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Caloric restriction augments ROS defense in *S. cerevisiae*, by a Sir2p independent mechanism

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Abstract
Aging is associated with increased production of reactive oxygen species (ROS) and oxidation-induced damage to intracellular structures and membranes. Caloric restriction (CR) has been demonstrated to delay aging in a variety of species. Although the mechanisms of CR remain to be clearly elucidated, reductions in oxidative damage have been shown to increase lifespan in several model systems. Contrary to the general belief that ROS production is reduced in CR, this article provides evidence that not only oxygen consumption but ROS production is enhanced in the calorie restricted condition. To understand the biological mechanism underlying the anti aging action of CR, the role of scavenging enzymes was studied. It was found that super oxide dismutase (SOD1 and SOD2), catalase and glutathione peroxidase (GPx) all are over expressed in CR. We further investigated the role of Sir2, a potential effector of CR response in the activation of scavenging enzymes. No marked difference was found in CR mediated activation of SOD and catalase in the absence of Sir2. Our results suggest that in CR scavenging enzymes are activated by a Sir2 independent manner.

Keywords: *Caloric restriction, ROS, yeast, SOD, catalase, Sir2*

Abbreviations: *YPD, Yeast extract Peptone Dextrose, OD, Optical Density, NR, Non restricted, CR, Caloric restrited, DCFDA, Dichloro fluorescein di acetate, NBT, Nitro Blue Tetrazolium, ROS, Reactive Oxygen Species, SOD, Superoxide Dismutase, GPx, Glutathione Peroxidase*

Introduction

The cause of aging process has been a subject of considerable speculation, however, all the different theories of aging also have some commonalities; cells are programmed with a discrete and finite life expectancy, with age cells lose their ability to replenish, accumulate damage and die. We are only beginning to understand the molecular mechanisms that mediate life-span extension however, the similarities between longevity regulatory pathways in organisms ranging from yeast to mammals suggest a conserved molecular strategy in all eukaryotes. The existence of conserved pathway(s) that increase resistance to damage and postpone aging indicates that it may be possible to prevent ageing related diseases by impeding the pathway(s) of cellular damage and aging.[1] The genetic basis of aging points the role of specific genes in the aging mechanism. Changes in the expression of specific genes may directly contribute to the aging process.[2,3] Studies in yeast suggest that inactivation of cAMP dependent PKA signalling pathways or limiting glucose concentration extends lifespan and alters expression of many genes.[4,5] Some of the longevity mutants identified in *C. elegans* and *Drosophila*, are related to insulin like signalling pathways. These genes regulate life span co-ordinately with reproduction, metabolism and free radical scavenging

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gene regulation, e.g. inactivation of insulin/IGF like daf2 pathway in *C. elegans* extends survival and increases thermo-tolerance and antioxidant defenses, by activating stress resistance transcription factor DAF16.[6] Longevity mutations in yeast like *sch9*, *ras2* and *cyr1* postpone super-oxide toxicity and mitochondrial damage.[7] These data indicate the possibility of interconnectivity between metabolism, gene expression, stress management and lifespan.

The phenomenon "caloric restriction" (CR) (a diet in which calories are limited by 30–40% compared with animals fed *ad libitum*) increases life span in a variety of species and has been the focus of much interest lately. CR increases life span in a variety of species including mammals. The extension in lifespan is in striking similarity to the effect of CR on aging in model organisms as *C. elegans*, *Drosophila* and yeast.[8,9] One of the earliest changes observed on the imposition of CR is a reduction in blood glucose levels. This and other features of CR have led to the suggestion that it depends on a change in the way glucose is metabolized.[10] Glucose levels call be readily manipulated in the growth medium of yeasts. It is known that glucose sensitive yeasts like *S. cerevisiae* are capable of fermentation even under aerobic conditions. When glucose level is high, fermentation is preferred[11,12] while at low levels yeast cells switch from fermentation to aerobic metabolism or respiration.[13] Shift from fermentation to respiration depending upon the nutrient availability is unique to yeast and does not happen in mammals. However, enhancement of longevity in yeast under glucose limited condition is not simply due to glucose derepression because of the following reasons. Low glucose media has been extensively explored independently by several groups (namely Michael Jazwinski and Leonard Guarente) to mimic CR. It has been found that extension of life span is proportional to the reduction of glucose (in the range of 2–0.1%)[14,15] in rich broth as well as in chemically defined medium. Moreover, enhancement of longevity is independent of specific nutrient as life extension is also obtained when amino acids concentration are lowered keeping glucose levels at 2%.[14] So it is reasonable to propose that glucose restriction in yeast closely resembles CR in mammals.

