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Enhancement of mitochondrial function correlates with the extension of lifespan by caloric restriction and caloric restriction mimetics in yeast

Kyung-Mi Choi [a], Hye-Lan Lee [a], Young-Yon Kwon [a], Mi-Sun Kang [b], Sung-Keun Lee [b], Cheol-Koo Lee [a,*]

[a] Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea
[b] Department of Pharmacology, College of Medicine, Inha University, Incheon 400-712, Republic of Korea.

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ABSTRACT

Caloric restriction mimetics (CRMs) have been developed to mimic the effects of caloric restriction (CR). However, research reports for the effects of CRMs are often times inconsistent across different research groups. Therefore, in this study, we compared seven identified CRMs which extend the lifespans of various organisms including caffeine, curcumin, dapsone, metformin, rapamycin, resveratrol, and spermidine to CR for mitochondrial function in a single model, *Saccharomyces cerevisiae*. In this organism, rapamycin extended chronological lifespan (CLS), but other CRMs failed to extend CLS. Rapamycin enhanced mitochondrial function like CR did, but other CRMs did not. Both CR and rapamycin worked on mitochondrial function, but they worked at different windows of time during the chronological aging process.

1. Introduction

The biology of aging has greatly expanded since scientists found ways to extend an organism's lifespan, for instance caloric restriction (CR). CR is a critical method to understand the mechanism of lifespan extension and the aging process in itself. Because CR accompanies the reduction of food consumption over a long period, simple chemical substitutes, called CR mimetics (CRMs), could be favorable in replacing the recipe. With this practical application, these CRMs are also useful for understanding the complexity of the aging process. Furthermore, these CRMs could be used as starting materials in developing drugs that prevent or ameliorate devastating aging-associated illnesses. The more methods have for increasing an organism's lifespan, the better understand the aging process by comparing the mechanisms of those methods.

Currently, dozens of chemicals have been demonstrated as lifespan extenders in different model organisms. Rapamycin, known as a chemical inhibitor of target of rapamycin (TOR), increased the lifespan of mice in a low dose [1] as well as that of yeast [2]. There are other CRMs for extension of lifespan working as a single model, or a few models, consistently. Resveratrol increased the lifespan of several organisms including that of yeast, worms, flies, and fish, but the targets of resveratrol are not yet clear. However, resveratrol failed to increase the lifespan of mice [3], though a low dose of dietary resveratrol produced a transcriptional change similar to that of CR [4]. In a similar way, a low dose treatment of resveratrol inhibited the cellular senescence of human mesenchymal stem cells, but a high dose of resveratrol accelerated their senescence [5]. A recent report showed that 4,4'-diaminodiphenylsulfone (also known as dapsone) which has been used to treat Hansen disease patients extended the lifespan of *Caenorhabditis elegans* through the inhibition of pyruvate kinase [6]. Metformin is known as an AMPK activator and has been used for treating diabetes patients. Metformin supplementation extended the lifespan of worms and rats. In a transgenic mouse with Huntington's disease, 2 mg/mL of metformin prolonged the lifespan of male mice, but at a higher concentration, it did not. Conversely, there was no longevity effect on female mice at either low or high doses [7]. When metformin was treated at an early age in SHR female mice, their lifespan was significantly extended [8]. Spermidine increased the lifespan of yeast, flies, worms, and human immune cells via the induction of autophagy [9]. Caffeine extended the lifespan of yeast through inhibition of TORC1 [10]. Curcumin increased the lifespan of flies, but the optimum dose of curcumin varied depending on sex and genetic background [11].

As mentioned above, CRMs can act either universally across different species or selectively on a single species at a different range of dose. So far, most CRMs work uniquely for the case of a particular species, gender, special genetic conditions, and ambient conditions. Therefore, if CRMs were applied to a single species, it might elucidate a possible common mechanism of lifespan extension by

Abbreviations: CR, caloric restriction; CRMs, caloric restriction mimetics; CLS, chronological lifespan; RLS, replicative lifespan; ROS, reactive oxygen species; MMP, mitochondrial membrane potential.

* Corresponding author. Fax: +82 2 921 1715.
E-mail address: cklee2005@korea.ac.kr (C.-K. Lee).

