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A Measurable Increase in Oxidative Damage Due to Reduction in Superoxide Detoxification Fails to Shorten the Life Span of Long-Lived Mitochondrial Mutants of *Caenorhabditis elegans*

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ABSTRACT

SOD-1 and SOD-2 detoxify superoxide in the cytoplasm and mitochondria. We find that, although several long-lived mutants of *Caenorhabditis elegans* have increased SOD levels, this phenomenon does not correlate with life span or growth rate. Furthermore, although disruption of *sod-1* or *-2* expression produces numerous phenotypes, including increased sensitivity to paraquat and increased oxidative damage to proteins (except in *daf-2* mutants), this fails to shorten the life span of these long-lived mutants. In fact, *sod-1(RNAi)* increases the life span of *daf-2* mutants and *sod-2(RNAi)* that of *clk-1* mutants. Our results suggest that increased superoxide detoxification and low oxidative damage are not crucial for the longevity of the mutants examined, with the possible exception of *daf-2*, where our results are inconclusive. These results are surprising because several of the long-lived mutants that we examined specifically affect mitochondrial electron transport, a process whose involvement in life-span determination is believed to be related to superoxide generation. We discuss the significance of our findings in light of the oxidative stress theory of aging.

A variety of *Caenorhabditis elegans* mutants display an increased, or shortened, life span compared to the wild type under standard conditions (Lakowski and Hekimi 1998; Guarente and Kenyon 2000; Hekimi *et al.* 2001b; Tissenbaum and Guarente 2002; Hekimi and Guarente 2003; Boehm and Slack 2005; Kenyon 2005). In life-span studies in a variety of organisms, it is frequently found that increased or decreased life span is associated with changes in the biology of reactive oxygen species (ROS) (Beckman and Ames 1998; Golden *et al.* 2002; Droge 2003; Hekimi and Guarente 2003; Balaban *et al.* 2005), and this is also true for a variety of *C. elegans* mutants (Vanfleteren and Braeckman 1999; Feng *et al.* 2001; Senoo-Matsuda *et al.* 2001; Hekimi and Guarente 2003; Shibata *et al.* 2003; de Castro *et al.* 2004; Kayser *et al.* 2004; Sedensky and Morgan 2006) and wild-type animals treated by RNA interference (RNAi) (Dillin *et al.* 2002; Lee *et al.* 2003). However, although these studies, as well as many other studies that have investigated the biochemical and molecular changes that accompany altered patterns of aging, provide strong arguments for the importance of ROS in life-span determination, it remains difficult to prove or disprove the hypothesis that ROS cause aging by damaging macromolecules. To date, there is conflicting evidence in favor of both views. For example, although overexpression of catalase in mouse mitochondria increases life span in some genetic backgrounds (Schriner *et al.* 2005), the life span of *Sod2* +/− mice, which demonstrably suffer increased oxidative stress (Mansouri *et al.* 2006), is not shortened (Van Remmen *et al.* 2003), nor does transgenic overexpression of the cytoplasmic SOD1 in mice increase the life span of the transgenic animals (Huang *et al.* 2000).

One way to manipulate ROS levels *in vivo*, and to test their effects on the life span of intact organisms, is by altering the expression of genes that code for proteins involved in ROS production or detoxification. This strategy has been followed in a variety of organisms, in particular by knocking out, or overexpressing, detoxifying enzymes such as superoxide dismutases (SOD) (Phillips *et al.* 1989; Parkes *et al.* 1999; Melov *et al.* 2001; Sun *et al.* 2002; Duttaroy *et al.* 2003; Fabrizio *et al.* 2003; Harris *et al.* 2003). In *C. elegans*, there are five distinct genes that code for SODs: *sod-2* and *sod-3* encode MnSODs that are localized in the mitochondrial matrix (Hunter *et al.* 1997), *sod-1* encodes the classical cytoplasm CuZnSOD (Giglio *et al.* 1994), *sod-4* encodes a membrane-bound and extracellular CuZn SOD (Fujii *et al.* 1998), and *sod-5* encodes a relatively uncharacterized, alternative cytoplasmic CuZnSOD (Jensen and Culotta 2005). We focus on *sod-1* and *sod-2* because they encode the primary and the best-characterized superoxide dismutases of the two categories: mitochondrial matrix MnSODs and CuZnSODs.

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Most, or all, long-lived *C. elegans* mutants display other phenotypes in addition to increased life span (Hekimi *et al.* 2001a,b; Hekimi and Guarente 2003). To test whether the increased life span of these mutants could be due to low ROS levels, we have investigated whether any of the phenotypes of long-lived mutants, including their life span, were correlated with the expression of SODs or could be suppressed by knocking down the SODs by RNAi. We focused on strains carrying one or several mutations in four well-characterized genes whose alteration can result in increased longevity and whose interactions have been previously studied. *daf-2* encodes an insulin-receptor-like tyrosine kinase (Kenyon *et al.* 1993); *clk-1* encodes a ubiquinone biosynthetic enzyme (Ewbank *et al.* 1997; Stenmark *et al.* 2001) and *clk-1* mutants display respiratory defects (Felkai *et al.* 1999; Miyadera *et al.* 2001; Braeckman *et al.* 2002a,b; Kayser *et al.* 2004); *isp-1* encodes the "Rieske" iron–sulfur protein (Feng *et al.* 2001), a catalytic subunit of mitochondrial complex *III*; and *ctb-1*, a gene encoded by the mitochondrial genome, encodes the cytochrome *b* of complex *III* (Feng *et al.* 2001). Mutations in *daf-2* and *clk-1* act synergistically to induce a very long life span (Lakowski and Hekimi 1996). In contrast, mutations in *isp-1* and *daf-2* are nonadditive (Feng *et al.* 2001), suggesting that mutations in these genes affect life span by a mechanism that is similar, although loss-of-function mutations in *daf-16*, which abolish the increased life span of *daf-2* mutants, do not abolish the increased life span of *isp-1* (Feng *et al.* 2001) or *isp-1 daf-2* mutants (J. Feng and S. Hekimi, unpublished observations). *ctb-1(qm189)* strongly suppresses many of the *isp-1* phenotypes, including slow development and behavior, but does not suppress the increased life span (Feng *et al.* 2001).

