Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H_2O_2 and superoxide dismutase activity

Ana Mesquita[a], Martin Weinberger[b], Alexandra Silva[a], Belém Sampaio-Marques[a], Bruno Almeida[a], Cecília Leão[a], Vítor Costa[c,d], Fernando Rodrigues[a], William C. Burhans[b,1], and Paula Ludovico[a,1]

[a]Instituto de Investigação em Ciências da Vida e Saúde (ICVS), Escola de Ciências da Saúde, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal; [b]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY 14263; [c]Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, 4150-180 Porto, Portugal; and [d]Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Departamento de Biologia Molecular, Universidade do Porto, 4099-003 Porto, Portugal

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The free radical theory of aging posits oxidative damage to macromolecules as a primary determinant of lifespan. Recent studies challenge this theory by demonstrating that in some cases, longevity is enhanced by inactivation of oxidative stress defenses or is correlated with increased, rather than decreased reactive oxygen species and oxidative damage. Here we show that, in *Saccharomyces cerevisiae*, caloric restriction or inactivation of catalases extends chronological lifespan by inducing elevated levels of the reactive oxygen species hydrogen peroxide, which activate superoxide dismutases that inhibit the accumulation of superoxide anions. Increased hydrogen peroxide in catalase-deficient cells extends chronological lifespan despite parallel increases in oxidative damage. These findings establish a role for hormesis effects of hydrogen peroxide in promoting longevity that have broad implications for understanding aging and age-related diseases.

aging | hydrogen peroxide | hormesis | antioxidant enzymes | oxidative damage

The longstanding free radical theory has guided investigations into the causes and consequences of aging for more than 50 y (1). However, the results of a number of recent studies have failed to provide support for the free radical theory or suggest that this theory is at best incomplete (2). Studies of naked mole rats, for example, demonstrated that this extremely long-lived rodent exhibits high levels of oxidative damage compared with mice or rats, whose lifespans are ≈1/10 that of naked mole rats (3). In addition, caloric restriction (CR), which extends the lifespans of a variety of eukaryotic organisms, promotes longevity in *Caenorhabditis elegans* by a mechanism that involves increased oxidative stress (4). In fact, in contrast to the destructive effects of reactive oxygen species (ROS), recent evidence indicates that in mammals, hydrogen peroxide (H_2O_2) and other forms of ROS function as essential secondary messengers in the regulation of a variety of physiological processes (reviewed in ref. 5). For example, H_2O_2 activates pro-survival signaling pathways mediated by p53, NF-κB, AP-1, and other molecules (6). Furthermore, increases in the intracellular steady-state production of H_2O_2 by *SOD2* overexpression can block the activation of cellular processes required for programmed cell death (7). However, a causal relationship between CR and effects on oxidative stress has been difficult to establish.

To better understand how CR impacts oxidative stress and longevity in the model organism *Saccharomyces cerevisiae*, in this study we examined intracellular levels of H_2O_2 and superoxide anions (O_2^-), which are two forms of ROS implicated in aging in all eukaryotes, under CR and other conditions. Our findings indicate that CR or inactivation of catalases extends chronological lifespan (CLS) by inducing elevated levels of H_2O_2, which activate superoxide dismutases that inhibit the accumulation of O_2^-. These findings establish a role for hormesis effects of H_2O_2 in promoting longevity induced by CR conditions that are likely to be conserved in complex eukaryotes. In catalase-deficient cells, increased H_2O_2 extends CLS despite parallel increases in oxidative damage. This violates a fundamental tenet of the free radical theory that posits oxidative damage as a primary determinant of aging.

Results

Caloric Restriction or Inactivation of Catalases Extends *S. cerevisiae* Chronological Lifespan by Increasing Intracellular Levels of H_2O_2. CLS of budding yeast is determined by measuring the survival of non-dividing stationary phase cells, which is impacted by conserved factors that affect aging of postmitotic cells of complex eukaryotes, including humans (8). As reported previously (9), CR by decreasing the concentration of glucose extended CLS of budding yeast (Fig. 1*A*). Surprisingly, however, the CLS-extending effects of CR were accompanied by an increase in the fraction of cells containing high levels of intracellular ROS, detected by staining cells with dihydrorhodamine 123 (DHR) (Fig. 1*B* and Fig. S1). CR-induced increases in ROS were also detected by staining cells with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Fig. S2). CR also induced the activity of the peroxisomal catalase Cta1p, as well as the cytosolic catalase Ctt1p (Fig. S3), which are the two main H_2O_2 scavenging enzymes in this organism. However, increased catalase activity did not contribute to the CLS-extending effects of CR, because CLS was also extended by mutational inactivation of *CTA1* and the longer CLS of Δ*cta1* cells was extended further by CR (Fig. 1*C*). Similar results were obtained upon mutational inactivation of *CTT1* or pharmacological inhibition of the synthesis of glutathione, which scavenges H_2O_2 (Fig. S4). These findings identify H_2O_2 as a form of intracellular ROS induced by CR in stationary phase budding yeast cells and establish that catalase activity or glutathione synthesis inhibits longevity in the chronological aging model.

