over time in the mutants tested affect life span, we measured chronological life span in WT (Fig. 5) vs. npt1/H9004 cells grown in 2.0 vs. 0.5% Glc. npt1/H9004 cells were chosen due to their known limitation in replicative life span (20), high H$_2$O$_2$ release rates, high exogenous H$_2$O$_2$ sensitivity, increased GSSG, and efficient response to CR. In Fig. 5, metabolic integrity in the late stationary phase was qualitatively measured using the FUN 1 probe (27), which is metabolized over time in live cells to a product fluorescent at 580 nm (33). We found that npt1 deletion limited FUN 1 metabolism in a manner partially reversed by CR. This indicates that oxidative stress in npt1/H9004 strains and the beneficial effects of CR on ROS release observed previously (Fig. 2) are reflected as decreased and improved metabolic integrity, respectively. In addition to measurements using FUN 1, we also determined the ability to resume cellular division once cells are removed from stationary phase growth conditions (Fig. 5B), a quantitative measurement of replicative integrity. The number of colonies generated by npt1/H9004 strains was lower than WT, in a manner
rescued by CR. Similar results, both using FUN 1 and colony counts, were observed with \textit{bna6}/H9004 cells (results not shown). Thus, we found that decreased chronological life span correlates with lower respiratory rates and higher H\textsubscript{2}O\textsubscript{2} release in these strains.

Matrix-soluble dehydrogenases are an important source of CR-sensitive ROS release

Our data demonstrate that mitochondrial respiration and NAD\textsuperscript{+} levels are critical for chronological longevity, in addition to their already known effects on replicative longevity (25). Our data also show that respiration and NAD\textsuperscript{+} content strongly affect redox balance. However, the increased mitochondrial H\textsubscript{2}O\textsubscript{2} production exhibited by \textit{bna6\Delta}, \textit{pnc1\Delta}, and \textit{npt1\Delta} strains is probably not a result of enhanced electron leakage from the mitochondrial electron transport chain, since levels and turnover of NADH (which provides these electrons) are lower. This observation suggests that electron leakage occurring upstream of the respiratory chain contributes toward CR-sensitive mitochondrial ROS production.

Recent work using mammalian tissue (14, 15) demonstrated that matrix-soluble dihydrolipoyl-containing dehydrogenases (pyruvate and, mainly, \alpha-ketoglutarate dehydrogenase) can also generate ROS, in a manner stimulated by low NAD\textsuperscript{+} availability. To investigate if these dehydrogenases were the source of ROS under our conditions, we measured O\textsubscript{2} consumption and H\textsubscript{2}O\textsubscript{2} release in a strain harboring a null allele of \textit{lpd1\Delta}, which does not display dihydrolipoyl dehydrogenase activity (38). As expected, \textit{lpd1\Delta} mitochondria present low O\textsubscript{2} consumption rates (9.82±0.54 O\textsubscript{2}/mg protein\textsuperscript{-1}·min\textsuperscript{-1}, in the early stationary growth phase), comparable to \textit{npt1\Delta} and \textit{bna6\Delta} mutants (see Fig. 2A). However, low respiratory rates in these mutants are not accompanied by increased H\textsubscript{2}O\textsubscript{2} release relative to WT strains (Fig. 6). Furthermore, we generated \textit{lpd1\Delta npt1\Delta} and \textit{lpd1\Delta bna6\Delta} double mutants and verified that the \textit{lpd1\Delta} null allele is epistatic over \textit{bna6\Delta} and \textit{npt1\Delta} null alleles, reverting increments in H\textsubscript{2}O\textsubscript{2} release observed in the single deletions (Fig. 6). This indicates that ROS release enhanced by lack of NAD synthesis occurs primarily at the level of dihydrolipoyl-containing dehydrogenases.