It is often difficult to interpret the effect of life-span-shortening treatments because a deleterious treatment could shorten life span without acting on the actual aging process. However, it is possible to exclude a major role in life-span determination of a particular molecular process (here superoxide detoxification) when a treatment is well defined and its molecular and phenotypic effects can be directly observed, yet the process does not impinge significantly on life span. Here we show that the increased life spans of mutants that have altered mitochondrial function and/or increased resistance to oxidative stress are not affected by reductions in superoxide detoxification and concomitant increases in oxidative damage.

MATERIALS AND METHODS

Strains used: The following strains were used: N2 (wild type), *isp-1(qm150)*, *isp-1(qm150); ctb-1(qm189)*, *clk-1 (qm30)*, *daf-2(e1370)*, *daf-2(e1370)*; *clk-1(qm30)*, *daf-2(e1370)*; *isp-1*(*qm150*), *daf-2 (e1370)*; *isp-1(qm150); ctb-1(qm189)*, and *mev-1(kn1)*. Worms were cultured at 20°.

Generation of *sod-1* and *sod-2* RNAi clones: Full-length *sod-1* and *sod-2* cDNA sequences were obtained by RT–PCR and were cloned into the L4440 feeding vector (pPD129.36). The resulting plasmids were transformed into bacteria DH10b and then retransformed into the RNase III-deficient feeding strain HT115 (DE3) (Shibata *et al.* 2003). Primer sequences can be obtained upon request.

Feeding RNAi: Single colonies of HT115 bacteria were inoculated in LB broth containing 50 μg/ml ampicillin (Amp) and grown overnight at 37°. Overnight cultures were diluted 1:100 and allowed to grow for 6–8 hr and then seeded directly onto NGM plates with Amp and 1 mm IPTG. Seeded plates were dried and induced overnight at room temperature. Animals that were grown on bacteria transformed with the empty vector were used as control. The technique used is essentially as described (Kamath *et al.* 2001).

Post-embryonic development: L4 stage hermaphrodite worms (P0) were placed onto newly prepared (fresh) RNAi plates and were incubated at 20° for 24 hr. Then young adults were transferred to fresh RNAi plates and were incubated for 24–48 hr to lay eggs. Unstaged eggs were placed onto RNAi plates and left for 3 hr to hatch. Larvae (F_1 progeny) that had hatched during that period were placed onto fresh RNAi plates (10/plate for a total of 50) at 20° and were monitored every 1.5 or 3 hr until maturity.

Life span: The aging experiments were begun as in post-embryonic development assays. F_1 worms were cultured on RNAi plates at 20° and examined every day until death. Animals were transferred to fresh RNAi plates twice each week.

Brood size: F_1 worms were placed singly onto fresh RNAi plates at 20° until they had matured and begun to lay eggs. The hermaphrodites then were transferred to RNAi plates daily and the progeny were counted. Worms that did not lay any eggs were defined as sterile.

Embryonic lethality: Eggs produced by F_1 worms during a limited time period (4–6 hr) were placed onto fresh RNAi plates and were monitored every day until hatching. The eggs that did not hatch were scored as dead embryos.

Paraquat resistance: Groups of L4 larvae were grown on control as well as on *sod-1* and *sod-2* RNAi plates until their progeny had reached the L4 stage. A total of 50 of those were transferred onto NGM plates with 4 mm paraquat (PQ) and kept for 72 hr at 20°, at which time the number of surviving worms was counted.

Paraquat-induced damage to proteins: L1 wild-type worms were transferred onto plates with different concentrations of paraquat (0.0, 0.1, 0.2, or 0.5 mm) and left to develop into adults. Their adult progeny was then harvested and used for protein extraction by freeze–thaw and dissolved in NET buffer. Protein concentrations were measured with the Bio-Rad (Hercules, CA) kit and then normalized to 5 μg/μl and used with the Oxyblot (Chemicon) kit and analyzed as described below.

Antibodies: Whole cDNA sequences of *sod-1* and *sod-2* were amplified from a *C. elegans* cDNA library (Invitrogen, San Diego) and cloned into the pGEX-5X-1 vector (Invitrogen). Bacterially expressed GST-fusion proteins were extracted and injected into two rabbits to obtain polyclonal antibodies. The terminal bleed of each rabbit recognizes the bacterial antigen and, in worm extracts, a predominant band at the expected size (18.5 kDa for SOD-1 and 24.5 kDa for SOD-2), whose intensity was drastically reduced upon specific RNAi treatment (*sod-1* or *sod-2* RNAi).

