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Maintenance of cellular ATP level by caloric restriction correlates chronological survival of budding yeast

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ABSTRACT

The free radical theory of aging emphasizes cumulative oxidative damage in the genome and intracellular proteins due to reactive oxygen species (ROS), which is a major cause for aging. Caloric restriction (CR) has been known as a representative treatment that prevents aging; however, its mechanism of action remains elusive. Here, we show that CR extends the chronological lifespan (CLS) of budding yeast by maintaining cellular energy levels. CR reduced the generation of total ROS and mitochondrial superoxide; however, CR did not reduce the oxidative damage in proteins and DNA. Subsequently, calorie-restricted yeast had higher mitochondrial membrane potential (MMP), and it sustained consistent ATP levels during the process of chronological aging. Our results suggest that CR extends the survival of the chronologically aged cells by improving the efficiency of energy metabolism for the maintenance of the ATP level rather than reducing the global oxidative damage of proteins and DNA.

1. Introduction

Chronological lifespan (CLS) measures the survival periods of non-dividing yeast cells that have passed the post-diauxic phase [1]. During the post-diauxic phase, unknown survival adaptation mechanism starts when yeast cells shift metabolism from fermentation to respiration, and the cells slow down their growth [2]. Caloric restriction (CR) may extend the lifespan of budding yeast by enhancing this survival adaptation. CR is a distinct regimen that extends the lifespan of various eukaryotic model organisms including mammals. Previous studies have shown that CR extends the CLS [3] as well as the replicative lifespan (RLS) of various yeast strains [4]. It has been hypothesized that these CR-induced effects on lifespan extension can be attributed to an increase of stress resistant responses, metabolic remodeling and mitochondrial activity, as well as a reduction of mitochondrial reactive oxygen species (ROS) production [5,6]. To support these hypotheses, several studies have successfully demonstrated reduced damage to intracellular macromolecules [7,8], enhanced repair of DNA damage [9] and decreased oxidative damage to proteins in mammals [5,10]. On the other hand, a small portion of ROS produced by enhanced mitochondrial metabolism due to CR might contribute to an extended lifespan by increasing the stress defensive mechanisms [11,12] through a hormetic effect in *Caenorhabditis elegans*. The contribution of CR-mediated ROS reduction to lifespan extension is still a controversial topic in the field of aging research.

We previously observed that the efficiency of the electron transport chain (ETC) as well as mRNA and protein levels of most ETC components was increased in the CR condition during the exponential growth of yeast [13]. In this study, we further investigated the importance of efficiency of energy metabolism during the chronological aging process of yeast.

2. Materials and methods

2.1. Yeast strain and culture

The yeast strain*Saccharomyces cerevisiae*, BY4741 (*MATa his3Δ1 leu2Δ met15Δ ura3Δ*) was used in this study. Freshly rejuvenated seeds were inoculated at 0.2 of OD_{600} for our chronological aging study, and grown at 30 °C in 2% or 0.5% glucose-containing YPD media, which were used for the control and CR conditions, respectively [13].

2.2. Labeling of fluorescent dyes for fluorescence-activated cell sorting (FACS) analysis

To evaluate the viability of chronologically aged yeast, total ROS production, mitochondrial superoxide generation, and mitochondrial membrane potential (MMP) using FACS, yeast cells were harvested at the indicated time points and labeled with specific fluorescent dyes. For analyzing the viability of chronologically aged

Abbreviations: CR, caloric restriction; CLS, chronological lifespan; ROS, reactive oxygen species; RLS, replicative lifespan; ETC, electron transport chain; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; 8-OHdG, 8-hydroxydeoxyguanosine; DNPH, dinitrophenylhydrazine.

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yeast and mitochondrial superoxide generation, harvested cells were washed in PBS and incubated with 5 μg/ml propidium iodide (PI) or 5 μM MitoSox for 20 min at 30 °C. For measurement of total ROS and MMP, cells were washed with PBS or 5% glucose-10 mM HEPES buffer (pH 7.4), respectively [14]. Then, cells were stained with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) containing PBS or 175 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) containing 5% glucose-10 mM HEPES buffer (pH 7.4), and then incubated at 30 °C for 1 h or 20 min, respectively. Stained cells were measured and analyzed using FACS calibur® (BD sciences, CA, USA) and CellQuest Pro® (BD sciences, CA, USA).