Following events have been observed in aging mammals, increase in free radical production, damage accumulation in DNA with age, progressive breakdown in accuracy of protein synthesis, cross linkage of macromolecules and hence reduction of functionality.[16] All of these events could be connected to increase in free radical generation and associated damage. Hence, the latest hypothesis considers free radical generation to be largely responsible for progressive aging. Oxidative damages are reduced in CR animals.[17] Although the mechanism is not clear, one hypothesis is that CR slows down metabolism, thereby decreasing the production of toxic by-products, mainly reactive oxygen species (ROS) and, in turn, decelerates aging.[8,18] If CR slows down metabolism, the production of ROS would decrease as a simple consequence. However, in caloric restricted organism metabolic rate does not slow down[19] and oxygen consumption (indirect estimation of metabolic rate) actually increases in caloric restricted yeast.[5] Since the experimental data does not support an overall reduction in metabolic rate, CR must be affecting some other existing cellular process(s) to manage oxidative stress. It has been hypothesized that CR might augment cellular processes by altering gene expression.[9,20] One possibility may be that under CR, electrons flow through the respiratory chain more efficiently and thus as a consequence reduces the production of ROS.[21–23] The other possibility is CR cells may posses an enhanced ability to detoxify ROS that slows oxidative damage in CR. Both the possibilities are consistent with oxidative damage theory and aging; however, it has not been validated experimentally. Hence in this report, we examined in detail the effect of CR on free radical generation and scavenging enzyme activity in yeast *S. cerevisiae*.

Materials and methods

Yeast strain and culture

The following strains were used in this study; CCFY100 (W303-1a *MATa ade2-1 ura3-1 trpl-289 leu2-3, 112 his3-11, 15 can1-100*),[24] JRY 4565 (W303-1b *MATa; ade2-1 ura3-1 trpl-289 leu2-3, 112 his3-11, 15 can1-100*).[25] Strains were grown at 30°C with shaking at 200 rpm in YPD-NR (non calorie restricted; 1% yeast extract, 2% peptone, 2% dextrose media), YPD-CR (calorie restricted; 1% yeast extract, 2% peptone, 0.5% dextrose media), YC-NR (non calorie restricted, yeast complete synthetic media containing yeast nitrogen base w/o amino acids 0.67%, succinic acid 1.0%, sodium hydroxide 0.6%, complete amino acid mix 0.125% with 2% dextrose), or YC-CR (calorie restricted, yeast complete synthetic media as in YC-NR with 0.5% dextrose), as specified appropriately. Selection plates for silencing assay (YC-Trp, YC + Can and FOA plates) were prepared as described in Roy and Runge 2000.[24]

Oxygen consumption and ROS assay

For oxygen consumption experiments, cells from the same colony were streaked on YPD-NR and YPD-CR plates. A single well grown colony from each plate was inoculated in 5 ml of YPD-CR and YPD-NR, respectively. An overnight culture was re-inoculated in 20 ml each of fresh YPD-CR and YPD-NR media at optical density 0.02–0.03 and grown till optical density 0.7–0.8 at 610 nm. Cells 10^7 were harvested,