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them. Here, in this study, we evaluated above-mentioned lifespan-extenders in a single species, *Saccharomyces cerevisiae*. We used a long-lived yeast strain BY4741, which allows us to exclude the limitation for short-lived strains regarding effectiveness. In particular, we investigated the effects of CR and seven CRMs on chronological lifespan (CLS) and mitochondrial function.

2. Materials and methods

2.1. Yeast culture and culture conditions

The BY4741 strain was used and cultured in YPD or synthetic complete (SC) medium as described previously [12]. All materials for the medium were purchased from BD Biosciences (CA, USA). The SC medium was composed of a 0.2% drop-out mix complete without yeast nitrogen base (US Biological, MA, USA), a 0.67% yeast nitrogen base without amino acids, and 2% glucose. This SC medium was prepared according to the manufacturer's instructions and it was adjusted to pH 6.0. For CRM-treated conditions, we added each CRM in order to make the indicated concentrations into 2% glucose-containing YPD media or SC media. All CRMs used in this study were purchased from Sigma (MO, USA).

2.2. CLS assay

Cellular viability was assessed using propidium iodide (PI) (Sigma, MO, USA) staining. We harvested samples at a density of 2×10^6 cells in 1 mL of phosphate buffered saline (PBS) at appropriate time-points. Cells were incubated for 20 min at 30 °C in the presence of 5 μg/mL PI. For each sample, 20,000 cells were analyzed by BD FACSCalibur or BD FACSVerse (BD, NJ, USA). The difference in flow cytometry equipment did not affect the viability data.

2.3. Reactive oxygen species (ROS) detection

Cells were prepared at a concentration of 2×10^6 cells per 1 mL PBS at the exponential (6 h), post-diauxic (2 days), and stationary phases (10 days). To measure total ROS generation, cells were incubated for 1 h in the presence of 200 μM 2',7'-dichlorofluorescin diacetate (H_2DCF) (Invitrogen, CA, USA) then they were analyzed using BD FACSVerse. To measure mitochondrial superoxide, cells were treated with 5 μM MitoSox Red (Invitrogen, CA, USA) and incubated for 20 min. MitoSox-stained cells were washed, resuspended in 1 mL PBS and analyzed using BD FACSCalibur.

2.4. Measurement of mitochondrial membrane potential (MMP)

Yeast cells were harvested under the same conditions and at the same time-points for the ROS measurements. The cells were suspended in a 10 mM Hepes buffer containing 5% glucose. Then, the cells were stained in 175 nM 3,3'-dihexyloxacarbocyaine iodide ($DiOC_6$) (Invitrogen, CA, USA) for 20 min as recommended in the manufacturer's instructions. The cells were then analyzed using BD FACSVerse.

2.5. ATP assay

For the ATP measurement, 2×10^7 cells were harvested at the same time-points for the ROS/MMP assessments. The harvested cells were washed in distilled water. After removing the water, the pellet was immediately frozen in liquid nitrogen and stored at −70 °C until the assay. Before the ATP measurement, cells were resuspended in water at a concentration of 2×10^5 cells/μL and lysed by boiling at 100 °C for 10 min. The ATP content was measured using an ATP colorimetric/fluorometric assay kit (BioVision, CA, USA) according to the manufacturer's instructions. Signals were detected using a Multilabel plate reader (Perkin Elmer, MA, USA).

2.6. Statistical analysis

We displayed data as a mean ± standard error of the mean (SEM) from three biological repeats. The statistical significance was assessed by a two-tailed *T*-test between the control (2% glucose or mock) and other conditions (CR or CRM-treated). We depicted the significance levels as $^{*}P < 0.05$ or $^{**}P < 0.01$.