Western blot analysis: After RNAi treatment, 100 young adult worms of each genotype were picked, lysed in two times loading buffer, and electrophoresed on 12% SDS–polyacrylamide gels (SDS–PAGE), and then blotted onto nitrocellulose membrane (Bio-Rad). After applying primary antibody (1:1000, rabbit polyclonal antibody against worm SOD-1 or SOD-2) and secondary antibody (1:10,000 mouse anti-rabbit

IgG, Invitrogen), the membranes were incubated with the ECL plus detection reagent (Amersham Biosciences) and scanned using a Typhoon trio plus scanner. Band densities were analyzed by ImageQuant TL V2003.03.

MnSOD activity assay: A total of 100 μl of pellets of young adult worms were ground into powder with a mortar in liquid nitrogen and mixed with 100–150 μl of extraction buffer (50 mM phosphate buffer, pH 7.8) and then were centrifuged at 4°, 10,000 × *g* for 10 min. The supernatant was used for electrophoresis on nondenaturing polyacrylamide gels (7.5%). After electrophoresis, the gel was soaked in 1.23 mM nitroblue tetrazolium for 10 min in the presence of 4 mM potassium cyanide to inhibit CuZn SOD activity and then soaked in 36 mM phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM TEMED for another 10 min. The gel was illuminated on a light box for ~10 min. The presence of the MnSOD proteins in the gel remained unstained, while the background of the gels was stained a bluish color.

O_2 consumption experiment: The rates of O_2 consumption of the wild type and *clk-1(qm30)* and *clk-1; sod-2*(RNAi) and *isp-1(qm150); ctb-1(qm189)* were measured. Worms were grown on RNAi feeding bacteria from L3 to L4 larvae to young adult of the next generation. Young adult worms were collected and washed with M9 to be bacteria free. Oxygen consumption and protein quantification were measured as in Feng *et al.* (2001).

Protein damage measurement by Oxyblot: One hundred adults from each strain or treatment or age class were picked, washed repeatedly, concentrated in 3 μl of M9 buffer, and frozen to −80° for storage. Before loading, 3 μl of lysis buffer and 4 μl 15% SDS were added into tubes. Samples were then treated by the freeze–thaw method to release protein. A total of 10 μl of DNP solution (Oxyblot) was added to the samples and kept at room temperature for 15 min, after which 7.5 μl of neutralization buffer (Oxyblot) was added. Samples (27.5 μl) were run in 7.5% SDS–PAGE gels, transferred to nitrocellulose (Bio-Rad), and blocked with 5% milk for 1 hr. Membrane was incubated with the first antibody (Oxyblot) (1:150) overnight at 4° and then for 1 hr with the second antibody (Oxyblot) (1:300) at room temperature. Membranes were incubated with the ECL plus detection reagent (Amersham Biosciences) and scanned using a Typhoon trio plus scanner. Band densities were analyzed by ImageQuant TL V2003.03. The densities of all bands in a given lane were added together and considered the Oxyblot value for the sample. Membranes were then incubated with 15% hydrogen peroxide for 30 min at room temperature and treated with a α-tubulin antibody to derive a density value for tubulin for each lane, with which an Oxyblot value normalized to tubulin was obtained. In Figure 3, the samples for which values are represented in a given panel were always run in the same gel and treated and analyzed together to reduce experimental variations. However, sometimes more than one sample of each genotype or condition was run on the same gel, which is why the sample size is not always identical for each genotype or condition. Each membrane contained at least two samples for each strain or treatment, including the control. The relative value for each sample, including for each control sample, was obtained by dividing the value of the sample by the average value for all control samples on the same blot, which is why the control bars in Figure 3 have SEM error bars.

Quantitative PCR measurements: Worms from RNAi treatment plates were washed into a 1.5-ml tube with M9 when they reached the young adult stage. After three washes with 100 μl M9, all M9 was carefully removed. Total mRNA was extracted using the Trizol reagent (Invitrogen) and then quantified with a spectrophotometer (Beckman Coulter). One microgram of total mRNA was reverse transcribed to cDNA using the Omniscript RT kit (QIAGEN, Valencia, CA). One-fortieth of the reverse transcription product was used as template to perform real-time PCR using the Quantitect SYBR Grenn PCR kit (QIAGEN) and an icycler apparatus (Bio-Rad Version 4.006). Each sample was done in triplicate. Data were analyzed with icycler software (version: 3.0.6070).

Statistical analysis: Mean post-embryonic rates were compared by using Student's *t*-test assuming unequal variances. Fertilities (self-brood size) were compared by using Student's *t*-test assuming equal variances. We performed log-rank statistics to determine if the adult life spans of the RNAi treatment groups were different from that of control groups. Embryonic viabilities and sensitivities to PQ were compared by a χ^2 test (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html).