washed and resuspended in 500 μl of fresh media. Oxygen consumption was measured starting at the point immediately after addition of the 500 μl sample to fresh media; using a polarographic oxygen electrode and readings recorded every 30 s. Results are reported as percent oxygen (O_2) consumed per minute. A blank reading was taken of simulated inoculated media. ROS produced per 10^7 cells was measured by measuring the amount of 2′,7′-dichloro fluorescin (DCF) present in the cells and dichlorofluorescin diacetate (DCFDA) assay.[26] Fluorescence was determined with a Perkin Elmer LS 50B fluorescence spectrometer with λ_{ex} 488 nm and λ_{em} 540 nm and excitation and emission slit widths 5 min and 10 min, respectively. Time dependent scans with an interval of 5 min were taken for a total time of 60 min. The standard curve for the calculation of DCF was prepared. To convert DCFDA to DCF, 0.5 ml of 1 mM DCFDA in absolute ethanol was added to 2 ml of 0.01 N NaOH and allowed to stand at room temperature for 30 min to give dihydro dichlorofluorescin (DCFH). The hydrolysate was then neutralized with 10 ml of 25 mM sodium phosphate buffer and stored on ice. This solution was discarded each day after use.[27,28] Each reaction mix contained 500 μl of this DCFH and increasing concentrations of 30% hydrogen peroxide (H_2O_2), and was incubated at 50°C for 90 min. Blank reactions were run in parallel without H_2O_2 and subtracted from those with H_2O_2. Concentration was found by extinction coefficient 91,000 $cm^{-1} M^{-1}$ at 502 nm. Fluorescence intensity of increasing concentration dilutions was measured. A control reaction containing 500 μl PBS with 5 μl of 1 mM DCFDA was set up to control for spontaneous air oxidation of DCFDA.

Assays for oxidative stress sensitivity and gene silencing

Cells from same single colony of master plate were streaked onto YC plates containing either 2% (NR plates) or 0.5% (CR plates) glucose. Three freshly grown colony (approximately 10^6–10^7 cells) were picked up with a sterile Pasteur pipette from both NR and CR plates. The colonies were resuspended separately in 1 ml water in microfuge tubes. Ten fold serial dilutions were made in the subsequent micro tubes. A total of 5 μl of each dilution (cell suspension) was applied onto the selection plates (YC, YC + 1 mM H_2O_2 or 1 mM Paraquat) to measure oxidative stress sensitivity and (YC, YC-Trp, YC + Can and FOA plates) to measure silencing.

Assays for scavenging enzymes

Glass bead homogenization was used for preparing cell lysates for GPx and SOD enzyme activity assays. The lysis buffer contained 3.35 mM sodium dihydrogen phosphate, 50 mM disodium hydrogen phosphate, 47.8 mM sodium chloride, 5 mM potassium chloride, 61 mM glucose, 0.1% TritonX-100 and 0.1 mM EDTA. For catalase assay 0.1% TritonX-100 and 0.1 mM EDTA were omitted from lysis buffer. For SOD activity, extracts were resolved on non-denaturating 12% polyacrylamide gel and stained by nitro blue tetrazolium (NBT). The gel was first soaked in 25 ml of 3 mM NBT for 15 min, then washed briefly and then soaked in dark in 30 ml of 100 mM potassium phosphate buffer pH 7.0, 30 mM TEMED and 28 μM riboflavin. Further gel was washed and then illuminated on light box for 15–20 min.[29] For catalase, extracts were resolved on non-denaturing 10% polyacrylamide gel. Activity was determined by incubating gel for 5 min in 5% methanol. Gel was washed three times with water followed by 10 min incubation in 10 mM H_2O_2. The gel was again rinsed with water and incubated in 1:1 mixture of freshly prepared 2% potassium ferric cyanide and 2% ferric chloride. Blue colour developed in the gel except at zones where H_2O_2 was decomposed by catalase. The gel was soaked in 10% acetic acid and 5% methanol solution to stop colour development.[30] Glutathione peroxidase was estimated by DAB staining. For DAB staining, gel was incubated in solution of 50 mM sodium citrate (pH 5.5) for 30 min. After this, it was kept overnight in a solution of 50 mM sodium citrate (pH 5.5), 1 mM DAB and 0.1% H_2O_2.[31] Gels were scanned using Bio-Rad GS 800 densitometer and quantified by Bio-Rad Quantity One version 4.4 software, as per instructions in user manual. Briefly, a rectangle was drawn over CR band(s) to fully enclose the band and then same area rectangles were used for two standards (value 0 and 1) as per software requirement. First Standard is a blank area on the gel for which a minimum arbitrary value approaching zero was assigned (0.0000000001). Second standard value was the control band (NR) for which value 1 was assigned. For background subtraction same rectangle was used. The quantification analysis report was then generated by software indicating fold increase or decrease with respect to control band. Values represented are average of three gels.