The longer CLS of Δ*cta1* cells was accompanied by an increased fraction of cells containing high levels of intracellular H_2O_2 under non-CR conditions (Fig. 1 *C* and *D*). Under CR conditions, the fraction of Δ*cta1* cells exhibiting high intracellular levels of H_2O_2 was similar to CR wild-type cells or non-CR Δ*cta1* cells at day 6 and lower at day 12 (Fig. 1*D*). Increased levels of H_2O_2 were also detected in Δ*ctt1* and in Δ*cta1*Δ*ctt1* compared with wild-type cells

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[1]To whom correspondence may be addressed. E-mail: wburhans@buffalo.edu or pludovico@ecsaude.uminho.pt.

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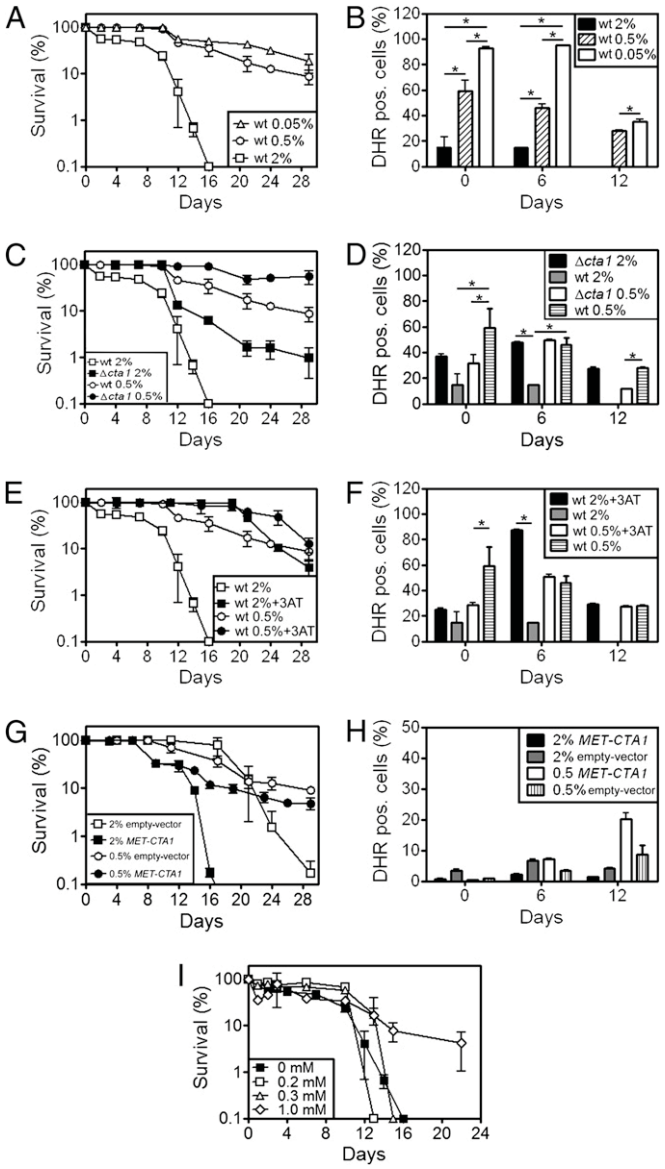

Fig. 1. Caloric restriction (CR) or inactivation of catalases extends *Saccharomyces cerevisiae* chronological lifespan by increasing intracellular levels of H_2O_2. Survival of (*A*) wild-type (BY4742) cells, (*C*) Δ*cta1* cells, (*E*) wild-type cells in the absence or presence of the catalase inhibitor 10 mM 3-amino-1,2,4-triazole (3AT), and (*G*) wild-type cells transformed with an empty vector or a plasmid that overexpresses *CTA1* (*"MET-CTA1"*). (*I*) Effects of ectopic exposure of wild-type cells to indicated concentrations of H_2O_2. Cell viability was measured at 2- to 3-d intervals beginning the day cultures achieved stationary phase (day 0) and is expressed as % survival compared with survival at day 0 (100%). Percentage of cells exhibiting high levels of intracellular ROS detected by FACS measurements of fluorescence of the probe dihydrorhodamine 123 (DHR) in (*B*) wild-type cells, (*D*) Δ*cta1* cells, (*F*) wild-type cells in the absence or presence of 3AT, and (*H*) wild-type cells transformed with an empty vector or a plasmid that overexpresses *CTA1* (*"MET-CTA1"*). Three to five biological replicas of each experiment were performed. Survival and DHR positive cell values are mean ± SD or mean ± SEM, respectively, in all experiments. Statistical significance (*$P < 0.05$) was determined by Student's *t*-test.