3. Results

3.1. Effect of CR and seven CRMs on lifespan at various concentrations

To compare the effects of CR and various CRMs on lifespan in a single model system, we cultured yeast cells under YPD medium containing various concentrations of glucose or chemicals including rapamycin, resveratrol, dapsone, metformin, spermidine, caffeine, and curcumin. The concentrations of the chemicals were set based on whether they crystallized in the culture media. For the CR condition, we examined four different glucose concentrations including 0.1%, 0.2%, 0.3%, and 0.5% as compared to a 2% non-CR condition (Fig. 1A). CR significantly increased CLS at all used concentrations up to 33%–55% on day 30 (Fig. 1A). We observed the maximum extension at the 0.5% CR condition. For the rapamycin treatment, the range from 100 nM to 10 μM concentrations led to a consistent extension of lifespan by ~87% on day 30, whereas low doses including 1 pM and 1 nM have no effect on lifespan on day 30 compared to no treatment (mock) (Fig. 1B). The lifespan of yeast cells grown in the presence of 1 nM–100 μM resveratrol or 1 pM–100 μM dapsone showed a minor effect and did not constitute any statistical significance ($p > 0.1$) (Fig. 1C and D). The highest treatments, 1 mM resveratrol and 1 mM dapsone, showed a minor level of toxicity (Fig. 1C and D). We also found that yeast treated with 1 nM–1 mM metformin (Fig. 1E), 1 nM–1 mM spermidine (Fig. 1F), 1 μM–1 mM caffeine (Fig. 1G), and 1 pM–10 μM curcumin (Fig. 1H) had not been affected in terms of its lifespan. An alternative explanation for the failure of these CRMs, could be ineffectiveness in a complex media condition such as YPD. To verify this possibility, we tested the effects of CR and the CRMs on lifespan under a simple condition of synthetic complete (SC) media. CR clearly increased CLS in SC media as well (Fig. S1). The cells under 0.2% glucose and 0.5% glucose showed a lifespan extension of up to 78% and 84%, respectively, compared to the 2% control on day 20 (Fig. S1). In case of rapamycin, 100 nM and 1 μM treatments successfully extended lifespan up to 42% and 35%, respectively on day 20; however, the lower doses including 1 pM and 1 nM did not extend lifespan (Fig. S1). Consistently with the results obtained in YPD, other CRMs showed no significant effect on the lifespan (Fig. S1). We also validated our CLS data by the PI assay using the CFU assay at the representative concentrations of CRMs. For CR conditions, 0.5% glucose was used as a standard CR treatment because the maximum extension of CLS was observed in this concentration in both YPD and SC media. For rapamycin, because 100 nM, 1 μM, and 10 μM treatments showed almost identical levels of CLS extension, we used 100 nM for measuring CLS. For other CRMs, we used the highest concentration among the levels for the determination of CLS by PI assay below toxic levels, thus 10 μM resveratrol, 10 μM dapsone, 100 μM metformin, 100 μM spermidine, 100 μM caffeine, and 10 μM curcumin for the test. As a result, we observed an agreement between the two methods, and only 0.5% CR and 100 nM rapamycin significantly extended CLS by the CFU assay (Fig. S2). We further