Results

In earlier studies, it has been shown that CR can be imposed in yeast by reducing the glucose concentration in the media from 2 to 0.5%. As cells continue to feed on yeast extract plus peptone, which are rich in amino acids, nucleotides and vitamins, the growth rate remains unaffected by low amount of glucose in the medium.[32] We have not observed any change in growth rate of CCFY100 strain grown in YPD-CR and YPD-NR medium over a period of 48 h (data not shown). However, Figure 1a shows more than two

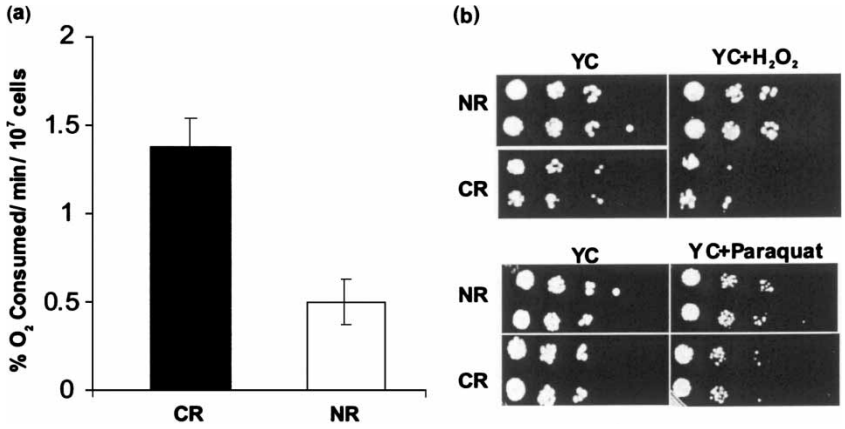

Figure 1. Caloric restricted cells consume more oxygen and are hypersensitive to oxidants. (a) Percentage of oxygen consumed by 10^7 cells in calorie restricted and non restricted media. (b) Spot assays for sensitivity to oxidants. Representative colonies from three or more assays are shown. Ten-fold serial dilutions of single yeast colonies were spotted onto different media to monitor sensitivity. YC shows the total number of cells spotted and the other media (YC + 1 mM hydrogen peroxide and YC + 1 mM Paraquat) show the extent of sensitivity. The cells were incubated at 30°C for 3 days.

folds increase in oxygen consumption under CR as compared to the NR state. We hypothesize that since the total number of oxygen molecules consumed by the cells per unit time is higher in case of CR grown cells than to NR, the amount of ROS generated might be more in CR grown cells. In that case, CR cells might be more sensitive to external oxidative stress. Because at a given point of time total amount of ROS (internal + external) will be more in CR grown cells. Plate assay was conducted to measure the CR and NR grown cells sensitivity to external oxidative stress using 1 mM H_2O_2 and 1 mM Paraquat. Results indicate that CR grown cells were more sensitive to external oxidative stress as compared to NR cells (Figure 1b). To quantify the generation of internal ROS, the DCF-DA based ROS assay was performed. However, DCFH in this assay can also be oxidized by cellular oxidants other then $O_2^{\bullet -}$. Hence, intracellular fluorescence due to DCFH oxidation has been generally considered as a representation of total cellular free radical concentration (reviewed in Ref. [33]). Since oxygen consumption is increased in CR we, therefore, consider that florescence generated by DCFDA oxidation is predominantly by ROS. ROS generation was measured for cells in control vs. calorie-restricted states, at three different time points, *viz* the early log, mid log, and the late log phases. It was found that the ROS generation was consistently higher in cells grown in the calorie restricted state and also found to be more pronounced in the mid and late log phases, as shown in Figure 2. The values provided are an average of results from at least three sets of independent experiments carried out on different days. Thus, from these sets of experiments, it was clear that yeast cells consumed more oxygen in a calorie restricted stage, and also produced more of the deleterious oxygen molecules. But calorie restriction has undoubtedly been a model for delayed aging in higher animals, and this has also been true for yeast. The question remains whether/how the caloric restricted cells are better capable of handling and getting rid of ROS molecules? The study was further extended to see the effect of CR on scavenging enzymes activity.