2.3. Protein preparation and immunoblot analysis

Lysates of harvested yeast cells were extracted in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 2 mM EDTA) supplemented with a complete protease inhibitor cocktail (Roche) and 1 mM PMSF. Cell lysates were cleared by centrifugation at 13,000 rpm for 15 min, and then they were subsequently quantified using a Coomassie Protein Assay Kit (Pierce). Each 20 μg sample of protein lysate was run on a 15% polyacrylamide gel, and separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). Carbonylation of extracted proteins was detected using OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs) according to manufacturer's instruction. For detecting ubiquitination, we incubated the membranes with anti-ubiquitin antibody (Cell Signaling Technology) for 12 h and subsequently with HRP-conjugated goat anti-rabbit IgG (Pierce) for 2 h in 2% non-fat dry milk/TBST. The immunoblot was visualized by ECL detection system (GE Healthcare). Intensity in each lane was quantified via densitometry using Quantity One software (Bio-rad).

2.4. Determination of ATP concentrations

We determined ATP levels in the cells using a BioVision ATP Colorimetric/Fluorometric Assay Kit. From each culture, 2×10^7 cells were harvested to determine ATP levels. Harvested cells were washed three times in distilled water and stored at −70 °C. To extract ATP from the frozen cells, we added 100 μl of distilled water to the frozen sample and lysed cells by boiling for 10 min. Lysed samples were centrifuged at 4 °C and 13,000 rpm for 5 min [15]. We mixed 25 μl of supernatant, 45.8 μl of ATP assay buffer, 0.2 μl of ATP probe, 2 μl of ATP converter and 2 μl of developer mix to each well of a 96-well plate provided in the kit. Next, we incubated the 96-well plate at room temperature for 30 min in the dark. We measured fluorescence using a Wallac VICTOR3™ Multilabel Plate Reader (PerkinElmer) at Ex/Em: 535/587 nm wavelength. The resultant raw data were converted into nM of ATP per 10^7 cells, and statistical significance was calculated using Student's *t*-test.

2.5. ELISA for 8-hydroxydeoxyguanosine (8-OHdG)

We harvested and lysed yeast cells at the indicated time point with breaking buffer containing 100 mM Tris–Cl (pH 8.0), 2% Triton X-100, 1% SDS, 100 mM NaCl and 1 mM EDTA using a Beadbeater (Biospec Products, OK, USA). Total DNA was then extracted using phenol–chloroform. Isopropyl alcohol-precipitated DNA was dissolved in distilled water and quantified in a UV–spectrophotometer. The quantity of 8-OHdG in each sample was measured by OxiSelect™ Oxidative DNA Damage ELISA Kit (Cell Cell Biolabs) according to the manufacturer's instructions. Absorbance was read using a PowerWaveXS microplate spectrophotometer (BioTek, VT, USA) at a primary wave length of 450 nm and converted to ng 8-OHdG per mg DNA. Statistical significance was tested using Student's *t*-test.

3. Results

3.1. CR extends CLS of budding yeast

To confirm the lifespan extension effect of CR on budding yeast BY4741, we measured viability and OD_{600} during the chronological aging process in the control and CR cultures. The viability was determined by the PI staining for dead cells. After five days of inoculation, PI-positive cells started to increase in the control culture compared to the CR culture. After 30 days, only 8.3% of yeast cells had survived under the control condition, while 81.7% of cells remained as PI-negative under the CR condition (Fig. 1A). The OD_{600} values (Fig. 1B) were similar to the earlier reports [3]