RESULTS

***sod-1* and *-2* expression in the wild type and after knockdown by RNAi:** We have raised antisera specific for *C. elegans* SOD-1 and SOD-2 (Figure 1A). RNAi treatment against each of these genes almost completely abolished detection of the respective proteins by Western blotting but did not alter the level of expression of the other protein (Figure 1A). SOD-2 and SOD-3 are homologous proteins of similar size but the antiserum that we raised against SOD-2 recognizes SOD-3::GFP only very weakly (not shown). However, *sod-2* RNAi virtually abolishes not only SOD-2 protein expression, but also all MnSOD activity (Figure 1A). Furthermore, *sod-2* RNAi severely reduces *sod-3* mRNA levels in *daf-2* and *clk-1* mutants where they are elevated (see below). We conclude that our *sod-2(RNAi)* treatment also severely affects *sod-3* expression. In any case, the *sod-2* RNAi treatment appears to virtually abolish superoxide detoxification in the mitochondrial matrix of the wild type.

SOD levels in long-lived mutants: Using our antisera, we quantified the levels of SOD-1 and SOD-2 in a variety of long-lived, slow-growing mutants (Figure 1B). For this part of the study only, we also examined double mutants because they were known to differ dramatically from each other in life span or growth rate (Wong *et al.* 1995; Lakowski and Hekimi 1996; Feng *et al.* 2001), which could help in determining whether SOD levels could be uncoupled from either, or each, of the two parameters. Several mutant strains (*isp-1*, *daf-2*, *daf-2 clk-1*, *daf-2 isp-1*, and *daf-2 isp-1; ctb-1*) show increased levels of both proteins, but no strain shows a significant increase of only one of them. Also, no strain shows a significant decrease in SOD levels in comparison to the wild type. All the strains examined are both long lived and slow growing, but to various degrees compared to the wild type (Wong *et al.* 1995; Lakowski and Hekimi 1996; Feng *et al.* 2001). Increased SOD levels likely reduce superoxide levels, which could impact both of these phenotypes. However, we did not observe any correlation between increased SOD levels and life span or growth rate. For example, although *isp-1* and *isp-1; ctb-1* mutants have essentially the same life span (Feng *et al.*

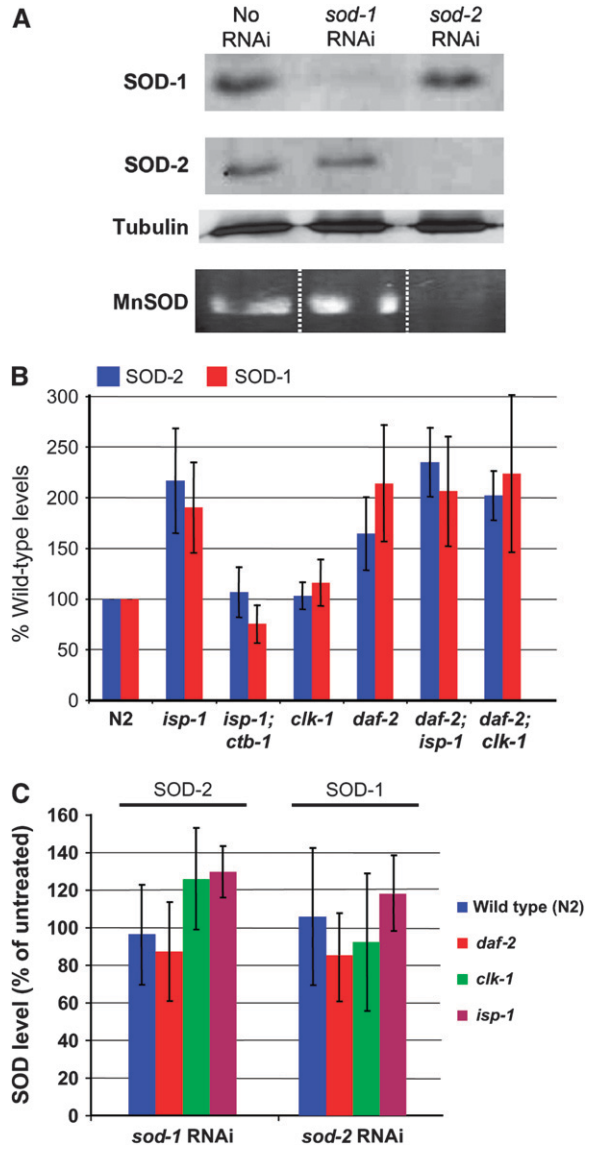

FIGURE 1.—Expression of SOD-1 and SOD-2 in different genetic backgrounds and after RNAi treatments. (A) Western blot and MnSOD enzymatic activity blot of wild-type (N2) worm extracts after treatment with RNAi against *sod-1* or *sod-2* or without treatment. The labels on the left indicate the specificity of the antiserum used or the activity. The interruption of the band in the first two lanes of the activity blot is an unwanted technical artifact of the gel. The three lanes are from the same blot. The dotted lines have been added to clarify the lanes because of the artifact. The same worm extracts were used in all three Western blot panels. (B) Quantification of the levels of SOD-1 and SOD-2 in different genetic backgrounds using Western blotting. Amounts are expressed relative to the wild type; $n = 5$ repeats of the experiment; error bars are SEM. (C) Quantification of the levels of SOD-1 and SOD-2 after treatment with RNAi against the other SOD gene in four genetic backgrounds. Amounts are expressed relative to untreated controls, for each genotype; $n = 5$ repeats of the experiment; error bars are SEM.