ROS are generated during the process of oxygen reduction. To maintain redox homeostasis, multiple enzyme defense system is present in the cells. As first line of defense, antioxidant enzymes that detoxify ROS are superoxide dismutase (SOD) and catalase. The second line of defense system includes reducing enzyme system glutathione peroxidase (GPx). thioredoxin (Trx) and glutaredoxin (Grx). Glutathione (GSH) reduces peroxides in the presence of glutathione peroxidase (GPx) and gets oxidized to GSSG. GSH is enzymatically restored by NADPH-dependent reduction of GSSG by GSH reductase (Figure 3a). Activity assay of the endogenous antioxidant enzymes related to the scavenging enzymes, SOD, catalase and GPx were assessed to ascertain the status of the enzymes in caloric restricted condition. For this, in-gel activity assay was performed for SOD, catalase and GPx. SOD was found to be over expressed in CR induced cells as compared to the non-restricted state of cells as shown in Figure 3b. The two forms of mitochondrial SOD (SOD 2) were found to be 1.11 ± 0.03 and 3.5 ± 0.74 folds overexpressed while the cytosolic SOD (SOD1) was found to be overexpressed by approximately 1.17 ± 0.04 folds. An in-gel assay for catalase showed marked difference in catalase activity in CR condition, as compared to NR. As shown in Figure 3b, CR grown cells showed two bands for catalase activity, as compared to only one in the non-restricted sample. A search through the *S. cerevisiae* genome database revealed that the yeast genome encodes two gene products of catalase, *CTA1* and *CTT1*, differing slightly in their molecular weights. The additional catalase activity band seen in

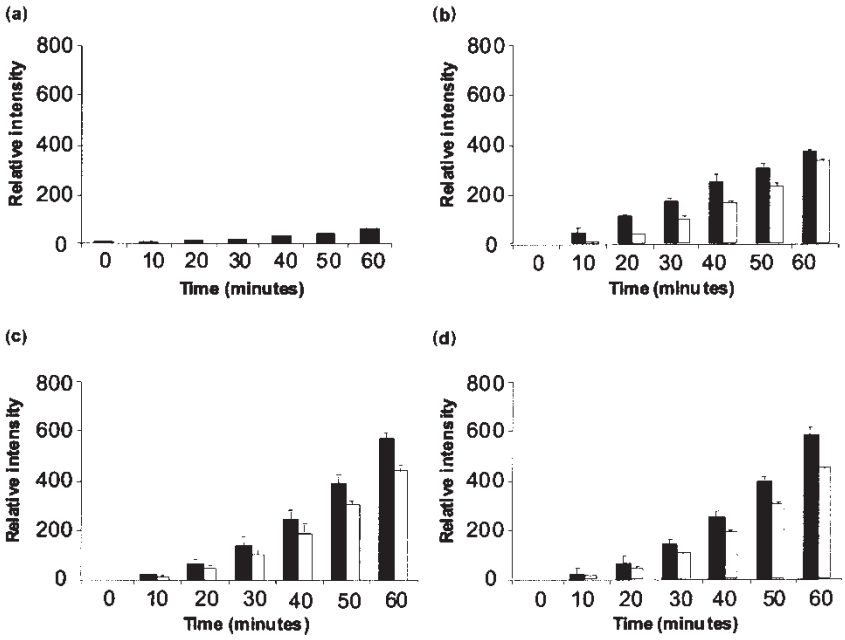

Figure 2. Caloric restricted cells have higher levels of intracellular ROS. Production of ROS expressed as relative intensity. (a) Air oxidation of DCF DA as a control. Production of ROS in calorie restricted (■ CR) and non restricted (□ NR) conditions during (b) early log, (c) mid log and (d) late log phases of cells growth in respective media.

case of CR cells corresponds to higher molecular weight *CTT1* that is expressed in the cellular response to stress.[34] GPx activity was tested by an enzymatic in gel assay as shown in Figure 3b and GPx was found to be over expressed by 1.45 ± 0.17 folds.

To understand the mechanism underlying activation of scavenging enzyme by CR following studies were performed. A "triple silencer" strain was used which can monitor increase or decrease in gene expression at rDNA as well as HM loci and telomeres.[24] We have measured the gene silencing at all three loci in CR and NR condition. Complete medium (YC) shows the total number of cells spotted on each plate; -Trp, medium selecting for *TRP1* expression in *hmrΔE* :: *TRP1* (where more growth means less silencing at this locus); +Can, medium selecting against *CAN1* expression (that is, for *CAN1* silencing in the rDNA where more growth means more silencing); and +FOA, medium selecting against *URA3* expression (that is, for *URA3* silencing at the chromosome VR telomere where more growth means more silencing). Results obtained from this set of experiments are shown in Figure 4a, it was found that in calorie restricted condition, the cells showed a marked increase in silencing at the *HM* and rDNA loci, while no change was apparent at the telomere locus.