(Fig. S5). Pharmacological inactivation of catalases by 3-amino-1,2,4-triazole (3AT) also extended CLS, and CR extended the CLS of 3AT-treated cells even further (Fig. 1*E*). Treatment with 3AT also increased the fraction of cells containing high levels of intracellular H_2O_2 (Fig. 1*F*). However, CR did not increase the fraction of 3AT-treated cells containing high intracellular levels of H_2O_2 further (Fig. 1*F*). Overexpression of *CTA1* had effects opposite to those associated with inactivation of catalases—it decreased CLS in non-CR and CR cells (Fig. 1*G*) and reduced the fraction of cells containing high levels of intracellular H_2O_2 (Fig. 1*H*). However, the fraction of cells containing high intracellular levels of H_2O_2 was also reduced (albeit to a lesser extent) in cells harboring an empty vector. This may reflect effects of the different medium required to maintain plasmids in these but not other experiments.

These observations suggest that H_2O_2 promotes longevity in the budding yeast CLS model. To address this possibility directly, we asked whether ectopic application of H_2O_2 over a range of concentrations from 0 to 1 mM would extend CLS of non-CR wild-type cells. The lowest H_2O_2 concentrations tested did not alter CLS compared with cells that were not exposed to H_2O_2 (Fig. 1*I*). In contrast, exposure to 1 mM H_2O_2 resulted in a significant increase in longevity (Fig. 1*I*). Overall these results indicate that H_2O_2 extends CLS of budding yeast and is an important component of the CLS-extending effects of CR.

CR is known to extend CLS by inhibiting the accumulation of acetic acid in culture medium (10). As reported earlier, buffering medium to eliminate acetic acid also extends CLS (10, 11). However, CR increased intracellular H_2O_2 in experiments performed in buffered medium (Fig. S6). This suggests that some of the CLS-extending effects of CR occur independently of reduced levels of acetic acid in medium. Furthermore, the CLS-extending effects of increased H_2O_2 detected in catalase mutants occurred in the absence of a change in medium pH compared with cultures of wild-type cells (Table S1). This establishes that the effects of increased H_2O_2 on CLS can occur independently of changes in levels of acetic acid in culture medium.

Elevated H_2O_2 Levels Induced by CR or Inactivation of Catalases Are Accompanied by a Reduction in Levels of Superoxide Anions. To determine whether CR or other experimental manipulations also increase levels of superoxide anions, we measured O_2^- levels in stationary phase cells using dihydroethidium (DHE), which can detect O_2^- (12). In non-CR conditions, O_2^- levels increased in wild-type cells from day 0 to day 3 of stationary phase, whereas H_2O_2 levels remained unchanged (Fig. 2*A*). However, CR caused a significant reduction in O_2^- levels compared with levels in non-CR cells, despite a pronounced increase in H_2O_2 in the same cells (Fig. 2*A*). Similar to the effects of CR in wild-type cells, O_2^- levels were decreased and H_2O_2 levels were increased in Δ*cta1* compared with wild-type cells at day 0 and day 3 of stationary phase (Fig. 2*A*). O_2^- levels in stationary phase Δ*cta1* cells were also reduced by CR conditions, comparable to the effects of CR on stationary phase wild-type cells (Fig. 2*A*). The reduction in O_2^- levels in CR Δ*cta1* cells was accompanied by an increase in H_2O_2 at day 0 (Fig. 2*A*). Treatment of wild-type cells with 3AT or inactivation of both catalases (Δ*cta1ctt1* cells) also reduced O_2^- levels at the same time that it increased intracellular H_2O_2 in the same cells (Fig. 2*B* and Fig. S5*B*). These findings suggest that the longevity-promoting effects of intracellular H_2O_2 in CR conditions or when catalases are inactivated are related to inhibition of the accumulation of O_2^-. Although buffering medium to eliminate the effects of acetic acid also inhibits the accumulation of O_2^- in wild-type cells in stationary phase (11), CR reduced levels of O_2^- in buffered medium (Fig. S6). Therefore, CR inhibition of O_2^- accumulation is mediated by both acetic acid-dependent and -independent mechanisms.