2001), only *isp-1* mutants have increased SOD levels (Figure 1B). Likewise, *daf-2 clk-1* mutants display the same levels of SODs as *daf-2* or *isp-1*, although their life span is substantially longer (LAKOWSKI and HEKIMI 1996; FENG *et al.* 2001). Similarly, for growth rate, we observe that, although *clk-1* grows much more slowly than *daf-2* (WONG *et al.* 1995; LAKOWSKI and HEKIMI 1996), *daf-2* has much higher levels of SODs, and although *daf-2; isp-1* mutants grow two times more slowly than *daf-2* single mutants (FENG *et al.* 2001), they have similar SOD levels (Figure 1B).

Interactions of SOD-1 and -2 levels with each other: The CuZn superoxide dismutase SOD-1 is expected to be expressed in the cytoplasm, but also in the mitochondrial intermembrane space (OKADO-MATSUMOTO and FRIDOVICH 2001; STURTZ *et al.* 2001; O'BRIEN *et al.* 2004). As hydrogen peroxide, which is the product of the reaction catalyzed by SODs, can cross membranes and affect cellular compartments distinct from that in which it was produced, we wondered whether the level of one type of SOD could affect the expression of the other type. We tested for this both in the wild type, as described above (Figure 1A), and in the mutants (Figure 1C). RNAi was as effective in reducing the level of the targeted SOD in the mutants as in the wild type (not shown). But we could detect no significant effects of RNAi on the levels of the untargeted SOD in the mutants or the wild type. The small effects of *sod-1(RNAi)* on SOD-2 levels appear minor, considering the much larger variations in SOD levels among genotypes (Figure 1B). These observations indicate that knocking down the expression of one *sod* gene does not induce the other and therefore suggests that superoxide detoxification is carried out independently in each cellular compartment.

Effect of *sod* RNAi on growth rate and other phenotypes in long-lived mutants: We treated our collection of long-lived mutants with RNAi against *sod-1* or *sod-2* and examined the effect of the treatment on the length of post-embryonic development (Table 1), adult life span (Table 2), embryonic lethality (Table 3), and fertility (Table 4). In the absence of any treatment, all of the long-lived mutants display slow post-embryonic development and, except for *daf-2*, altered fertility. Some show increased embryonic lethality. Treatment with *sod-1(RNAi)* has only relatively mild effects, sometimes deleterious and sometimes slightly suppressive. For example, it appears to be deleterious for the fertility of the wild-type and *daf-2* mutants, but slightly increases the fertility of *clk-1* (Table 4). In fact, *sod-1(RNAi)* appears to be slightly suppressive for several phenotypes of *clk-1* mutants, as noted previously (SHIBATA *et al.* 2003). However, in general, we believe that the relatively small magnitude of the effects observed here with *sod-1(RNAi)* precludes a clear interpretation of their meaning. On the other hand, *sod-2(RNAi)* appears to be clearly deleterious as it strongly slows down development of *clk-1* and *isp-1* (Table 1), dramatically increases embryonic

TABLE 1

Effects of *sod-1* and *sod-2* RNAi on post-embryonic development rate

Strain	Control	*sod-1* RNAi	*sod-2* RNAi
Wild type (N2)	46.0 ± 1.9 $n = 146$	46.6 ± 2.1 $n = 134$	46.8 ± 2.1 $n = 148$
daf-2(e1370)	74.0 ± 3.5 $n = 135$	76.1 ± 3.6 $n = 120$	72.6 ± 2.7 $n = 133$
isp-1(qm150)	108.7 ± 7.6 $n = 138$	100.2 ± 7.1 $n = 148$	116.0 ± 6.7 $n = 144$
clk-1(qm30)	83.3 ± 4.7 $n = 129$	74.4 ± 2.3 $n = 148$	111.1 ± 15.3 $n = 81$

Post-embryonic development rate is expressed in hours to reach adulthood after hatching. All values found for treated animals *vs.* untreated animals are significantly different at $P < 0.05$ using Student's *t*-test assuming unequal variances. The control plates contained bacteria with the empty vector.

lethality of these two mutants (Table 3), and decreases fertility, especially of *isp-1* mutants (Table 4).

Effect of *sod* RNAi on sensitivity to paraquat: No function other than superoxide detoxification has been reported for superoxide dismutases, which strongly suggests that any phenotypic effect of *sod* RNAi treatment on mutants is due to an increase in ROS resulting from poor superoxide detoxification. To investigate this further, we have examined how *sod-1* or *sod-2* RNAi affects survival after treatment with PQ, a superoxide generator that is frequently used to establish alterations

TABLE 2

Effects of *sod-1* and *sod-2* RNAi on adult life span

Strain	Control	*sod-1* RNAi	*sod-2* RNAi
Wild type (N2)	17.5 ± 5.6 Maximum: 29 $n = 150$	16.2 ± 4.8 Maximum: 28 $n = 142$ $P = 0.008$	18.0 ± 5.8 Maximum: 29 $n = 150$ $P = 0.236$
daf-2(e1370)	33.0 ± 11.0 Maximum: 53 $n = 150$	37.4 ± 11.3 Maximum: 66 $n = 140$ $P = 0.0002$	33.5 ± 9.2 Maximum: 52 $n = 150$ $P = 0.266$
isp-1(qm150)	23.3 ± 8.2 Maximum: 50 $n = 260$	22.1 ± 8.2 Maximum: 50 $n = 227$ $P = 0.198$	22.6 ± 7.9 Maximum: 45 $n = 267$ $P = 0.171$
clk-1(qm30)	18.6 ± 6.4 Maximum: 37 $n = 150$	18.7 ± 5.4 Maximum: 37 $n = 150$ $P = 0.625$	23.9 ± 7.7 Maximum: 43 $n = 150$ $P < 0.0001$
mev-1(kn-1)	13.3 ± 3.8 Maximum: 23 $n = 63$	14.3 ± 3.5 Maximum: 23 $n = 67$ $P = 0.137$	12.8 ± 3.1 Maximum: 24 $n = 55$ $P = 0.442$

Adult life span is expressed in days. *P*-values are determined using the log-rank statistics. Values indicating a lack of significance are also shown.

