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Higher Respiratory Activity Decreases Mitochondrial Reactive Oxygen Release and Increases Life Span in *Saccharomyces cerevisiae**

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Increased replicative longevity in *Saccharomyces cerevisiae* because of calorie restriction has been linked to enhanced mitochondrial respiratory activity. Here we have further investigated how mitochondrial respiration affects yeast life span. We found that calorie restriction by growth in low glucose increased respiration but decreased mitochondrial reactive oxygen species production relative to oxygen consumption. Calorie restriction also enhanced chronological life span. The beneficial effects of calorie restriction on mitochondrial respiration, reactive oxygen species release, and replicative and chronological life span could be mimicked by uncoupling agents such as dinitrophenol. Conversely, chronological life span decreased in cells treated with antimycin (which strongly increases mitochondrial reactive oxygen species generation) or in yeast mutants null for mitochondrial superoxide dismutase (which removes superoxide radicals) and for *RTG2* (which participates in retrograde feedback signaling between mitochondria and the nucleus). These results suggest that yeast aging is linked to changes in mitochondrial metabolism and oxidative stress and that mild mitochondrial uncoupling can increase both chronological and replicative life span.

The only intervention known to increase average and maximum life span in mammals is caloric restriction (CR),[1] a reduction of 25–60% in calorie intake without essential nutrient deficiency. This diet not only extends life span but also delays many unwanted effects of aging and age-related pathologies. CR is highly effective in a wide range of organisms, increasing life span by up to 50% in some species (reviewed in Refs. 1–3). Unfortunately, the mechanisms through which it results in increased life span are still controversial (see Ref. 4 for a critical review).

A leading hypothesis on the mechanism through which CR prevents aging is that this process decreases reactive oxygen species (ROS) generation and, hence, the oxidation of cellular components (5–8). Indeed, aging is usually accompanied by oxidative damage of DNA, proteins, and lipids (9, 10). CR promotes a metabolic shift resulting in more efficient electron transport in the mitochondrial respiratory chain (1, 5). Faster and more efficient electron transport may lead to lower production of ROS by mitochondria, one of the major intracellular ROS sources. This occurs because of reduced leakage of electrons from the respiratory chain and/or lower oxygen concentrations in the mitochondrial microenvironment (11, 12). Indeed, artificially increasing mitochondrial respiration using uncouplers such as 2,4-dinitrophenol (DNP) strongly prevents mitochondrial ROS release (11). Furthermore, CR decreases ROS release/O_2 consumed in isolated mammalian mitochondria (13), possibly because of enhanced expression of mitochondrial uncoupling proteins (14, 15). Despite this evidence supporting a correlation between ROS-induced damage and aging, a clear cause-effect relationship has been hard to establish, and conflicting results are often presented in the literature (see Ref. 4 for a critical review).

Saccharomyces cerevisiae has been used as a model system to study mechanisms of life span modulation. Two types of life span may be measured in *S. cerevisiae*: chronological and replicative (10, 16–18). Chronological life span is measured in the stationary growth phase, in which reproduction rates are low. Under these conditions, cells gradually senesce in a manner that may be related to ROS removal capacity (19, 20). However, factors influencing chronological longevity (or aging in nondividing cells) are expected to be different from those influencing replicative life span, which is defined by the number of generations a yeast cell produces when in logarithmic growth phase (16). Possible shared pathways and differences in these forms of aging have not been thoroughly explored to date, and it is unclear which form of life span relates best to longevity in multicellular organisms.