Induction of Superoxide Dismutase Activity by Intracellular H_2O_2. Ectopic application of sublethal concentrations of H_2O_2 in budding yeast induces the transcription of genes encoding both the cytosolic Cu/Zn-dependent and the mitochondrial Mn-dependent superoxide dismutases *SOD1* and *SOD2* (13, 14) as well as an increase in levels of the corresponding proteins (13). Ectopic H_2O_2

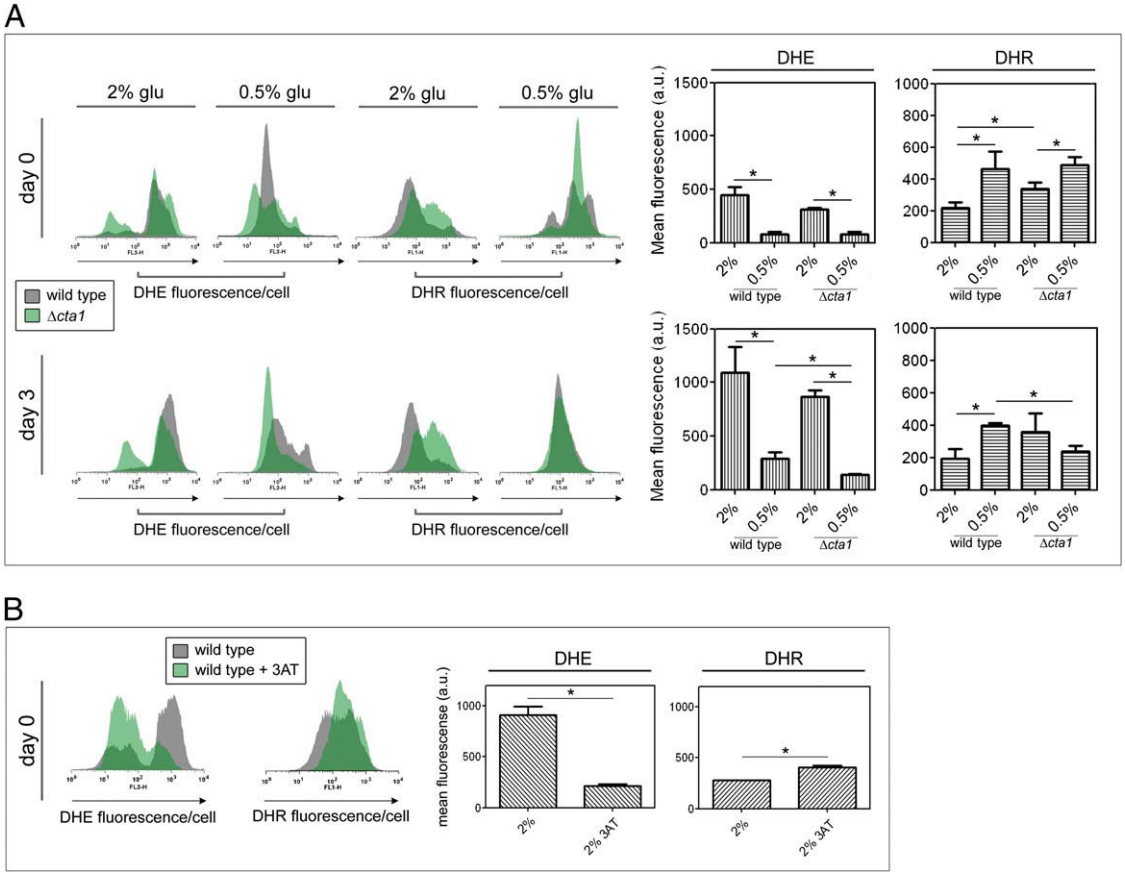

Fig. 2. The longevity-promoting effects of high intracellular H_2O_2 levels induced by CR or inactivation of catalases are accompanied by a reduction in the chronological age-dependent accumulation of superoxide anions. (*A*) FACS measurements of superoxide anions using the probe dihydroethidium (DHE) in parallel with measurements of H_2O_2 using dihydrorhodamine 123 (DHR) in wild-type (gray histograms) and Δ*cta1* (green histograms) cells at day 0 and day 3 of stationary phase. Bar graphs indicate mean ± SD fluorescence/cell (arbitrary units) measured in 25,000 cells/sample in three independent experiments. (*B*) FACS measurements of superoxide anions (DHE) and H_2O_2 (DHR) in wild-type cells in the absence (gray histograms) or presence (green histograms) of 10 mM 3-amino-1,2,4-triazole (3AT) at day 0 of stationary phase. Bar graphs indicate mean ± SD fluorescence/cell (arbitrary units) as described above. Statistical significance (*$P < 0.05$) was determined by Student's *t*-test.