Sir2 is the only factor that is indispensable for silencing at all three silent regions.[35] To find out whether Sir2

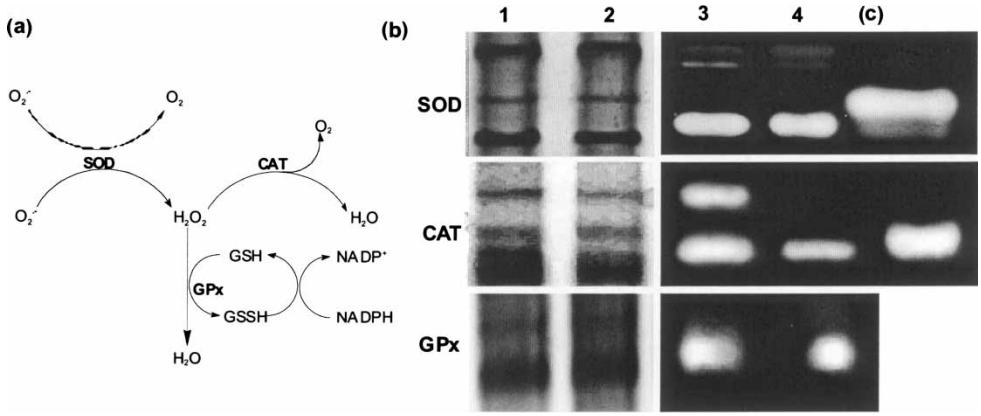

Figure 3. Cellular redox system in yeast and activity assay of the endogenous antioxidant enzymes. (a) A schematic diagram of SOD, catalase and GPx's involvement in maintaining cellular redox homeostasis. (b) For activity assay 50 ug of total protein were loaded in all the lanes. Lanes 1,3, are loaded with protein samples from CR grown cells; lanes 2,4, are loaded with protein samples from NR grown cells. In SOD and CAT activity gel lane C is loaded with 1 unit of bovine SOD (Sigma) and 3 units of catalase (Sigma) as a control, respectively. Lanes 1 and 2 are stained with coomassie brilliant blue, while lanes 3–5 are tested for respective enzyme activity.

play any role in CR mediated activation of scavenging enzymes, in-gel activity assay for SOD and catalase was carried out in Sir2 deleted strain in caloric restricted and non-restricted condition. As shown in Figure 4c two mitochondrial SOD (SOD2) were found to be 1.62 ± 0.14 and 2.44 ± 0.27 folds overexpressed while the cytosolic SOD (SOD1) was 1.25 ± 0.09 folds over-expressed. Similarly, there was no alteration of catalase expression in the absence of Sir2 compare to wild type (Figures 3b and 4c). We also measured the oxygen consumption of sir2 deleted strain (JRY 4565) in CR and NR conditions. Oxygen consumption is increased in sir2 deleted strain as compared to CCFY. However, oxygen consumption in CR grown cells are significantly higher than NR grown cells as shown in Figure 4b.

Discussion

In general calorie-restricted state exhibits a delayed aging phenotype, which is better capable of handling oxygen stress. According to the leading theory, one should expect a hypometabolic state in yeast cells under CR conditions[8,9] similar to the findings of life-span extension of Clk mutants of *C. elegans*. On the contrary, under CR conditions the rate of oxygen consumption was found to be elevated by 2 folds. This suggested that since respiration is higher in case of CR grown cells, so should be the amount of ROS. However, the "free radical generation and damage" theory of aging hypothesize that aging is a result of cumulative damage of cellular macromolecules owing to the production of ROS. Further, calorie restricted state may show delayed aging due to reduced formation of these reactive molecules. Thus it was expected that the cells grown in CR conditions should show a decrease in the ROS content. Results obtained from our experiments were in contrast to the CR and reduced ROS production hypothesis that CR functions by slowing metabolism and thereby slowing the generation of ROS.[8,9,16]