Replicative life span has been more extensively studied in yeast, and a hypothesis relating CR and changes in life span to altered gene expression has been developed using this model. Guarente and co-workers (21) have shown that replicative life span extension in *S. cerevisiae* can be achieved by decreasing the culture media substrate content, a condition mimicking CR. Yeast replicative life span extension promoted by CR depends on the activity of the *SIR2* gene. *SIR2* codes for a histone deacetylase and prevents the formation of extrachromosomal rDNA circles (ERCs), which accumulate during replicative aging (16, 22). Because Sir2p activity depends on nicotinamide adenine dinucleotide as a substrate, the effect of CR in yeast

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[1] The abbreviations used are: CR, caloric restriction; DNP, 2,4-dinitrophenol; ERC, extrachromosomal rDNA circle; ROS, reactive oxygen species; SOD, superoxide dismutase; YPD, yeast extract/peptone/dextrose medium.

may be related to an increase in the NAD^+/NADH ratios in restricted cells due to higher respiratory rates (23, 24). Lower glucose levels increase respiration, shifting the preferred fermentation pathway toward oxidative phosphorylation (reviewed in Ref. 4).

Guarente and co-workers (23) found that CR in yeast did not enhance the resistance of these cells to exogenous oxidants, such as paraquat or H_2O_2, or alter the expression of antioxidant enzymes, a finding presented as an indication for the lack of a ROS effect in replicative aging. However, oxidative stress is the result of an imbalance between ROS removal and ROS formation, which was not measured under their conditions. Furthermore, these authors detected increased respiratory rates in CR yeast (23), which may alter mitochondrial ROS release rates, as discussed above. It is thus important to reconsider a possible participation of changes in mitochondrial ROS release levels in the replicative life span effects of CR.

Other aspects that warrant investigation are the comparison of replicative and chronological aging and the effects of factors known to influence replicative life span on chronological life span. CR and *SIR2* have been extensively shown to enhance replicative life span by decreasing ERCs, but their effects on chronological life span have not, to our knowledge, been determined to date. Retrograde feedback between nucleus and mitochondria also plays a role in replicative life span by decreasing ERCs, as indicated by the fact that deletion of *RTG2*, a gene that plays a central role in relaying retrograde response signals, decreases replicative life span (25). However, the effect of *RTG2* on chronological life span is also unknown.

To analyze further the role of mitochondrial activity in yeast longevity, we measured the effects of CR on mitochondrial respiration and ROS release. We also tested the effects of well established regulators of mitochondrial ROS release and genes involved in the regulation of replicative aging on chronological life span, using a recently developed fluorescence technique. Finally, we uncovered links between respiration, ROS release, and aging in yeast by demonstrating that CR and mitochondrial uncoupling can affect both chronological and replicative life span.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—*S. cerevisiae* W303–1A cells (R. Rothstein, Columbia University, New York, NY) were used in most experiments. EUROFAN BTY4741 wild type strain and strains harboring null mutations of *SIR2*, *SOD2*, and *RTG2*, named here ΔSIR2, ΔSOD2, and ΔRTG2, respectively, were used in Fig. 2, *C* and *D*. Cells were cultured at 30 °C with continuous shaking in standard YPD medium (26) containing 0.5 or 2% glucose.

Mitochondrial Isolation—Mitochondria were prepared from yeast strain W303–1A cultures grown in YPD to early stationary phase by the method of Faye *et al.* (27), except for the use of zymolyase 20,000 units/g (ICN) instead of glusulase to convert cells to spheroplasts. Mitochondria isolated in this manner present intact inner membranes and respiratory complexes (28).

Oxygen Consumption—O_2 consumption was followed at 30 °C in isolated mitochondrial suspensions incubated in 0.6 M sorbitol, 20 mM Tris-HCl (pH 7.5), and 0.5 mM EDTA in the presence of 2% ethanol, 0.5 mM malate, and 0.5 mM glutamate, using a computer-interfaced Clark electrode operating in an air-tight chamber with continuous stirring.

Hydrogen Peroxide (H_2O_2) Release—H_2O_2 production was measured as described elsewhere (28) by measuring the oxidation of 50 μM Amplex™ Red (Molecular Probes®) in the presence of 1.0 units/ml horseradish peroxidase (Sigma). The incubation media contained 0.6 M sorbitol, 20 mM Tris-HCl (pH 7.5), and 0.5 mM EDTA, using 2% ethanol, 0.5 mM malate, and 0.5 mM glutamate as substrates. The rate of Amplex™ oxidation was recorded at 30 °C using a Hitachi F-4500 fluorescence spectrophotometer equipped with continuous stirring, operating at excitation and emission wavelengths of 563 and 587 nm, respectively.