TABLE 3

Effects of *sod-1* and *sod-2* RNAi on embryonic lethality

Strain	Control	*sod-1* RNAi	*sod-2* RNAi
Wild type (N2)	3.9 $n = 178$	2.9 $n = 173$	1.9 $n = 213$
daf-2(e1370)	0 $n = 201$	2.0* $n = 200$	1.5 $n = 200$
isp-1(qm150)	6.3 $n = 313$	8.1 $n = 358$	30.6*** $n = 411$
clk-1(qm30)	2.5 $n = 316$	5.8* $n = 328$	50.3*** $n = 349$

Embryonic lethality is expressed in percentages of unhatched eggs. Mutant embryonic lethalities were compared to the wild-type level by the χ^2 nonparametric test. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

in ROS resistance. We determined PQ resistance by treating worms for one generation with RNAi and then by testing the ability of adults to survive a relatively high concentration of PQ (4 mM) (Figure 2). We find that both *sod-1* and *sod-2* RNAi increase the sensitivity to killing by PQ of both the wild type and the mutants, with the exception of treatment of *daf-2* with *sod-2* RNAi, which has no effect. Furthermore, the mutants are more resistant than the wild type to killing by PQ in every condition, except for treatment of *isp-1* with *sod-1* RNAi, which is as effective as in the wild type. We conclude that the level of reduction of SOD-1 or SOD-2 expression by RNAi treatment effectively decreases superoxide detoxification. However, since we do not know the exact nature of the damage that kills the animals, the relative effects of *sod-1 vs. sod-2* RNAi in this experiment does not tell us which treatment produces more overall oxidative stress or damage or how it might relate to aging. For the same reason, the increased resistance of the mutants to PQ confirms only the generally observed correlation between altered life span and altered resistance to imposed acute oxidative stress.

TABLE 4

Effects of *sod-1* and *sod-2* RNAi on fertility

Strain	Control	*sod-1* RNAi	*sod-2* RNAi
Wild type (N2)	319.4 ± 30.4 $n = 20$	267.0 ± 54.2** $n = 20$	296.7 ± 59.7 $n = 20$
daf-2(e1370)	298.4 ± 23.5 $n = 15$	266.8 ± 19.5** $n = 15$	279.1 ± 15.4* $n = 15$
isp-1(qm150)	68.5 ± 21.4 $n = 50$	66.8 ± 20.1 $n = 50$	51.4 ± 30.7*** $n = 50$ 5% sterile
clk-1(qm30)	146.5 ± 54.5 $n = 10$	195.5 ± 35.4* $n = 10$	167.8 ± 25.0 $n = 10$

Fertility is scored as self-brood size. *P*-values were determined by using Student's *t*-test assuming equal variances. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

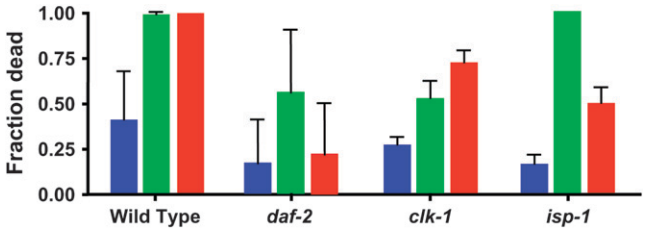

FIGURE 2.—Sensitivity of the wild-type, *daf-2*, *clk-1*, and *isp-1* mutants to PQ after *sod* RNAi treatment. The fraction of worms that die after 72 hr of treatment with 4 mM PQ was scored. Treatment of *sod-1(RNAi)* is shown in green and *sod-2(RNAi)* in red; no RNAi treatment is shown in blue. The experiment was repeated three times; error bars are SEM. The wild type and all three mutants were sensitized to PQ by RNAi treatments (all differences are significant at $P < 0.01$ by a χ^2 test), except for *daf-2*, which is insensitive to *sod-2(RNAi)*. Furthermore, after RNAi treatment, all three mutants are somewhat more resistant to PQ treatment than the wild type ($P < 0.01$; $P < 0.025$ for *clk-1*), except for *isp-1*, which is as sensitive to *sod-1(RNAi)* as the wild type.