Thus, from this study, it was clear that yeast cells consumed more oxygen in a CR stage, and also produced more of the deleterious oxygen molecules. Then, how cells are getting rid of the ROS molecules? In normal cells, there is a balance between the production and scavenging of ROS. Oxidative stress occurs when the rate of scavenging is less then production of ROS and rate of cellular antioxidant depletion exceeds the rate of replenishment for sufficient duration. With age anti-oxidant replenishment and scavenging mechanism slows down causing ROS accumulation and damage. SOD, catalase and GPx play a central role in defending against oxidative damage.[18] These enzymes work by removing superoxide radicals, H_2O_2 and organic hydroperoxides. It has been reported previously that

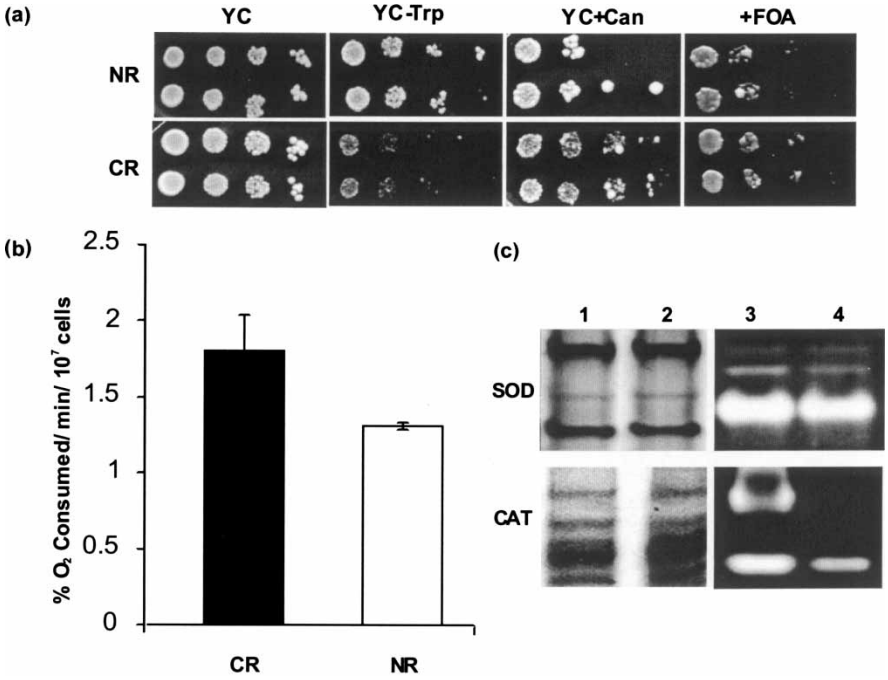

Figure 4. Caloric restriction alters silencing and augments SOD and catalase activity in the absence of Sir2. (a) Spot assays for silencing at HMR, rDNA and telomeres. Ten-fold serial dilutions of single yeast colonies were spotted onto different media to monitor silencing. Representative colonies from three or more assays are shown. YC shows the total number of cells spotted and the other media (-Trp, YC lacking tryptophan, +Can canavanine medium lacking arginine and FOA, 5-fluoro-orotic acid medium) show the extent of silencing. The cells were incubated at 30°C for 3 days. (b) Percentage of oxygen consumed by 10^7 sir2 deleted (JRY 4565) cells in calorie restricted and non restricted media (c) SOD and catalase activity in sir2 deleted strain. A total of 50 ug protein were loaded in all the lanes. Lanes 1,3, are loaded with protein samples from CR grown cells; lanes 2,4, are loaded with protein samples from NR grown cells. Lanes1 and 2 are stained with coomassie brilliant blue, while lanes 3–5 are tested for respective enzyme activity.

under CR SOD and catalase gene expression as well as activity is upregulated in rat liver.[36,37] However, no reports were found in literature about the upregulation of SOD, catalase and GPx in relation to CR in yeast cells.

SOD1 is cytosolic, and SOD2 is an intra mitochondrial, free radical scavenging enzyme that dismutates ROS to H_2O_2 and molecular oxygen and is the first line of defense against accumulation of superoxides produced as a by product of oxidative phosphorylation. Removal of the superoxide radical by SOD1/ SOD2 and H_2O_2 by GPx prevents formation of reactive hydroxyl radicals, which are postulated to be responsible for oxidative cellular injury. Data from this study suggest that under a caloric state there was an inherent increased activity of both the SOD enzymes, as a mechanism to cope up with extra ROS produced. This also suggests that oxygen free radicals may act as second messengers to regulate SOD activity. The additional catalase activity band seen in case of CR cells could be the higher molecular weight *CTT1* that is involved in the cellular response to stress. It has been reported that transcription of the *CTT1* gene of *S. cerevisiae* is controlled by oxygen via heme, by nutrients via cAMP and by heat shock.[34,38] *CTT1* is only induced to all appreciable extent when at least two of the three factors contributing to its expression activate the *CTT1* promoter.[34] Thus, in the calorie restricted stage, since the oxygen stress is more and the nutrients are scarce this over expression of *CTT1* gene is seen.