Yeast Chronological Life Span—Yeast were cultured with continuous shaking for 4 days at 30 °C. Viability was assessed in the stationary phase using the fluorescent FUN® 1 (Molecular Probes) probe. This method provides faster and more reliable results than colony counts (29). Culture quantities were determined by measuring the absorbance at 600 nm. ~2×10^8 cells were added to 1 ml of reaction buffer consisting of 5 μM FUN® 1, 2% glucose, and 10 mM HEPES, pH 7.5. FUN® 1 determines yeast metabolic activity through fluorimetric analysis. Only metabolically active cells can convert the bright green fluorescent probe into an intravacuolar orange-red compound in a manner independent of fermentation or respiratory metabolism (29). The fluorescent conversion was detected using a Hitachi F-4500 fluorescence spectrophotometer with 470 nm excitation and 535 and 580 nm emission wavelengths. Data are expressed as the difference in 580 and 535 nm emissions over time, in arbitrary fluorescence units.

Yeast Replicative Life Span—Replicative life span measures the number of generations a yeast cell is capable of generating by budding (30) and was determined as described previously (31). Briefly, 1 μl of cells grown logarithmically overnight in liquid YPD or YPD supplemented with 10 nM DNP was plated on YPD and YPD + 10 nM DNP plates. A group of unbudded cells was separated from the rest by micromanipulation (TDM400™ micromanipulator and Nikon Eclipse E400 microscope) and allowed to produce buds. Fifty of these buds were removed and used as the starting mother cell population. The number of daughter cells (generations) for each mother cell was counted by following cell division and separating daughter cells. Cells were grown at 30 °C during the day and at 8 °C overnight. Each experiment involved ~50 mother cells and was carried out three times independently. There was no significant variability among the independent repetitions. Statistical significance of life span differences was determined using a Mann-Whitney Rank sum test.

RESULTS

ROS Release and O_2 Consumption in CR and Control S. cerevisiae Mitochondria—Because CR increases mitochondrial respiratory rates (23), we examined the possibility that CR alters ROS production in isolated yeast mitochondria. To do so, we measured the release of H_2O_2, a membrane-permeable ROS, in suspensions of mitochondria isolated from *S. cerevisiae* grown in YPD containing 2 or 0.5% glucose, a condition previously shown to extend replicative life span (21). Interestingly, although oxygen consumption rates tended to be larger in CR mitochondria (Fig. 1*A*), the release of H_2O_2 was not directly proportional to the oxygen consumption rates measured (*panel B*). In fact, H_2O_2 release/O_2 consumption ratios in yeasts grown in 2% glucose were significantly higher than those of CR mitochondria (*panel C*), indicating that CR alters the quantity of H_2O_2 generated per O_2 consumed. As a result, despite the fact that yeasts grown in 0.5% glucose display O_2 consumption rates larger than those observed in 2% glucose (23), their total mitochondrial ROS release may be lower. Indeed, the uncoupler carbonyl cyanide 3-chlorophenylhydrazone, which artificially enhances respiration, decreased H_2O_2 production in *S. cerevisiae* mitochondria by 27% (*panel D*), as observed previously in animal tissues (11, 12). DNP (5 μM), a structurally unrelated uncoupler, also lowered H_2O_2 release by 25–30% (results not shown), whereas antimycin A, a respiratory inhibitor, strongly enhanced H_2O_2 release (*panel D*).

Respiration and ROS in Yeast Chronological Life Span—Yeast CR has been shown to increase replicative life span (21), but its effects on chronological life span have not been determined to date. To measure chronological life span, we grew cells in stationary phase and marked them with the fluorescent FUN® 1 probe, which is gradually metabolized in aerobic or anaerobic live cells, leading to a fluorescence peak at 580 nm when excited at 470 nm. Metabolically inactive cells do not process the probe and fluoresce at 535 nm. Thus, the difference in 580 and 535 nm fluorescence is proportional to the live/dead cell contents (29).