also induces transcription of the superoxide dismutase sodA in *Escherichia coli* (15) and the transcription and activity of MnSOD, but not Cu/Zn-SOD in rat cells (16). To determine whether induction of Sod1p and/or Sod2p activity underlies the reduction in O_2^- levels that accompanies increases in intracellular H_2O_2, we measured the activities of Sod1p and Sod2p under CR and non-CR conditions in wild-type and *CTA1* mutant cells. CR or *CTA1* deletion resulted in minor increases in Sod1p activity at day 0 that were not detected at day 3 of stationary phase (Fig. 3 *A* and *B*). However, at day 6 both CR and deletion of *CTA1* increased the activity of Sod1p (Fig. 3 *A* and *B*). CR or deletion of *CTA1* increased the activity of Sod2p at day 0 compared with wild-type cells in non-CR conditions (Fig. 3 *A* and *B*). Larger increases in Sod2p activity were induced by CR conditions in wild-type cells at day 3 and day 6 (Fig. 3 *A* and *B*). Deletion of *CTA1* also induced large increases in Sod2p activity at day 3 and day 6 under non-CR conditions and under CR conditions at day 3 (Fig. 3 *A* and *B*). Similar observations were made in Δ*ctt1* cells at these same time points (Fig. S7). Exposure of wild-type cells to 1 mM H_2O_2 also resulted in an increase in Sod2p but not Sod1p activity (Fig. 3*C*). These findings indicate that intracellular H_2O_2 induced by CR or by inactivation of catalase activity or ectopic exposure to H_2O_2 induces superoxide dismutase activity in budding yeast, especially Sod2p activity. The more robust induction of Sod2p activity in these experiments is consistent with earlier reports that ectopic exposure to H_2O_2 induces higher levels of Sod2p compared with Sod1p (13, 16, 17).

Effects of Increased H_2O_2 Induced by Caloric Restriction or by Inactivation of *CTA1* on Oxidative Damage to Macromolecules. According to the free radical theory, oxidative damage to macromolecules is a primary factor in aging. Our observations show that under different experimental conditions, H_2O_2 levels are increased, whereas O_2^- levels are decreased in the same cells (Fig. 2). To determine the overall impact these divergent changes in different types of ROS have on oxidative damage, we examined levels of protein carbonylation, which is a form of oxidative damage, under different experimental conditions. Protein carbonylation was increased in Δ*cta1* cells compared with wild-type cells at day 0 of stationary phase (Fig. 4*A*). A similar increase in protein carbonylation was observed in stationary phase Δ*ctt1* cells at this time point (Fig. S8). CR conditions also increased protein carbonylation in wild-type cells at day 0. Nevertheless, CR conditions decreased protein carbonylation in wild-type and Δ*cta1* cells at day 6 (Fig. 4*A*).

We also measured changes in cellular autofluorescence, which is produced by global oxidative damage to proteins and lipids (18, 19). Similar to the increased protein carbonylation detected in Δ*cta1* compared with wild-type cells, autofluorescence of Δ*cta1* cells was increased at day 0 and day 3 (Fig. 4). Comparable results were obtained in Δ*ctt1* cells (Fig. S8). In contrast, CR conditions