CR mediates life span extension. However, the hypothesis that the CR reduces the production of ROS does not hold true, at least for yeast. Rather, ROS production actually increases under CR and so does the oxygen consumption. From the findings of this study, we propose that one of the anti aging mechanism(s) of CR works by augmenting the activity of endogenous system for protection against the increased free radical production. Lin *et al.*, (2002) reported increased oxygen consumption and speculated about higher ROS production in caloric restricted yeast, however, they did not find any increase in expression of SOD or catalase genes in micro array experiments. SOD1, SOD2, CTA1, CTT1 induced only 1.0, − 1.1, 1.0 and 1.2, respectively. This data suggests higher activity of SODs or catalases (this study) are probably not because of change in regulation at transcriptional level. It could be due to alteration in post transcriptional level rather than transcriptional level. However, more detailed experiments are needed to establish the fact conclusively.

Many other genes are also expressed differentially by CR.[5,20] Identification of mechanism(s) for differential expression will help in better understanding of the pathways involved in CR mediated enhancement of longevity. Silencing in *S. cerevisiae* is a chromatin-mediated alteration of gene expression within specific chromosomal domains. These domains include telomeres, the silent mating type loci *HMR* and *HML*, and the ribosomal RNA (rDNA) gene array. There is a strong correlation between increased rDNA silencing and life span.[39] SIR2 encodes a component of the Sir2–Sir3–Sir4 silencing complex that catalyses the formation of heterochromatin. Sir2 is a limiting component of yeast longevity.[40] The role of Sir2 in longevity regulation appears to be conserved.[40] Multiple lines of evidence indicate Sir2 as a key protein in yeast lifespan regulation. Sir2 homologues are found in a wide array of organisms, ranging from bacteria to humans, and increased dosage of the Sir2 homologue, sir2.1, can extend the life span of the nematode *C. elegans*.[41] Metabolic shift activates sir2.1 which in turn activates the *C. elegans* forkhead transcription factor DAF-16.[42] DAF-16 and its mammalian homologue FOXO3a are involved in the transcription of oxidative stress genes. Previous work in *S. cerevisiae* has shown that metabolic shifts alter gene silencing.[42] Deletion of sir2 prevents lifespan extention and CR enhances silencing at rDNA locus in wild type cells.[5] Authors concluded Sir2p deacetylase activity enhanced by CR. However, they have not ruled out the possibility of relocalization of silencing complex from telomere to internal locus. Triple silencer strain can monitor relocation of silencing complex, if it happens.[24] In calorie restricted condition, the cells showed a marked alteration in silencing at the *HM* and rDNA loci without decreasing the silencing at telomeres. This result (Figure 4a), rule out the possibility of redistribution of silencing complex from telomere to internal locus. Increase in silencing at HMR and rDNA are probably by the same mechanism like *C. elegans*, activating Sir2 enzymatic activity. Our study shows that, oxygen consumption is increased in sir2 deleted strain as compared to CCFY. Increased oxygen consumption in NR grown cells could be due to the changes in certain physiological processes affected by sir2 deletion. However, oxygen consumption of sir2 deleted strain in CR is significantly increased compared to NR control. This increase indicates that CR mediated increase in oxygen consumption does not depend upon Sir2 activity. Consequently, activation of SOD catalase and GPx are also independent of the enhanced Sir2 activity.

In conclusion, our results are in contrast to the popular hypothesis that CR functions by slowing down metabolism and decreasing ROS generation. On the contrary, it shows in caloric restricted condition cells consume more oxygen and subsequently produce more free radical. To balance the situation some unknown mediator augments expression of scavenging enzymes. The enhanced level of cellular scavengers, annul the free radicals produced and reduce the cellular damage resulting in life span extension as compared to non restricted conditions. Our results provide evidence for a Sir2 independent augmentation of ROS defense mechanism under CR.

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