We observed that cells cultured under CR conditions (0.5% glucose) in stationary phase present a larger proportion of live cells than yeast grown in 2% glucose (Fig. 2*A*), indicating that CR also increases chronological life span. To verify the effects of respiration and ROS release on chronological life span, we used DNP as a mild uncoupler (to avoid cell death due to excessive

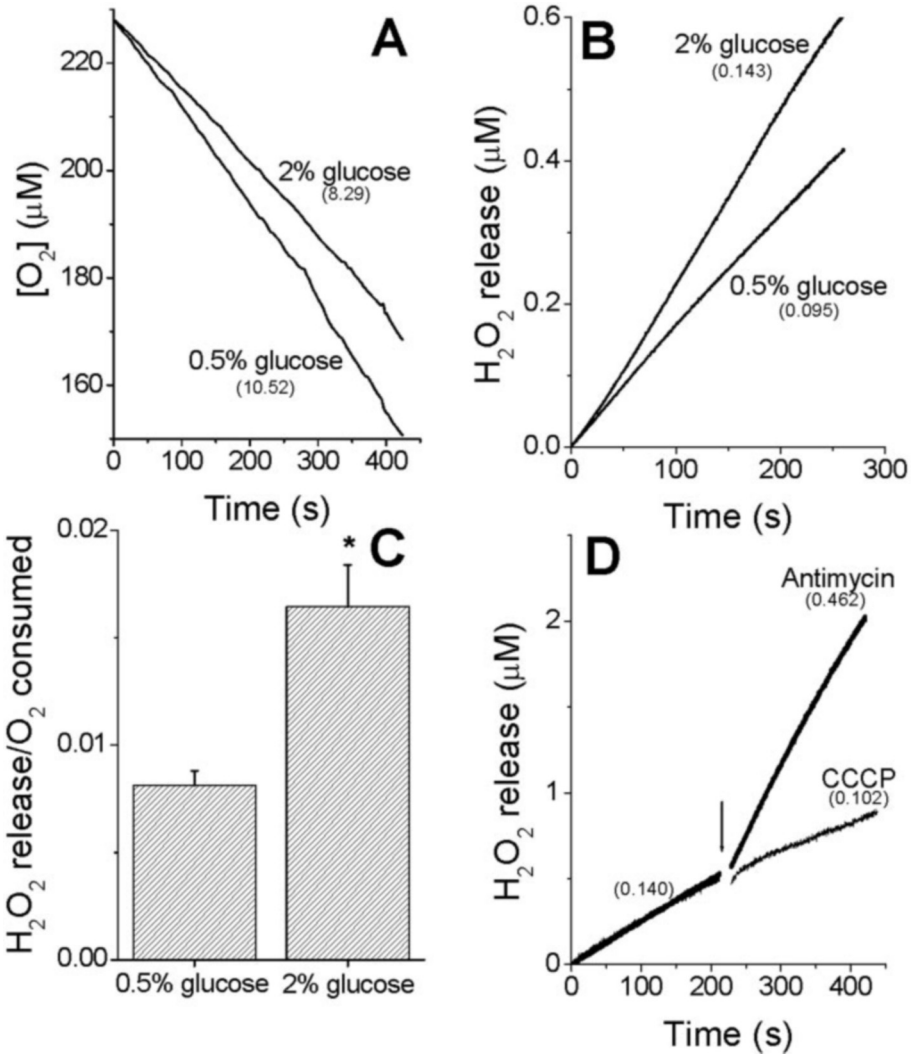

FIG. 1. **CR and uncoupling decrease mitochondrial H_2O_2 release/O_2 consumed.** Mitochondria were isolated from W303–1A *S. cerevisiae* grown in the presence of 2 or 0.5% glucose as described under "Experimental Procedures." Respiratory rates (*A*) and H_2O_2 release rates (*B*) were measured in parallel. The average ± S.E. H_2O_2 detected/O_2 consumed of three separate experiments, such as those in *panels A* and *B*, is depicted in *panel C* (*, $p < 0.01$ relative to 0.5% glucose, pairwise Tukey test). *D*, H_2O_2 release from mitochondria isolated from cells grown in 2% glucose was measured, and 0.5 μM carbonyl cyanide 3-chlorophenylhydrazone or 0.5 μg/ml antimycin A was added where indicated. Numbers in parentheses indicate O_2 consumption and H_2O_2 release rates in μM/min. *A*, *B*, and *D*, representative experiments of at least three similar repetitions.

H^+ transport) and antimycin A to block respiration (Fig. 2*B*). We found that low doses of DNP (1–10 nM) significantly increase 2% glucose live cell contents, a result indicative of enhanced survival. This effect was not observed in cells grown in 0.5% glucose (results not shown). Higher DNP doses (100 nM, not shown, to 1 mM, Fig. 2*B*) did not affect or slightly decreased stationary phase viability relative to control cells, probably because of perturbed energy metabolism. On the other hand, the respiratory inhibitor antimycin A consistently and strongly increased dead cell contents at every concentration tested (Fig. 2*B* and results not shown). These results are in agreement with the hypothesis that ROS affect yeast viability during the stationary phase (20).