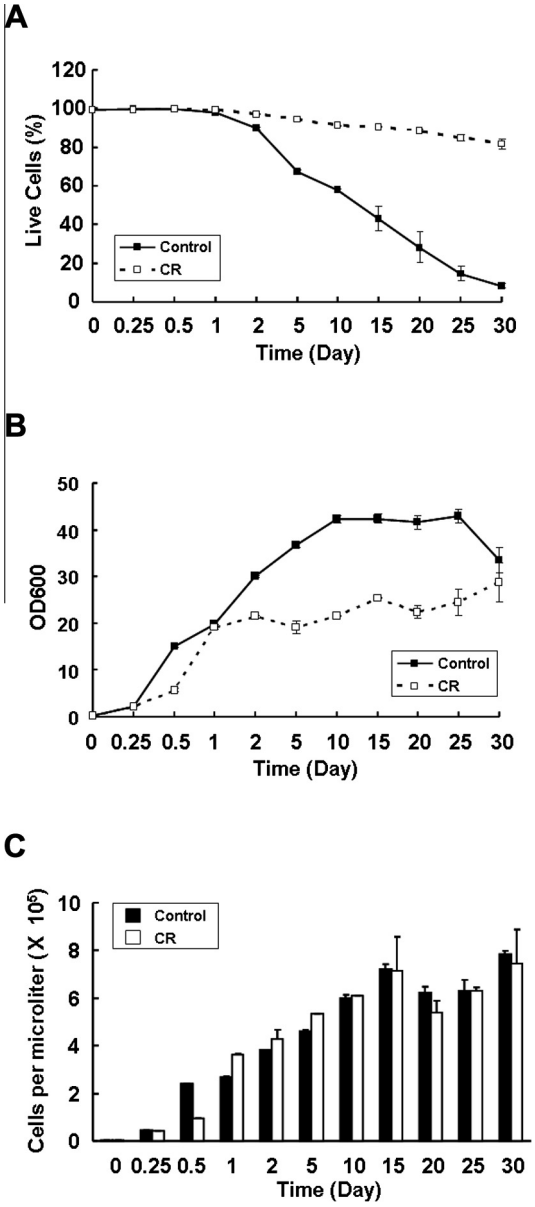

Fig. 1. CR extends CLS of yeast. (A) Yeast viability was assayed by FACS analysis following PI staining under a control or CR condition ($^*p < 0.05$, $^{**}p < 0.01$). The values were presented in the graph as mean ± SD ($n = 3$ batches in each group). (B) OD_{600} under each condition was measured with a spectrophotometer ($n = 3$). (C) Cell number was counted with a hemocytometer ($n = 3$).

in both culture conditions. Glucose has been considered as a major growth factor as well as a preferable carbon and energy source in yeast [16]. Indeed, the OD_{600} value was 50% lower in the CR culture than in the control culture during most of the stationary phase. However, we observed similar cell counts per unit volume in both the CR and control cultures (Fig. 1C). In fact, the FACS analysis showed that the cells in the CR culture were smaller and less complex than those in the control culture (data not shown) explaining the lower OD_{600} measurement in the CR culture.

3.2. CR reduces the generation of intracellular ROS and mitochondrial superoxide

Mitochondria produce ROS as by-products of oxidative phosphorylation. The increase in intracellular ROS levels by stimulus can shorten the lifespan of yeast cells [17,18], and the reduction of oxidative stress contributes to an increase in the lifespan of various model organisms [19–22]. Previous reports showed that CR reduces cellular ROS generation in both animals and yeast during the log phase [13,23].

We measured the generation of total ROS and mitochondrial superoxide during the chronological aging process both in the control and CR conditions. In the CR condition, the generation of both total ROS (Fig. 2A) and mitochondrial superoxide (Fig. 2B) was significantly reduced compared to the control after Day 2. CR maintained the lower total ROS during the log and early diauxic phase, eventually reducing it more than twofold during the stationary phase, and maintaining the reduction constantly afterward (Fig. 2A). Also, CR caused little reduction of the mitochondrial superoxide during the log and early diauxic shift. However, CR gradually reduced mitochondrial superoxide to a great extent after Day 2 (Fig. 2B).

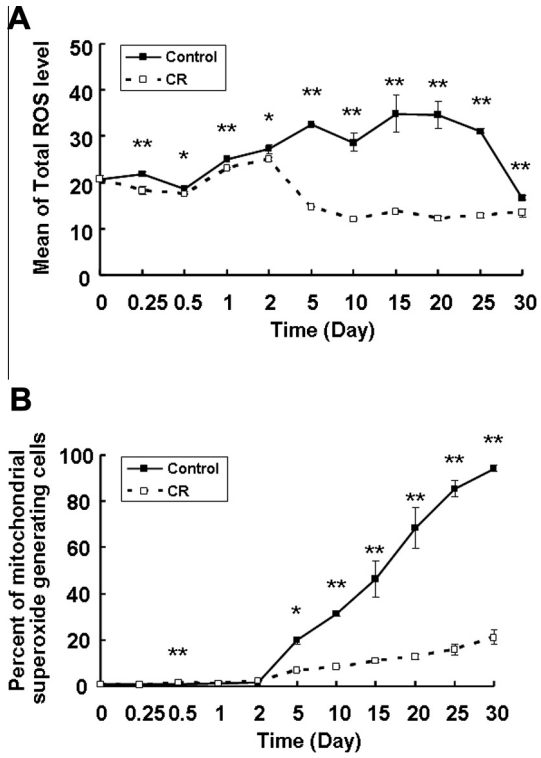

Fig. 2. CR-induced effects on total ROS and mitochondrial superoxide generation. (A) CR reduced total ROS generation. Total ROS levels were determined by H_2DCFDA and FACS analysis ($^{*}p < 0.05$, $^{**}p < 0.01$; $n = 3$). (B) CR reduced mitochondrial superoxide generation. Levels of mitochondrial superoxide were determined by MitoSOX™ and FACS analysis ($^{*}p < 0.05$, $^{**}p < 0.01$; $n = 3$).