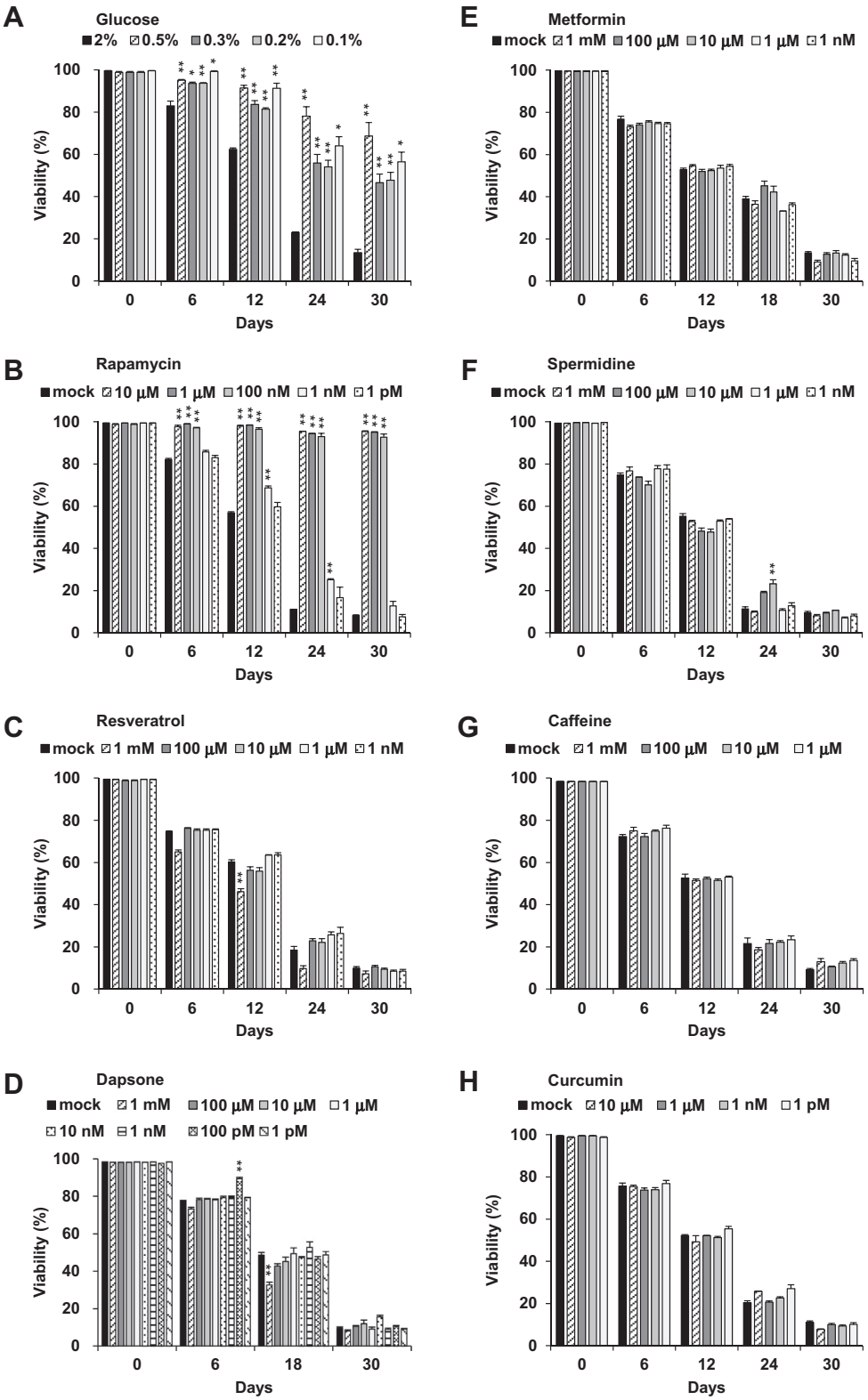

Fig. 1. Effect of CR and seven CRMs on the CLS of yeast in YPD medium. Cellular viability was measured by calculating the proportion of PI-negative cells using a flow cytometer. (A) The viability of cells grown under 2% glucose (the control condition) or lower glucose (the CR conditions). (B–H) The viability of cells grown under no treatment (mock) or various concentrations of CRMs including rapamycin (B), resveratrol (C), dapsone (D), metformin (E), spermidine (F), caffeine (G), and curcumin (H).

examined the effect of these CRMs at the representative concentrations on replicative lifespan (RLS), but we did not observe significant effects on RLS even for 0.5% CR and 100 nM rapamycin (Fig. S3).

3.2. Reduction of ROS generation correlated with the extension of CLS

ROS has been suggested as an aging-inducing factor, and CR reduced oxidative stress to retard aging and extend lifespan. To

determine whether the effect of CR and various CRMs on lifespan shows a correlation with ROS generation, total levels of ROS (hydrogen peroxide) and mitochondrial superoxide were measured using H_2DCF and MitoSox Red, respectively. Yeast cells were grown under the representative conditions for CLS and harvested at three different growth phases including the exponential (6 h), post-diauxic (day 2), and stationary phases (day 7). As shown in Fig. 2A, the fluorescence intensity of H_2DCF was strongly detected after 6 h of culture, and after the time was up, the signal had dramatically decreased. Rapamycin almost completely suppressed ROS generation at the 6 h mark whereas other treatments did not affect ROS generation. These results strongly suggest that the reduction of the total ROS measured using H_2DCF in the early stages of growth can be a proprietary marker of rapamycin. We also measured mitochondrial superoxide generation (Fig. 2B). In contrast to the total ROS, mitochondrial superoxide was detected in small quantities at the 6 h mark and on day 2, but it was dramatically increased on day 10. For the case of mitochondrial superoxide, both CR and rapamycin treatments greatly prevented its generation. The proportion of cells generating mitochondrial superoxide dropped 4-fold by CR and 25-fold by rapamycin. Other treatments failed to prevent the generation of mitochondrial superoxide. Rapamycin is a strong preventer of oxidative stress.