Confirming the idea that mitochondrial ROS determine chronological life span, the null mutant of mitochondrial superoxide dismutase (ΔSOD2), which is incapable of dismutating intramitochondrial superoxide radicals to H_2O_2, showed decreased chronological life span relative to its wild type strain BTY4741 (Fig. 2*C*). A *rtg2* mutant, which has been previously shown to present decreased replicative life span (25) due to defective retrograde (mitochondria-nuclear) signaling, also presented decreased chronological life span (Fig. 2*C*). This result indicates more parallels between chronological and replicative life span in yeast.

However, aspects affecting chronological and replicative life span were not identical. Although the BTY4741 strain also presented increased chronological life span in response to CR, deletion of *SIR2*, which is essential for the beneficial effects of CR in replicative life span (22, 23), did not strongly decrease the effects of CR on chronological life span (Fig. 2*D*).

Mild Uncoupling and Replicative Life Span—Because we found that mild uncoupling reproduces the effects of CR on mitochondrial respiration, H_2O_2 release, and chronological life span, we tested its effect on replicative life span. In three independent experiments involving 50 yeast mother cells each, we found that 10 nM DNP led to a small but reproducible and statistically significant increase of ~15% in replicative life span (see Fig. 3 for a representative experiment). Thus, mild uncoupling mimics CR and increases both chronological and replicative life span.

DISCUSSION

The role of mitochondrial metabolism, respiration, and ROS in life span and the beneficial effects of CR have been the focus