3.3. Reduction of ROS by CR does not affect oxidative damage on DNA and proteins

Several studies have suggested that oxidative damage to the genome and proteome might be a major cause of aging. We also observed that CR reduced both total ROS and mitochondrial superoxide in yeast. This caused us to expect less damage to protein and DNA. We detected carbonylation and ubiquitination of proteins in whole cell lysate via immunoblot using specific antibodies. Based on densitometric analysis of resultant immunoblot images, the carbonylation levels of total protein consistently remained during chronological aging in the CR condition; however, the levels were slightly reduced at Day 15 and Day 20 in the control condition without a statistical significance (Fig. 3A).

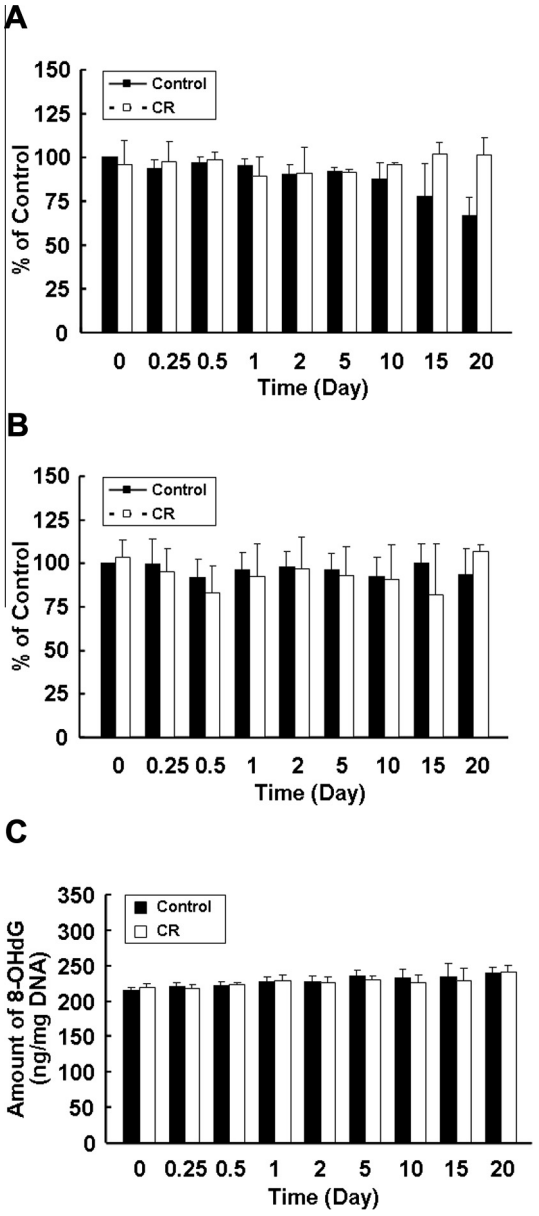

Fig. 3. Reduced total ROS and mitochondrial superoxide by CR does not affect the oxidative damage in protein and DNA. CR did not alter the levels of protein carbonylation (A), ubiquitinated protein (B) or 8-OHdG (C). Carbonylation or ubiquitination of total protein was detected via immunoblot using the anti-DNPH or ubiquitin-specific antibody ($n = 3$). The level of 8-OHdG of total DNA was investigated via ELISA using an anti-8-OHdG monoclonal antibody. Calculated amounts of 8-OHdG were plotted as mean ± SD ($n = 3$).

Ubiquitination levels were consistent during chronological aging in both the control and CR conditions (Fig. 3B). Although the levels of 8-OHdG in genomic DNA slightly increased during chronological aging, there were no statistically significant differences between the two conditions (Fig. 3C). Our data show that the reduction of both total ROS and mitochondrial superoxide in the CR condition did not relate to the reduction of oxidative damage on cellular proteins and DNA in yeast.