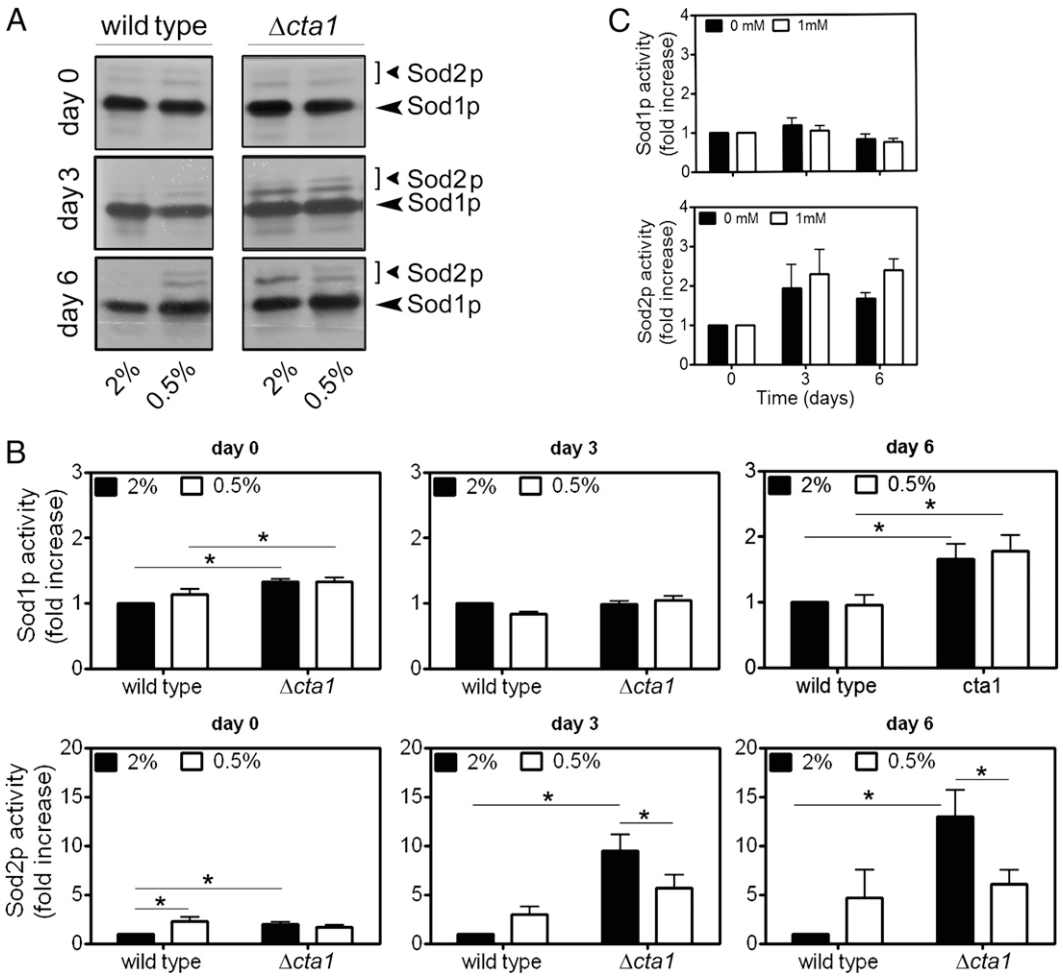

Fig. 3. Induction of superoxide dismutase activity by intracellular H_2O_2. (*A*) In situ determination of superoxide dismutase activity in stationary phase wild-type and Δ*cta1* cells measured as previously described. MnSOD (Sod2p) activity was assessed in the presence of 2 mM potassium cyanide, which inhibits Sod1p, but not Sod2p activity (Fig. S7*B*). (*B*) Quantification of fold increases in Sod1p and Sod2p activity under indicated conditions in wild-type and Δ*cta1* cells. Sod1p and Sod2p activity at each time point was normalized to activity in wild-type cells under non-CR conditions (2% glucose). (*C*) Quantification of fold increases in Sod1p and Sod2p activity in wild-type cells after ectopic exposure to 1 mM H_2O_2. Sod1p and Sod2p activity at each time point was normalized to activity in wild-type cells under non-CR conditions (2% glucose). Values indicate mean ± SEM from three independent experiments. Statistical significance (*$P < 0.05$) was determined by Student's *t*-test.

led to a reduction in autofluorescence of wild-type cells at these time points, similar to the effects of CR on protein carbonylation in wild-type cells at day 6 (Fig. 4). CR conditions also reduced the autofluorescence of Δ*cta1* (Fig. 4*B*) and Δ*ctt1* cells (Fig. S8). Together, these findings establish that CR extends CLS in parallel with a reduction in oxidative damage to macromolecules in stationary phase budding yeast cells despite the induction of higher levels of H_2O_2. In contrast, the CLS-extending effects of inactivating catalases are accompanied by parallel increases in levels of both H_2O_2 and oxidative damage, especially under non-CR conditions.

Discussion

Our study leads to the surprising conclusions that despite the induction of catalase activity by CR conditions that promote longevity, catalases are proaging factors in the budding yeast chronological aging model and both CR and catalase inactivation promote longevity in this model by inducing oxidative stress in the form of H_2O_2. Although previous studies showed that glucose restriction increases ROS that promote longevity both in budding yeast (20) and in *C. elegans* (4), specific types of ROS were not identified in these studies. In our study, the effects of genetic or pharmacological inactivation of catalases, catalase overexpression, and ectopic exposure to H_2O_2 combined with the use of probes that can distinguish between H_2O_2 and O_2^- definitively establish H_2O_2 as a longevity-promoting form of ROS induced by CR. The absence of toxic effects of H_2O_2 in these experiments reflects the well-documented dual roles of ROS in both survival and cell death pathways reported in other systems. Although H_2O_2 can induce programmed cell death (21), the intracellular concentrations that induce the beneficial hormesis-like effects in our study are likely to be far less than the concentrations that induce toxic effects. Furthermore, stationary phase cells are more resistant to H_2O_2 and other stresses compared with cells in exponential phase growth.