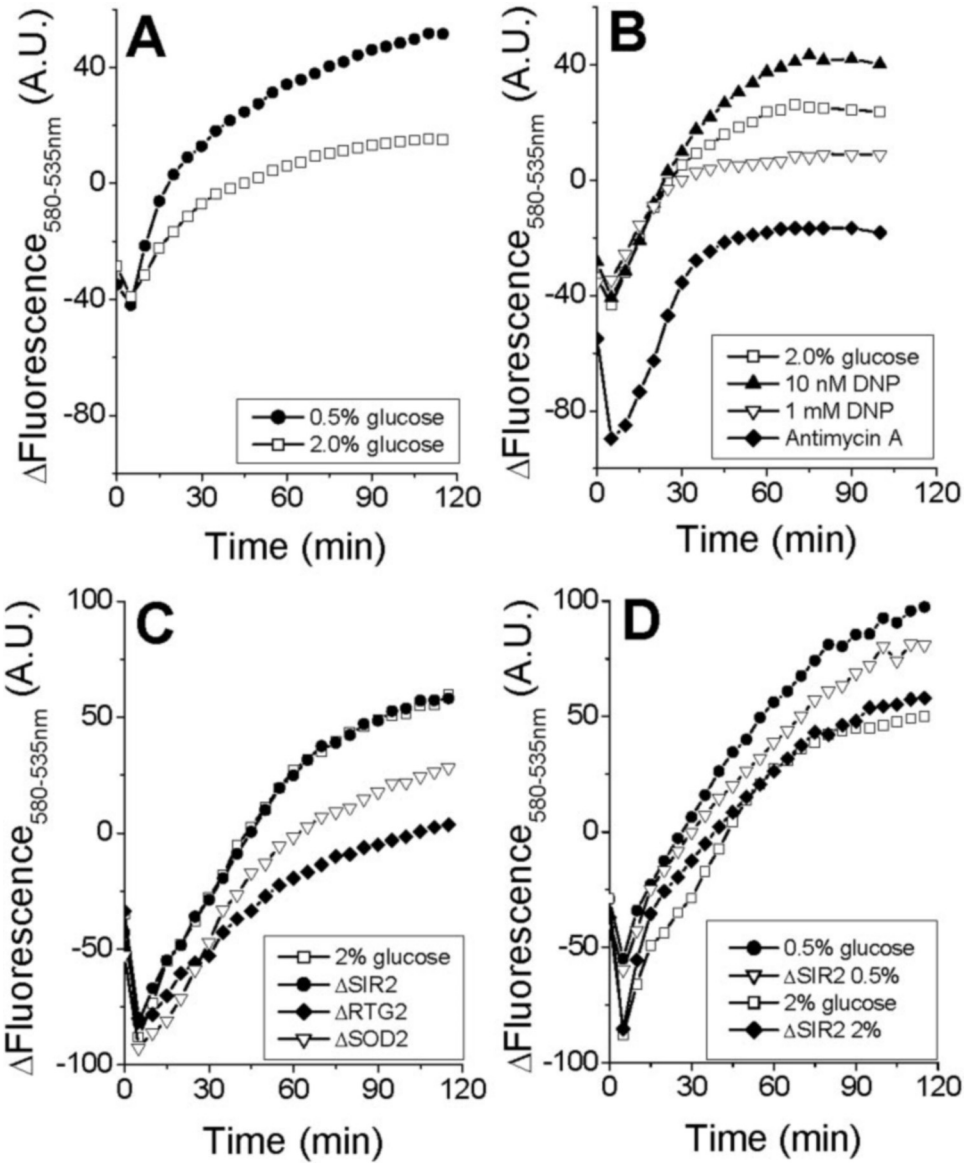

FIG. 2. **CR and mild uncoupling enhance chronological life span.** The difference between metabolized red-orange FUN® 1 fluorescence (living cells) and bright green fluorescence (dead cells) was measured every 5 min and plotted over time until stable levels were obtained (see "Experimental Procedures"). The initial decrease in values occurs because of FUN® 1 incorporation by the cells, whereas fluorescence differences after stabilization are proportional to live cell counts. *A* and *B*, the fluorescence of W303–1A cells grown for 4 days in 2% glucose (*B*, all traces) or 0.5% glucose, as shown, in the presence of DNP (at the concentrations shown) or 0.1 μg/ml antimycin A where indicated. *C* and *D*, BTY4741 wild type or null mutants of *SIR2, SOD2,* and *RTG2* cells were grown for 4 days in 2% (*C*, all traces) or 0.5% glucose as shown. The results presented are representative experiments of at least three similar repetitions.

of many studies. Although most research using animals has found an inverse correlation between levels of mitochondrial ROS and life span (reviewed in Refs. 5–8), a causative effect of ROS-promoted oxidation in limiting life span has been hard to establish because of the inconsistent and/or nonexistent effects of antioxidants (4, 32).