Effect of life-span mutants and *sod* RNAi on ROS damage to proteins: We have used a Western blotting method (Oxyblot; see MATERIALS AND METHODS) to quantify oxidative damage to proteins (carbonyl formation) as the most sensitive method currently available for *C. elegans* to measure ROS damage (SHACTER 2000; DALLE-DONNE *et al.* 2003; KAYSER *et al.* 2004; YASUDA *et al.* 2006). We have examined damage in response to increasing doses of PQ to establish that the method is capable of yielding a graded response (Figure 3A). Treatment with paraquat is well recognized as increasing oxidative stress. The 0.5-mM condition was nearly lethal (not shown). Note that Figure 3A shows the result of treating wild-type worms with PQ throughout development, which is a more severe treatment than growth on PQ only at the adult stage, as in the experiments of Figure 2, and thus requires lower doses of PQ. The results suggest that the Oxyblot measurements indeed measure oxidative stress and that a 1.5-fold increase corresponds to a degree of increase in damage that is close to the maximum compatible with survival. We have also examined the levels of carbonyls in mutants in comparison to the wild type (Figure 3B) and in response to *sod* RNAi treatments (Figure 3, C–F).

Figure 3B shows that in the absence of any treatment only *daf-2* mutants have significantly reduced levels of oxidative damage. There is also a similar tendency for *clk-1* and *isp-1* mutants, but the effect is minor. Figure 3, C–F, shows that *sod-1* RNAi increases damage in all strains except in *daf-2* mutants where, surprisingly, it tends to lower damage. Possibly, in this mutant, the excess superoxide produced by the reduction in detoxification induces an overcompensating protective mechanism involving different detoxifying enzymes. It is also of note that the reduction in damage produced by *sod-1* RNAi in *daf-2* mutants is consistent with the slight increase in life span produced by this treatment (Figure 4). Figure 3, C–F, also shows that *sod-2* RNAi increases damage significantly in the wild type and in *isp-1* mutants, but is without effect in *daf-2* and *clk-1* mutants. In the case of *clk-1*, this might be the result of a form of developmental compensation (see below). It is important to note that the effects of *sod-1* RNAi on *clk-1* and of *sod-2* RNAi on *isp-1* increase damage ~1.4-fold, while there appears to be only a minor reduction in damage in untreated *clk-1* and *isp-1* mutants compared to the wild type (Figure 3B). Thus, the level of damage in treated mutants must be similar to, or higher than, that in the wild type.

mev-1 encodes a subunit of mitochondrial complex *II*; *mev-1* mutants have a short life span and are hypersensitive to paraquat (ISHII *et al.* 1998), which has suggested to previous researchers that their short life span was due to elevated mitochondrial oxidative stress. We have found that both *sod-1* and *sod-2* RNAi are capable of substantially increasing carbonyl damage in *mev-1* mutants (Figure 3G). These results suggests that our RNAi treatments do strongly increase oxidative stress by a degree that is rather greater than that by which the *mev-1* mutation increases oxidative stress.

In the experiments described in this section of the RESULTS, for each repeat of the experiments that compared RNAi-treated and mock-treated animals (Figure 3, C–G) all the samples were analyzed on the same gels to reduce experimental variation. However, we also wanted to make sure that we could relate the relative differences between treated and untreated animals to those differences that were observed between untreated wild-type and mutant animals (Figure 3B). Thus, for this, as a control, we prepared samples from *clk-1* or *isp-1* mutants treated with *sod-1* or *sod-2* RNAi and ran them together on the same gels with samples from untreated wild-type animals (Figure 3H). We found that, in both *sod-1* and *sod-2* RNAi-treated *isp-1* and *clk-1* mutants, the level of carbonyls was indeed as high or higher as in untreated wild-type animals (Figure 3H).

In the experiments described above, we treated young animals and compared their carbonyl levels. However, it is possible that protein oxidation changes with chronological age. To test this, we chose to examine and compare carbonyl levels in the wild type and *isp-1* at a specific time: the time (13 days) at which 15% of the wild-type animals but none of the mutants had died (Figure 3I). We chose *isp-1* because our experiments suggest that their carbonyl levels at a young age are not significantly different from those of the wild type (Figure 3B), yet were increased by *sod* RNAi treatments, albeit without effects on life span (see below). Furthermore, the robust difference in life span between the wild type and *isp-1* allowed for measurement that indeed compared chronological with physiological age. We found that even at the time of 15% mortality for the wild type there was no difference in carbonyl levels