3.3. Energy status correlated with the extension of CLS

A major source of ROS is mitochondria, where the cellular energy ATP is also produced. In this context, we further evaluated the energy status of cells treated with CR or CRMs by measuring both MMP and ATP. Rapamycin significantly increased MMP up to 2-fold at the 6 h mark, and then went back to the MMP level of the control condition on day 2 and day 10 (Fig. 3A). CR did not increase the MMP level at the 6 h mark, but CR increased MMP on day 2 and day 10 up to approximately 60% above the control condition (Fig. 3A). Rapamycin increased MMP in the early growth phase (at the 6 h mark), however, CR increased MMP in the later growth phase (on day 2 and after day 2). None of the other CRMs affected MMP during the same period (Fig. 3A). Interestingly, the patterns of MMP changes contrasted the patterns of ROS changes by rapamycin and CR, respectively. Consistent with the MMP changes, both rapamycin and CR significantly increased ATP production at corresponding time-points. Rapamycin increased production of ATP more than 2-fold at the 6 h mark, and CR increased the production of ATP up to 80% higher on day 10 (Fig. 3B). Other CRMs did not change the production of ATP (Fig. 3B).

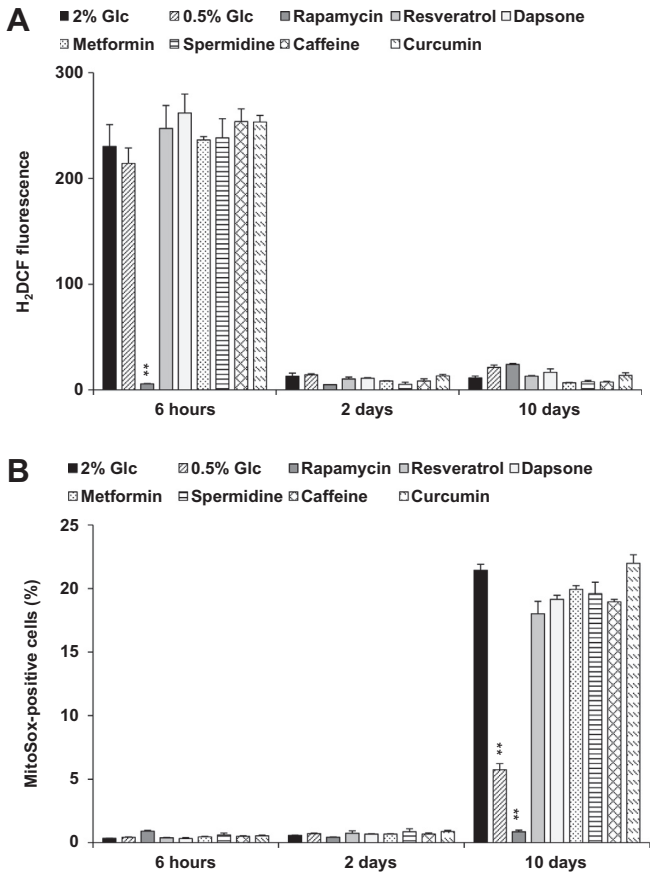

Fig. 2. Effect of CR and seven CRMs on ROS production. (A) The total ROS including H_2O_2 was detected using H_2DCF in the YPD media after treating a single concentration showing a maximum effect on CLS. For the CRMs that did not increase CLS, we used the highest concentration tested so long as it did not show any toxicity. (B) Superoxide derived from mitochondria was detected using MitoSox Red under the same conditions for the total ROS measurement.