Our study also shows that the mechanism by which H_2O_2 exerts its prolongevity effects in budding yeast involves H_2O_2 induction of SOD activity. Hormesis theories posit that mild oxidative and other stresses induce adaptive responses that protect against further increases in stress (22). Our data identify SODs as targets of a H_2O_2-induced stress response triggered by CR that confers hormesis-like protective effects against aging by reducing levels of O_2^-. This is consistent with previous reports that overexpression of SODs extends budding yeast CLS (23, 24). Although

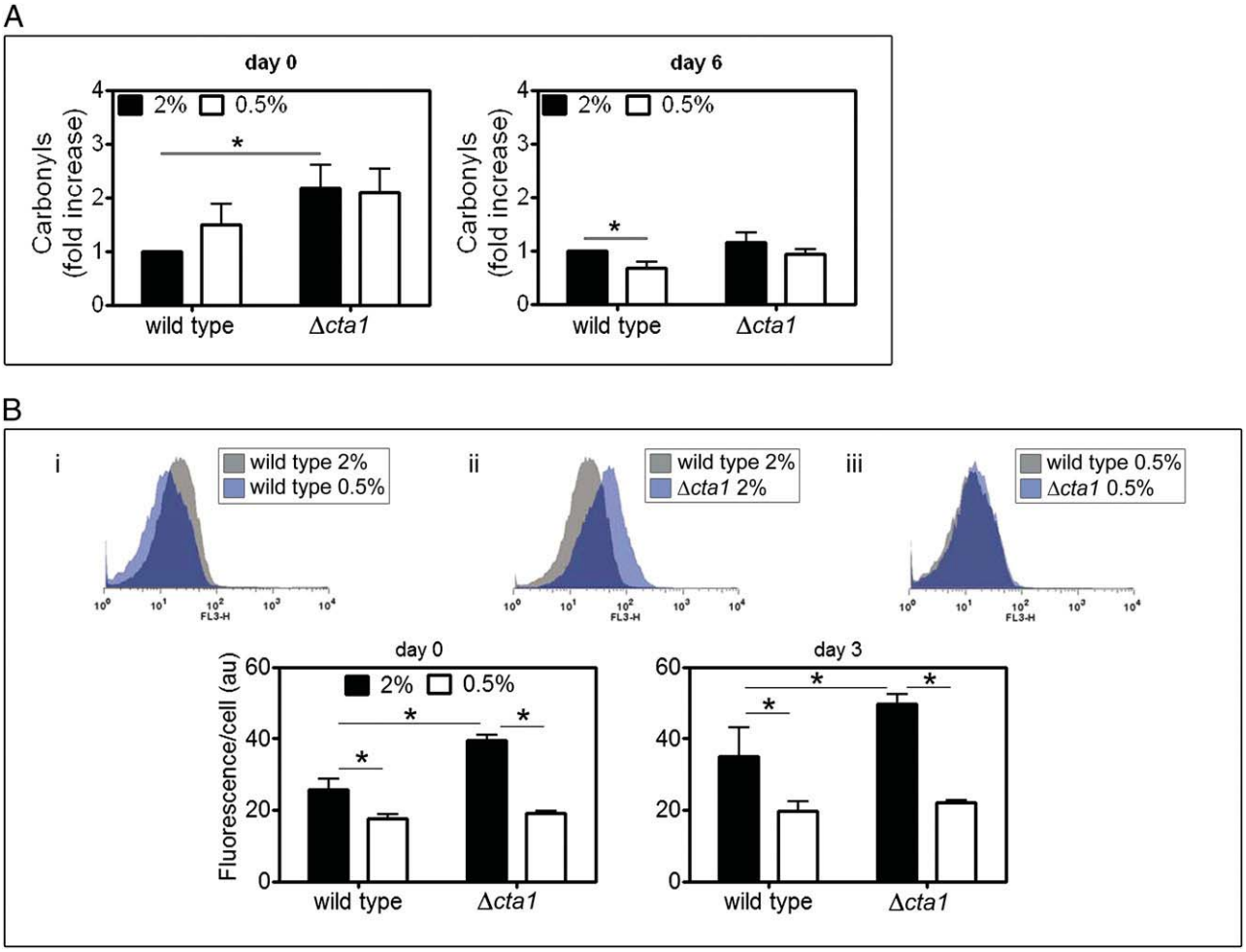

Fig. 4. Effects of increased H_2O_2 induced by CR or by inactivation of *CTA1* on oxidative damage to macromolecules. (*A*) Oxidative damage was assessed by measuring levels of oxidized proteins (carbonyls) in stationary phase wild-type and Δ*cta1* cells under non-CR and CR conditions. Levels of carbonyls were normalized at each time point to wild-type cell values under non-CR conditions (2% glucose). (*B*) Oxidative damage to proteins and lipids measured as autofluorescence of stationary phase wild-type and Δ*cta1* cells under non-CR and CR conditions. Histograms are representative of data collected at day 3. Values indicate mean ± SEM from three independent experiments. Statistical significance (*$P < 0.05$) was determined by Student's *t*-test.