3.4. CR increases MMP

The loss of MMP causes failures in cellular energy metabolism [24,25]. Thus, we measured MMP at the indicated time points both in the control and CR conditions during the chronological aging process. From the beginning up to Day 5, the MMP level was consistently higher (10–25%) in the CR condition than in the control condition. At Day 10, the MMP level became similar in both conditions, but after Day 10, the MMP levels dramatically increased in the CR condition up to 90% (Fig. 4A). As shown in the FACS plots (Fig. 4B), the MMP peaks of each condition demonstrated a similar distribution until Day 10; however, the MMP peaks in the control condition became flat and moved toward the direction of low value after Day 10.

3.5. CR maintains ATP level during the chronological aging process

CR dramatically decreased total ROS and mitochondrial superoxide generation, and increased MMP in this study. In addition, we measured the change of ATP level from the chronologically aged cells (Fig. 4C). In the control condition, ATP level was 3.49 nM/10^7 cells at 6 h after exponential growth began. After passing the exponential growth phase, ATP level in the control condition was at the maximum concentration (7.07 nM/10^7 cells) at Day 1. After reaching the maximum point, ATP level was maintained until Day 5, and then it rapidly declined to 2.28 nM/10^7 cells on Day 30. On the other hand, ATP levels in the CR condition were maintained between 3.66 and 5.79 nM/10^7 cells up to Day 30. ATP levels in the CR condition were lower than in the control condition until Day 5, but after Day 5, the ATP levels in the CR condition were higher than in the control condition.

4. Discussion

Many reports have suggested the possible mechanisms to extend yeast CLS by CR focusing on oxidative stress [26,27], and nutrient sensing signaling [28,29]. In our previous studies, we found that genes related to mitochondria were increased at the transcriptional and translational levels by CR during the exponential phase [13,30]. These reports indicate the importance of the mitochondrial function in CR-mediated lifespan extension of yeast during its exponential growth phase [13]. In the current study, we further demonstrated that CR maintains cellular ATP level by elevating MMP via increasing mitochondrial efficiency.

Glucose is known not only as an energy and carbon source, but also for facilitating the growth and proliferation of yeast [16]. In general, organisms under CR tend to be smaller. We observed 50% less OD_{600} in the CR condition than in the control at the stationary phase. However, the number of cells in a unit volume in the CR condition was similar to the number in the control condition (Figs. 1B and 1C). This indicates that CR did not interfere with the proliferation of cells but that it did interfere with growth because of a limited availability of glucose [31].

The free radical theory of aging focuses on oxidative damage to cellular components, such as DNA, proteins and lipids by ROS [32,33]. Also, many reports have indicated that caloric restriction could reduce oxidative damages to cellular components in various models when induced by stimuli of oxidative stress [10,34,35]. ROS lead to irreversible protein carbonylation [36]. Carbonylated proteins are mainly degraded in the proteasome; however, highly carbonylated proteins can form proteolysis-resistant aggregates [37,38]. The ubiquitin–proteasome pathway modulates the turnover of proteins in cells, and CR is known to increase the activity of this pathway in rodents [39]. ROS, induced by many factors