4. Discussion

In our previous reports, CR increased the components for the mitochondrial electron transport chain and the expression of genes for mitochondrial function during the exponential growth phase [12,13]. In this study, we observed that CR affected mitochondrial

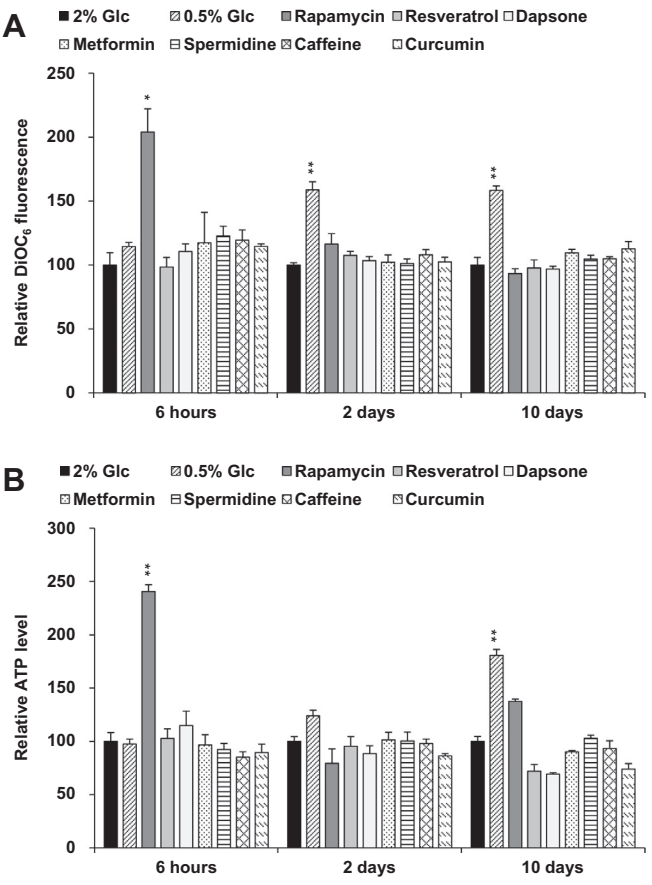

Fig. 3. Effect of CR and seven CRMs on MMP and ATP. (A) The relative fluorescence signals of $DiOC_6$ for MMP. Signals of CR or the treatment of CRMs with respect to 2% YPD (arbitrarily set to 100) were shown. CRMs were treated at the identical concentration for the ROS detection. (B) The relative levels of ATP in cells under CR or CRMs treatments with respect to cells under 2% YPD without treatment (arbitrarily set to 100) were shown. Concentrations of treatments were identical for the detection of ROS and MMP. Asterisk marks for statistical significance indicated bars exhibiting higher than 50% change from the control.