Further questions involving the role of ROS in life span have been uncovered by studies using *S. cerevisiae* as a model for aging and longevity (30). These studies, which focused on replicative life span, show that CR does not enhance the expression of redox-related genes or resistance against oxidative stress (23). Although the authors suggest this evidence excludes a role for ROS in the replicative life span-extending effects of CR, they demonstrate that mitochondrial metabolism and respiration play a role in this process. By intensifying respiration, CR increases intracellular NAD^+/NADH ratios and the activity of Sir2p, which prevents the accumulation of ERCs and loss of replicative ability in the logarithmic growth stage (24) (see Fig. 4). Recently, the mammalian *SIR2* orthologue, *Sirt1*, has been shown to be up-regulated as a result of CR (33).

In this study, we have attempted to establish a more integrative link between mitochondrial metabolism, ROS, and both chronological and replicative life span. We began by measuring ROS release levels in mitochondria from yeasts grown under control and CR conditions and found that CR significantly decreases ROS release/O_2 consumed (Fig. 1). This finding suggests that even though CR yeast do not present more antioxidant defenses or increased resistance against exogenous oxidants (23), their redox balance is improved by lower levels of mitochondrial ROS release. The effects of CR on ROS release could be mimicked by artificially increasing respiration with uncouplers, whereas respiratory inhibition strongly enhanced ROS release, indicating the CR effect occurs as a result of respiratory stimulation. Yeast growth in 2% glucose represses the synthesis of electron transport chain components (23, 34). As a result, electrons may accumulate at intermediate levels of

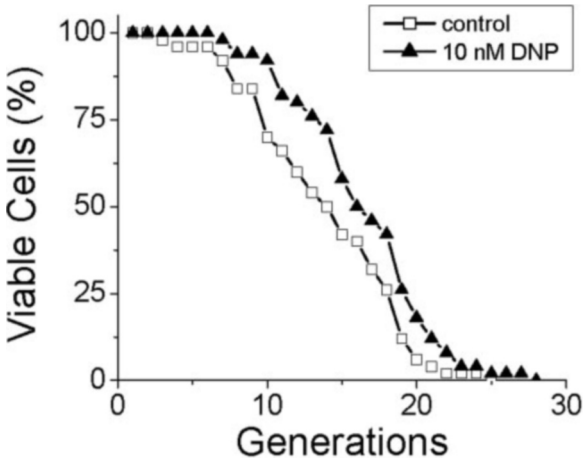

FIG. 3. **Mild uncoupling enhances replicative life span.** W303–1A cells were incubated overnight in the presence or absence (control cells) of 10 nM DNP and then plated on YPD medium containing 10 nM DNP or with no further additions (*control*). Mother cells were separated by micromanipulation, and the number of generations was counted in each group. The generation average of three experiments for control cells was 13.6 ± 0.20 and 15.6 ± 0.26 for cells treated with 10 nM DNP. The differences in the median values between the two groups are greater than would be expected by chance ($p = 0.016$, Mann-Whitney Rank sum test). The experiment shown is representative of three similar repetitions.