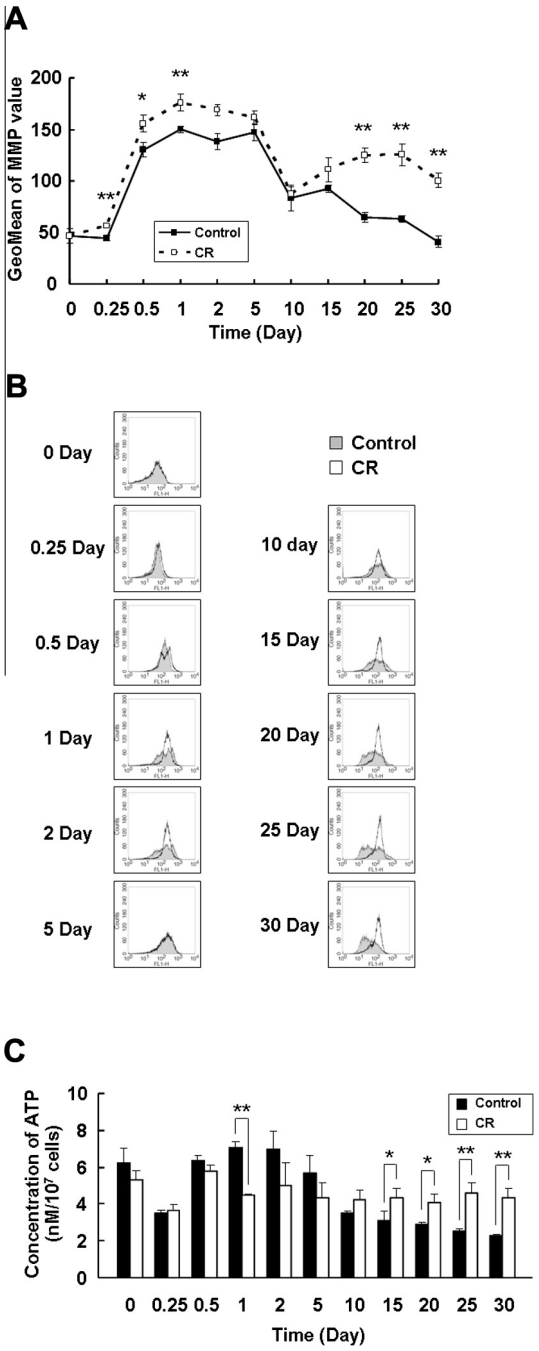

Fig. 4. CR enhances MMP. (A) Under the CR condition, MMP was elevated at the log phase and stationary phase. Relative levels of MMP were investigated by $DiOC_6$ and FACS. Twenty thousand events were acquired and analyzed ($^{*}p < 0.05$, $^{**}p < 0.01$; $n = 3$). (B) Histograms of $DiOC_6$ fluorescence for control and CR cells were merged. The shaded area is for the control, and the empty area is for CR-treated cells. (C) CR maintains cellular ATP levels during the chronological aging process. Yeast cell lysates from the control and CR groups were assayed for ATP levels ($^{*}p < 0.05$, $^{**}p < 0.01$; $n = 3$).

including ionizing radiation, and aging, produces 8-OHdG which is a mutation-prone DNA-base-modified product in genomic and mitochondrial DNA [40]. 8-OHdG has been measured as a marker for oxidatively damaged DNA. In this study, our data demonstrated that CR dramatically reduced total ROS (Fig. 2A) and mitochondrial superoxide generation (Fig. 2B) at the stationary phase. However, interestingly, the data from analysis of total protein carbonylation (Fig. 3A), ubiquitination (Fig. 3B) and 8-OHdG (Fig. 3C) did not indicate significant differences as a result of CR. This indicates that yeast under both culture conditions have sufficient potential to eliminate the produced ROS before they cause macromolecular damage during the chronological aging.

Mitochondria play a central role in cellular bioenergetics and are a major site for generation of free radicals in cells [41]. Indeed, aging is associated with a decline in mitochondrial function [42], and mitochondrial dysfunction by free radicals could be a major contributor to aging [43,44]. Also, several reports have showed that mitochondrial DNA is an important target of free radicals during aging [45,46]. In yeast, the accumulation of oxidative damage to mitochondria and mitochondrial proteins is associated with both chronological and replicative aging [47]. MMP is the driving force for mitochondrial ATP synthesis [48]. Because a decrease of MMP and mitochondrial mass was observed during the aging process in mice [49], the relationship between aging and cellular energetics could be important. In this study, we measured both cellular ATP level and MMP during the chronological aging process of yeast in the CR or non-CR conditions. The cells in the CR condition had significantly higher MMP than those in the control condition during the chronological aging process (Fig. 4A). The FACS histograms clearly show the difference of MMP between the cell populations of the control and CR conditions (Fig. 4B). We also measured ATP levels (Fig. 4C) at the identical time points for MMP. The change between MMP and ATP during the chronological aging process was similar in pattern at both conditions. The ATP levels in the CR condition remained steady from Day 1 to Day 30, while the levels in the control condition gradually decreased. The high ATP level in the control condition compared to the CR condition during the log and early stationary phases can be explained by the high content of glucose in the initial control YPD media. However, surprisingly, the real difference in ATP concentration between the two conditions was not large (less than 1.5-fold) at the early stages of the chronological aging process, in spite of the quadruple difference of glucose concentration at the start in the media. Thus our results suggest that CR enabled the efficient operation of cellular energy metabolism even with a limited energy source. In addition, CR enhanced the efficiency of mitochondrial ETC for ATP production along with the reduction of mitochondrial superoxide production. This CR-mediated transformation in energy metabolism might contribute to maintaining a consistent intracellular ATP level.

Taken together, our results demonstrate that CR lowers the generation of total ROS and mitochondrial superoxide, which is a sign of efficient ETC, and increases MMP. Eventually, these CR-mediated changes drive production and maintenance of cellular ATP during the chronological aging process, which guarantee the cellular longevity of yeast in the CR condition.

Acknowledgments

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