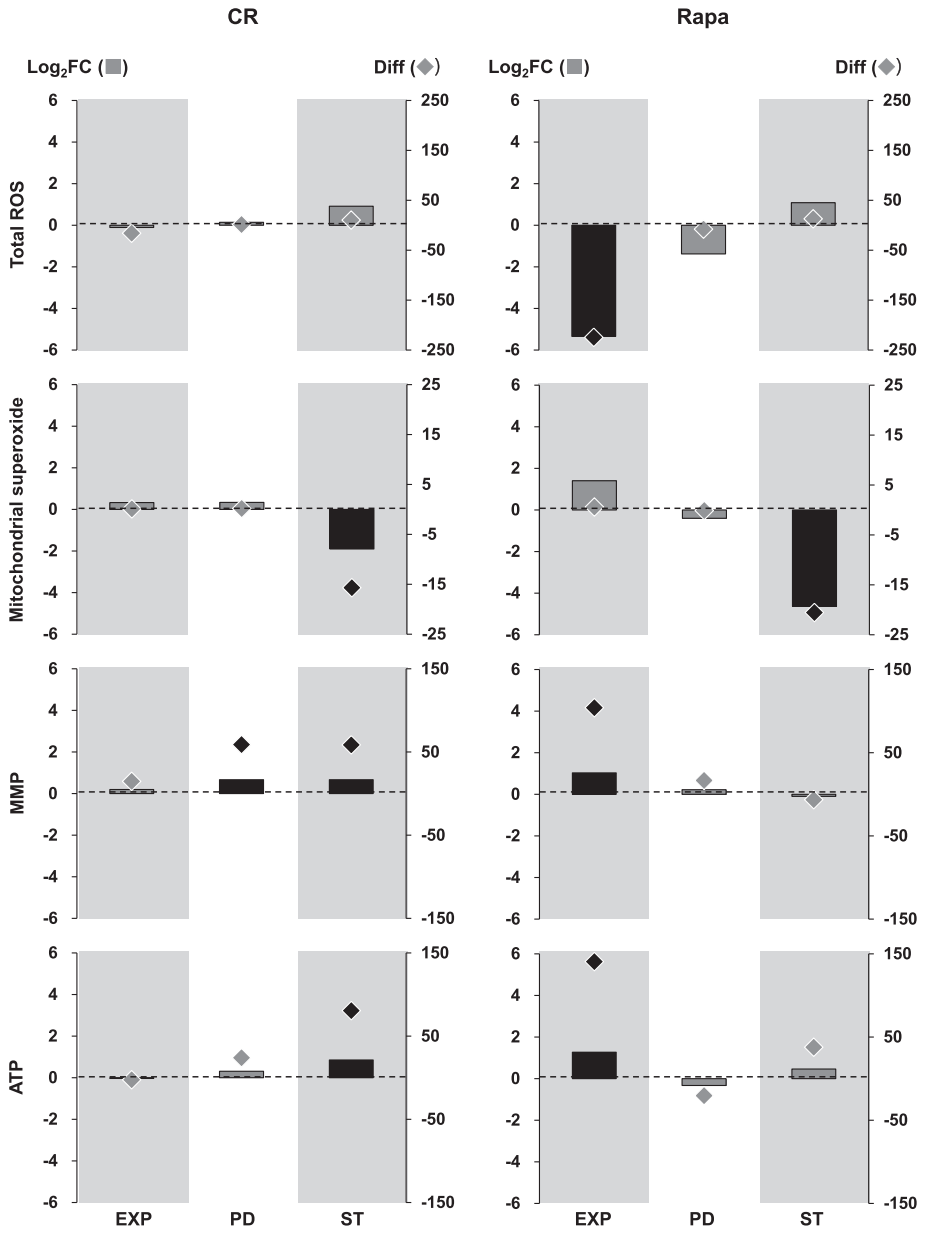

Fig. 4. CR and rapamycin regulate mitochondrial function at different time points. The effect of CR and rapamycin (Rapa) on total ROS, mitochondrial superoxide, MMP, and ATP at the exponential (EXP), post-diauxic (PD), and stationary phases (ST). The diamond and rectangle symbols indicate the $\log_2$ fold-change ($\log_2$FC) and the difference of raw signals (Diff), respectively. The FC between the treatment (either CR or Rapa) and the control (2% YPD) was calculated (FC = the raw fluorescence signal of treatment/ that of the control) and scaled to $\log_2$. The difference (Diff) was calculated (Diff = the raw fluorescence signal of treatment - that of the control) to distinguish the values that provide misunderstandings such as having a high FC but small Diff (this means the FC was exaggerated because of low raw signals). A black fill indicates a statistically significant change ($P < 0.05$) and a gray fill indicates no statistical significance.

function during the late stage of growth including the diauxic shift and the stationary phase. CR significantly increased MMP and ATP, but reduced the generation of mitochondrial superoxide. Therefore, we used these parameters as indicators of lifespan extension in yeast. Here, seven identified CRMs were tested for these parameters along with CLS. Among these CRMs, only rapamycin extended CLS. Rapamycin also increased MMP and ATP, and reduced ROS during the chronological-aging process. These identical enhancements of the mitochondrial function were also observed by CR, but in different working windows of time. Other CRMs that failed to extend CLS did not change any of these parameters related to the mitochondrial function.

Rapamycin has been suggested as a CR-mimicking compound, which works via similar mechanism by CR to increase lifespan. Our observations in this study also showed that rapamycin and CR could extend lifespan through a shared mechanism involved in mitochondria (Fig. 4). But there were differences in the extents of the effects and the working windows of time between these two methods. There was a distinguished parameter between rapamycin and CR. Although both rapamycin and CR suppressed the generation of mitochondrial superoxide at the stationary phase (Fig. 2B), only rapamycin prevented the production of the total ROS during the exponential growth measured by H_2DCF reactive to H_2O_2 (Fig. 2A). On the other hand, there was a clear difference between CR and rapamycin for the extent of lifespan extension depending on the culture media (Fig. 1 and Fig. S1). Rapamycin (range from 100 nM to 10 μM) extended CLS more powerfully than CR (the most effective CR condition in this study was 0.5%) in the YPD culture (Fig. 1A and B). In contrast, CR showed a stronger effect than rapamycin on the SC culture. CR (0.5% glucose) increased

lifespan approximately two times higher than rapamycin (maximum effect at 100 nM) on day 20 (80% vs. 42% compared to the control, respectively) (Fig. S1).