CR can extend CLS by inhibiting the accumulation of acetic acid (10), the hormesis-like mechanism by which H_2O_2 produced by CR and catalase inactivation extends CLS by inducing SODs and reducing O_2^- reported here does not depend on changes in levels of acetic acid. Additional mechanisms likely exist by which CR extends CLS independently of the hormesis-like mechanism identified here.

The induction of H_2O_2 by CR reflects a mechanism by which CR extends lifespan that is likely conserved in *C. elegans* and other organisms, including humans. Consistent with this possibility, ectopic exposure of human skin keratinocytes to low concentrations of H_2O_2 extends their replicative lifespan (25). Furthermore, activation of SODs by H_2O_2 is conserved in rats (16). The replicative lifespan-extending effects of ectopic exposure of human keratinocytes to H_2O_2 are accompanied by an increase in telomere length (25). Superoxide anions inhibit telomere elongation (26) and superoxide dismutases can extend the replicative lifespan of mammalian cells by promoting telomere maintenance (27). Reduced levels of O_2^- are also detected in concert with elevated levels of H_2O_2 in wild-type but not *SOD2*-defective mouse cells driven into quiescence by contact inhibition (28). This suggests that inhibition of superoxide anions by H_2O_2 may be a conserved feature of the quiescent state that is enhanced by CR. The discovery that H_2O_2 reduces superoxide anions by inducing SOD activity in budding yeast also may be relevant to other aspects of human health in addition to aging. For example, it was recently reported that in a mouse model of inflammatory responses in the lung, genetic or pharmacological inactivation of catalase in neutrophils induces intracellular H_2O_2 that inhibits the superoxide-dependent inflammatory responses of these cells (29, 30). The findings reported here predict that H_2O_2 exerts its antiinflammatory effects in mouse neutrophils by inducing SOD activity, leading to a reduction in levels of O_2^- that promote inflammation.

On the basis of the free radical theory of aging, one might predict that hormesis-like effects that promote longevity would protect against oxidative damage to macromolecules. Consistent with this prediction, the CLS-extending effects of CR are accompanied by decreased oxidative damage, despite increased levels of H_2O_2. However, similar to the increased oxidative damage observed in long-lived naked mole rats (3), the increased oxidative damage we detected in catalase mutant cells in association with increased longevity represents a clear violation of the free radical theory postulate that oxidative damage to macromolecules is an important component of aging. Presumably, the high levels of H_2O_2 detected in catalase mutants are responsible for the increased oxidative damage that accompanies their longevity phenotype.

In summary, our findings establish that CR or inactivation of catalases induces oxidative stress in the form of H_2O_2, which

promotes longevity despite increased oxidative damage to macromolecules by inducing superoxide dismutases that reduce levels of O_2^-. This mechanism likely underlies recent findings that challenge the validity of the free radical theory and provides a different paradigm for understanding how oxidative stress impacts aging and health.

Materials and Methods

Strains, Media, Chronological Lifespan, and Treatments. *S. cerevisiae* strain BY4742 cells (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and the respective knockouts in the *CTT1* and *CTA1* genes (European *Saccharomyces cerevisiae* Archive for Functional Analysis, EUROSCARF) were used for all experiments. Cell stocks were maintained in YEPD agar medium containing 0.5% yeast extract, 1% peptone, 2% agar, and 2% glucose. All experiments were performed in synthetic complete (SC) medium containing glucose as a carbon source and 0.67% yeast nitrogen base without amino acids (Difco Laboratories) supplemented with excess amino acids and bases for which the strains were auxotrophic (50 μg/mL histidine, 50 μg/mL lysine, 300 μg/mL leucine, and 100 μg/mL uracil) with the exception that uracil was absent from medium in experiments that used the *CTA1* overexpressing strain, the control strain transformed with an empty vector or the double mutant *CTA1CTT1*. CLS was measured in different conditions as described in Supplementary Information. Construction of the *CTA1* overexpressing strain or the double mutant *CTA1CTT1* strain, treatment with pharmacological inhibitors of catalases or glutathione synthesis, measurements of intracellular reactive oxygen species, oxidative damage and SOD activity were performed using standard techniques: details are provided in Supplementary Information. Statistical analysis was performed according to the description in *SI Materials and Methods*.

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