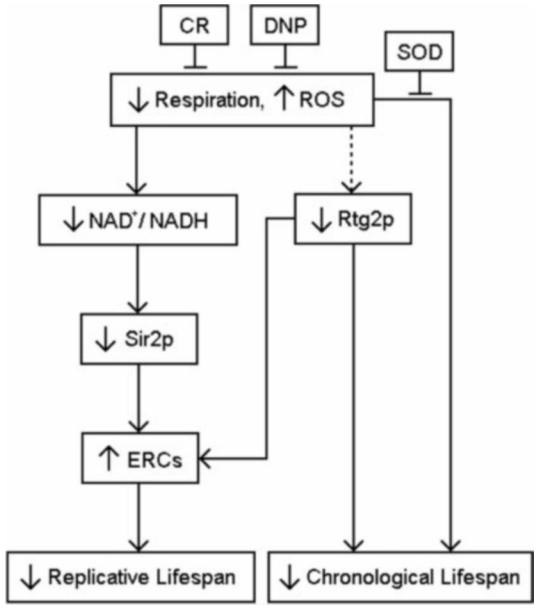

FIG. 4. **Schematic representation of the proposed role of mitochondria in yeast life span.** Decreased respiratory rates increase NAD^+/NADH ratios, leading to a lower Sir2p activity and ERC accumulation, which limits replicative life span. In addition, lower respiratory rates increase ROS production, which diminishes chronological life span, in a manner prevented by superoxide dismutase (*SOD*). Rtg2p deletion decreases chronological life span and increases ERC accumulation leading to reduced replicative life span. CR and mild uncoupling promoted by DNP increase respiration and limit mitochondrial ROS release, enhancing both chronological and replicative life span.

the respiratory chain, favoring electron leakage and ROS formation. In CR yeast, glucose repression of mitochondrial respiration is reduced, stimulating electron transport and preventing ROS formation. In this manner, the effects of CR are similar to those of mild uncoupling that decreases mitochondrial ROS by enhancing respiration and preventing the accumulation of electrons at early steps of the transport chain where they can reduce oxygen monoelectronically, generating superoxide radical anions (11, 12).

To verify whether mild uncoupling was the cause of the beneficial effects of CR, we measured the effects of low concentrations of the uncoupler DNP on life span in *S. cerevisiae*. We initially studied chronological life span, which has been the focus of fewer studies in the area. We found that CR increases chronological life span in two yeast strains (W303–1A and BTY4741), indicating that chronological and replicative life spans share some common pathways (Fig. 2). Furthermore, the effects of CR could be mimicked by low doses of DNP, whereas respiratory inhibition decreased cell viability under these conditions, suggesting the CR effect is related to changes in respiration and ROS release promoted by this treatment. Because the deletion of mitochondrial superoxide dismutase also decreased cell viability, it seems chronological life span is limited by mitochondrial ROS production, as suggested previously (17, 20).

Further support for a role of mitochondrial metabolism in the determination of chronological life span was obtained by the finding that ΔRTG2, a mutant strain deficient in retrograde signaling, also displays reduced chronological life span. The deletion of this gene has previously been shown to affect replicative life span (25), bringing further support for the existence of some common pathways in these processes (see Fig. 4). However, there are clear differences between the two mechanisms of aging in yeast. The null *sir2* mutant, which still responds to the effects of CR on chronological life span (Fig. 3), represses the effect of CR on replicative life span (23). This result indicates chronological life span is not limited by ERC accumulation, as expected in a non-dividing cell. Further support for this notion was provided by the finding that a null mutant of *PNC1*, which affects NAD^+/NADH levels and ERC accumulation (35), displayed an increase in chronological life span similar to that observed in wild type cells when incubated under CR conditions (results not shown).

Because mild uncoupling with DNP promoted the same respiratory, ROS, and chronological life span effects as CR, we tested its effects on replicative life span. The finding that DNP leads to an ~15% increase in replicative life span indicates that mild uncoupling efficiently mimics CR (which increases replicative life span by ~24% (21)) and improves life span in both dividing cells and those in stationary phase.

Based on our results, we propose a model which relates the effects of mitochondrial respiration and ROS release with chronological and replicative life span (Fig. 4). The finding that mild uncoupling, like CR, enhances both forms of life span suggests this may be a viable intervention to prevent aging in more complex organisms. Indeed, CR has been shown to promote a decrease in protonmotive force and ROS release in rats (36). Furthermore, individual mice with longer life spans have larger respiratory rates and proton leaks (37), supporting the idea that CR causes mild uncoupling that is responsible for the prevention of aging. Although the use of DNP as an uncoupler has many unwanted toxic effects, mammals contain naturally occurring pathways that lead to mild uncoupling, such as mitochondrial ATP-sensitive K^+ channels (38) and uncoupling proteins (39, 40). These pathways, when activated, decrease H_2O_2 release/O_2 consumption ratios and could prove useful in further studies designed to establish a link between mild uncoupling and longevity.

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