Mitochondria are major sites for ATP during respiration. We measured the change of MMP (Fig. 3A) and the cellular ATP level (Fig. 3B) in CR and CRM-treated conditions. Among these conditions, only CR and rapamycin altered the levels of MMP and ATP. CR increased MMP (~60% more than the control) on day 2 and day 10, but not at the 6 h mark (Fig. 3A). Consistent with this trend, CR increased cellular ATP on day 2 (24%) and day 10 (81%) compared to the control (Fig. 3B). Interestingly, rapamycin increased both MMP and ATP (~2-fold) only at the 6 h mark, but not on day 2 and day 10 (Fig. 3A and B). It might be interesting to know whether this difference of timing may or may not relate to the different mechanisms of these two treatments for lifespan extension. Other CRMs did not have an effect on these parameters at all.

In our results, one unexpected outcome is the ineffectiveness of most CRMs on the CLS of budding yeast at the tested concentrations (Fig. 1 and Supplemental data). The lifespan-extending effects of dapsone, metformin, and curcumin have been demonstrated in other organisms including worms, flies, and rodents, but have not yet been tested in yeast. All of these CRMs failed to extend the CLS of budding yeast, which might result from the natural differences in complexity between unicellular and multicellular systems. For example, the effect on the lifespan of rodents by metformin revealed a range of variation depending on the age of onset, gender, and disease conditions (reviewed in [14]). Resveratrol also failed to extend the CLS of budding yeast in our result. Resveratrol was first reported as a potential CRM in yeast, which extended the RLS of yeast and increased the activity of Sir2 [15]. However, recent studies have questioned whether resveratrol indeed activates sirtuins or slows down the aging process [3,16]. There was a report that 4 mM spermidine extended the CLS of yeast [9]. However, 1 M spermidine did not extend CLS, as shown in our study. Although caffeine has been claimed to increase the CLS of yeast at 400 μM treatment by colony forming unit (CFU) assay [10], the actual increase in quantity was very small (increased the median survival for only 20 h) [10]. In our assay with 1 mM caffeine of PI-staining method for CLS [17], caffeine showed no effect on CLS. Our study may have failed to detect such a minuscule change in our assay.

Along with CLS, RLS has been used for the measurement of yeast lifespan. These two methods deliver different biological characteristics [18]. CLS reflects a broad spectrum of survivorship under exhausted media during a long period of time. Therefore, CLS reflects diverse aspects of cellular aging. However, RLS counts the number of progeny from a single cell until the virgin cell loses its fecundity. Therefore, RLS is a parameter for reproduction. In our results, both CR and CRMs did not extend RLS at the representative concentrations. We measured RLS for each treatment blindly on different days. Both BY4741 and BY4742 were confirmed as long-lived haploids by RLS [19]. Especially Kaeberlein et al., thoroughly tested RLS for BY4742 at different experimental trials. They showed that the mean RLS was from 23.4 to 30.4 (range = 7) at time-independent trials. Our RLS data for CR and CRMs for BY4741 showed a mean RLS from 29.3 to 38.5 (range = 9.2). Our RLS range showed a similar error range as shown by Kaeberlein et al. Although our data indicates no effects as a result of the treatments on RLS, further studies are needed, such as the optimization of treatments, because our working concentrations were determined by CLS.

In conclusion, we found that rapamycin extended the CLS of yeast via identical parameters for mitochondrial function as CR did. However, rapamycin worked on these parameters at different windows of time. This observation emphasized the differences between the mechanisms utilized for the extension of the lifespan of rapamycin and CR (Fig. 4). Further studies on these different time frames could lay a resolution for the various possible mechanisms of lifespan extension caused by these well-identified methods.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.049.

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