To investigate if dihydrolipoyl dehydrogenases were also important ROS sources in WT cells, we compared ROS release rates in WT mitochondria using different substrates. In intact mitochondria, the limited matrix space allows products of enzymatic reactions to accumulate and act as substrates for other enzymes, so individual contributions of each reaction toward ROS release from concentrations of substrates. Figure 4. \textit{npt1\Delta} deletion results in reduced resistance to exogenous H\textsubscript{2}O\textsubscript{2}. WT and mutant strains were grown in 2.0 or 0.5% Glc-containing liquid media. After 16 h, 2·10\textsuperscript{6}, 4·10\textsuperscript{7}, and 8·10\textsuperscript{8} cells were plated sequentially (from left to right) on solid minimum media in the presence or absence of 0.6 or 0.9 mM H\textsubscript{2}O\textsubscript{2}, as shown. Plate growth was photographed after 36–40 h growth at 30°C.
generation cannot be determined. To circumvent this situation, we measured ROS release in mitochondria in which membranes were permeabilized by the pore-forming compound alamethicin, which allows for free substrate passage, but does not release mitochondrial matrix enzymes (14). The use of different substrates under these conditions allows for the comparison of ROS release rates by individual mitochondrial sources, since the products of enzymatic reactions are largely diluted.

We found (Fig. 7) that the addition of α-ketoglutarate and pyruvate (substrates for dihydrolipoyl dehydrogenase-containing enzymes) but not malate to WT permeabilized mitochondria resulted in substantial ROS formation. In lpd1Δ cells, no H2O2 release was measured after the addition of these substrates, indicating that the release was, indeed, dependent on the activity of Lpd1p. Succinate was added to compare ROS formation by these matrix-soluble dehydrogenases with respiratory chain ROS release, since NADH cannot be added due to interference with all horseradish peroxidase-based measurements. In mammals, succinate leads to large quantities of ROS formation in most tissues, since it can feed electrons to coenzyme Q in Complex III and (by reverse electron transport) to Complex I, where superoxide formation occurs (10). Surprisingly, under our conditions electron leakage promoted by succinate at the respiratory chain was substantial but still slightly lower than that observed with α-ketoglutarate. These results confirm that although the electron transport chain generates ROS, ROS generated by dihydrolipoyl dehydrogenase-containing enzymes α-ketoglutarate and pyruvate dehydrogenase are the main source of these species in WT cells.

**DISCUSSION**

Aging studies in *S. cerevisiae* have uncovered a complex control system for replicative life span involving suppression by Sir2p family proteins of toxic ribosomal DNA circle accumulation in dividing cells (for review, see 22). CR alters replicative life span by regulating Sir2p activity in a manner dependent on fluctuations in NAD+/NADH levels promoted by changes in respiratory rates (20, 21, 25).

However, Sir2p family proteins are not the only determinants of *S. cerevisiae* life span. Some groups (23, 39) have found that CR increases replicative life span even in sir2Δ cells. Furthermore, although *S. cerevisiae* CR enhances both replicative and chronological longevity, sir2Δ mutations do not decrease chronological life span (27, 40). Interestingly, chronological life span, but apparently not replicative, is limited by mitochondrial oxidative stress (27–29, 34).

In animals, there is ample evidence that ROS participate in aging processes, including enhanced levels of oxidative markers with age and in short-lived animals,
inverse correlations between levels of mitochondrial ROS release and life span and involvement of oxidative stress in many age-associated diseases (for review, see refs. 4, 12, 13, 41–43). Furthermore, CR in animals prevents mitochondrial ROS release and oxidative stress markers accumulated with aging (for reviews, see ref. 4, 12, 13). Considering the complexity of the aging process, it is not surprising that it is regulated by multiple factors both in simpler model organisms such as yeast and in multicellular animals. Other factors proposed to mediate aging are metabolic rates, telomere loss, loss of DNA repair and genome stability, and aggregated protein accumulation (for review, see ref. 44). Most likely, all these factors play a role in aging, acting in concert. The genetic, metabolic, and oxidative processes involved in S. cerevisiae aging support the use of this model, since it bears a closer resemblance to multifactor aging processes in animals.

We have previously demonstrated using S. cerevisiae that the link between the beneficial effects of CR in chronological and replicative aging is the increase in respiratory rates that results from Glc limitation. Indeed, artificially increasing respiration by using a proton ionophore enhances both replicative and chronological life span (27). Here, we investigate the effects of respiratory rates on mitochondrial and cellular redox state and uncover the mechanisms through which ROS metabolism is altered under conditions that change yeast longevity.

We found that respiratory rates of a variety of yeast strains grown in distinct Glc concentrations are inversely correlated with the release of mitochondrial H$_2$O$_2$, a relatively stable and membrane-permeable ROS (Fig. 2). Supporting the idea that increased mitochondrial H$_2$O$_2$ release is reflective of cellular oxidative imbalance in vivo, GSSG and total glutathione contents increase in cell types and growth conditions in which mitochondrial ROS release is highest (Fig. 3). These findings are in line with measurements of protein carbonylation in S. cerevisiae indicating that this form of oxidative damage is prevented by CR (45). In addition, we found that cellular oxidative stress promoted by lack of CR and/or defects in NAD$^+$ synthesis resulted in limited chronological longevity (Fig. 5). This result further supports the idea that yeast chronological longevity is limited by mitochondrially generated ROS.

The strong correlation between O$_2$ consumption and H$_2$O$_2$ release measurements (Fig. 2) suggests they are related in a cause/effect manner. Indeed, there is ample evidence in the literature that ROS generation in mitochondria from animals and plants is prevented by increasing O$_2$ consumption (for reviews, see refs. 10, 46–48). Previously, two main reasons for reduced mitochondrial ROS generation promoted by enhanced electron transport have been presented: 1) enhanced O$_2$ consumption creates a lower oxygen tension microenvironment, preventing the donation of electrons from complexes I and/or III to oxygen that leads to superoxide radical formation; or 2) enhanced electron transport results in lower life times of the reduced forms of respiratory complexes that can generate superoxide anions (48, 49). However, neither of these explanations seems plausible in the case of enhanced ROS release observed in npt1Δ, bna6Δ and pnc1Δ cells, since the levels of total and reduced NAD are lower, feeding a smaller quantity of electrons into the respiratory chain (see Fig. 8). As a result, we focused our attention on sites upstream of NAD$^+$ reduction which could generate ROS.
Recent studies by the groups of Beal and Adam-Vizi (14, 15) suggest that dihydrolipoyl dehydrogenase-containing enzymes, in particular α-ketoglutarate dehydrogenase, are major sources of mitochondrial ROS in mammals. Indeed, these groups found that superoxide radical generation by these enzymes is augmented by the lack of NAD\textsuperscript{+} or by high NADH/NAD\textsuperscript{+} ratios. The product of the mammalian Dld gene, which encodes the E3 subunit of α-ketoglutarate dehydrogenase, was identified as the probable source of ROS generated by this enzyme using heterozygous knockout mice. This concept is consistent with the finding that flavoenzymes are ROS sources (50). Within these enzymes, the absence of NAD\textsuperscript{+} keeps lipoamide dehydrogenase in the reduced state because the cellular environment is reductive. Consequently, there is an increased probability of lipoamide dehydrogenase reactions with oxygen, generating ROS.

We found support for the idea that dihydrolipoyl dehydrogenase generates ROS by testing strains that do not express Lpd1p, the E3 subunit of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase in yeast (38). LPD1 deletion completely reversed the increased ROS release levels found in npt1\textDelta and bna6\textDelta cells (Fig. 6). Furthermore, in experiments comparing ROS release rates induced by different substrates in alamethicin-permeabilized mitochondria (Fig. 7), we found substantial ROS formation promoted by pyruvate and very pronounced ROS formation induced by α-ketoglutarate. These results unequivocally indicate that dihydrolipoyl dehydrogenase-containing enzymes pyruvate and α-ketoglutarate dehydrogenase are very important mitochondrial ROS sources. Interestingly, ROS formation by these enzymes is strongly controlled by NADH/NAD\textsuperscript{+} levels (18, 19) and will thus decrease when higher respiratory rates are present, such as under CR growth conditions (25).

It is important to stress that our study does not rule out the existence of other mitochondrial ROS sources such as NADH dehydrogenases and respiratory complex III (see Fig. 8). Indeed, succinate is capable of generating significant amounts of ROS in permeabilized mitochondria. However, ROS release levels in the presence of α-ketoglutarate and pyruvate are, together, larger than those induced by succinate (Fig. 7). This finding, and evidence that ROS release by this enzyme in npt1\textDelta and bna6\textDelta cells can limit life span, highlight the importance of dihydrolipoyl dehydrogenase within redox metabolism and emphasize the necessity of additional research concerning the causes and effects of mitochondrial ROS generation.

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