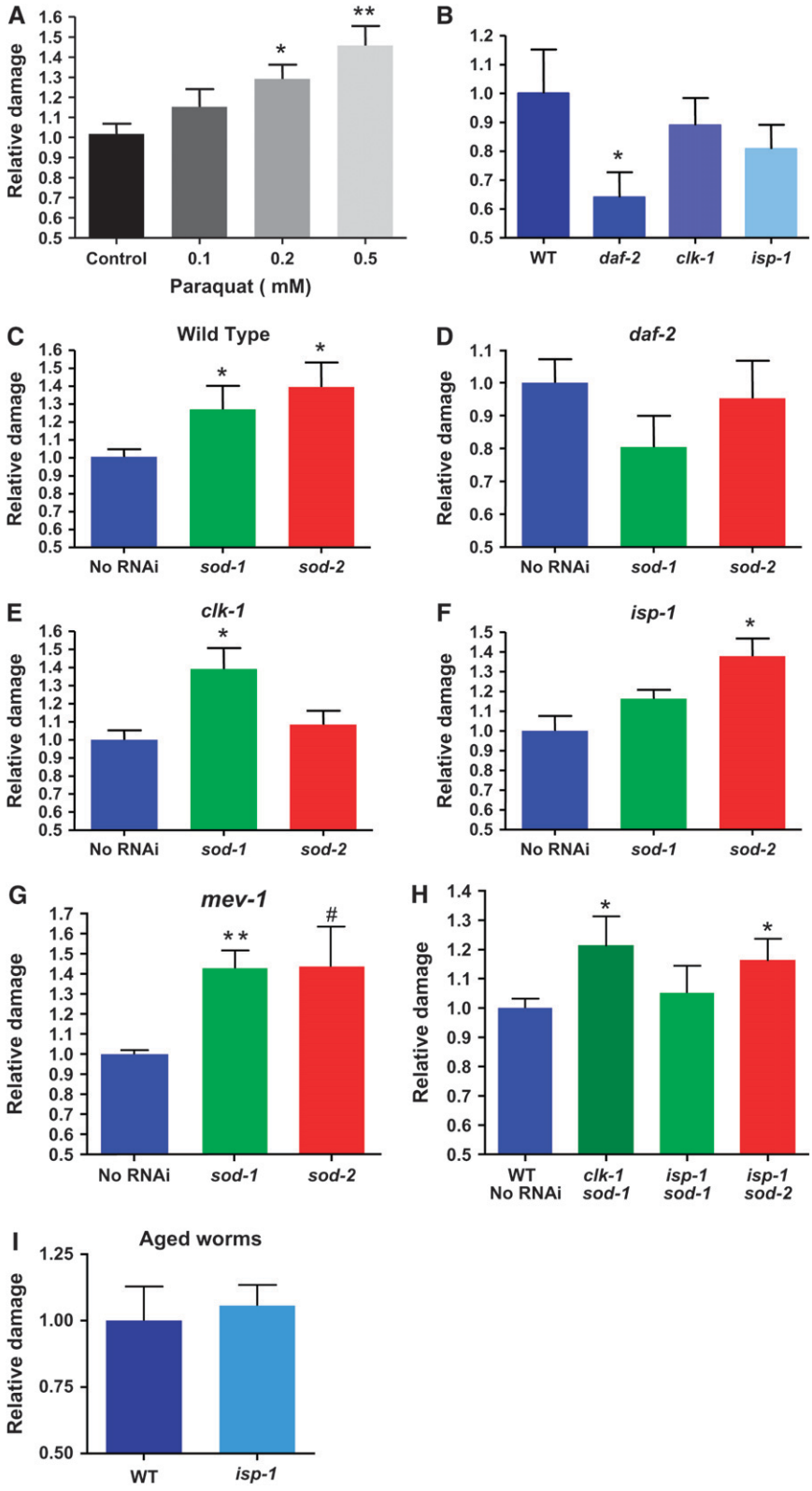

FIGURE 3.—Oxidative damage to proteins after treatment with paraquat in mutant strains at various chronological ages and after RNAi against superoxide dismutases. Oxidative damage to proteins was measured by Oxyblot and is expressed as relative to control. The sample sizes given below in the legend indicate the number of independent experiments. Statistical significance is given relative to the wild type or no RNAi condition ($^{*}P \leq 0.05$; $^{**}P < 0.01$, $^{\#}P = 0.07$). The error bars are SEM. (A) Treatment of wild-type worms with paraquat ($n = 5$); n represents repeats of the experiment. (B) The oxidative damage levels at the young adult stage in different strains [wild type ($n = 7$); *daf-2* ($n = 8$); *clk-1* ($n = 7$); *isp-1* ($n = 8$)]. Although there is a tendency for less damage in all three long-lived mutant strains, it is significant only in *daf-2* mutants. (C) Reduction of either SOD-1 or SOD-2 levels by RNAi leads to significant increases in oxidative damage in the wild type ($n = 8$; no RNAi $n = 7$). (D) Neither *sod-1* nor *sod-2* RNAi significantly affects protein damage levels in *daf-2* mutants ($n = 8$). However, there is a tendency for a reduction of damage after *sod-1* RNAi treatment. (E) Reduction of SOD-1 levels but not SOD-2 levels affects oxidative damage in *clk-1* mutant ($n = 7$). (F) Reduction of both SOD-1 and SOD-2 levels increases oxidative damage in *isp-1* mutants, although only the increase produced by SOD-2 is statistically significant ($n = 7$). (G) Reduction of both SOD-1 and SOD-2 levels increases oxidative stress in *mev-1* ($n = 3$ for each condition). (H) Reduction of both SOD-1 and SOD-2 levels increase carbonyls in mutants to levels that are higher than in the wild type in a direct comparison ($n = 7$ except for *isp-1* treatment with *sod-2* RNAi for which $n = 5$). (I) The carbonyl levels in aged *isp-1* mutants (13 days old; $n = 10$) are not reduced compared to wild type ($n = 5$).

between the wild-type and *isp-1* mutants. Thus, no spontaneous reduction in oxidative damage to proteins in the mutants can explain why *isp-1* mutants survive longer than the wild type.

Effect of *sod* RNAi on the life span of long-lived mutants: Although we did not find any correlation between the levels of SODs and life span in the long-lived mutant strains that we have examined (Figure 1B), the reduction of a given SOD activity should produce an increase of superoxide in the compartment where the SOD is expressed. Indeed, this must be why these reductions induce various deleterious phenotypes

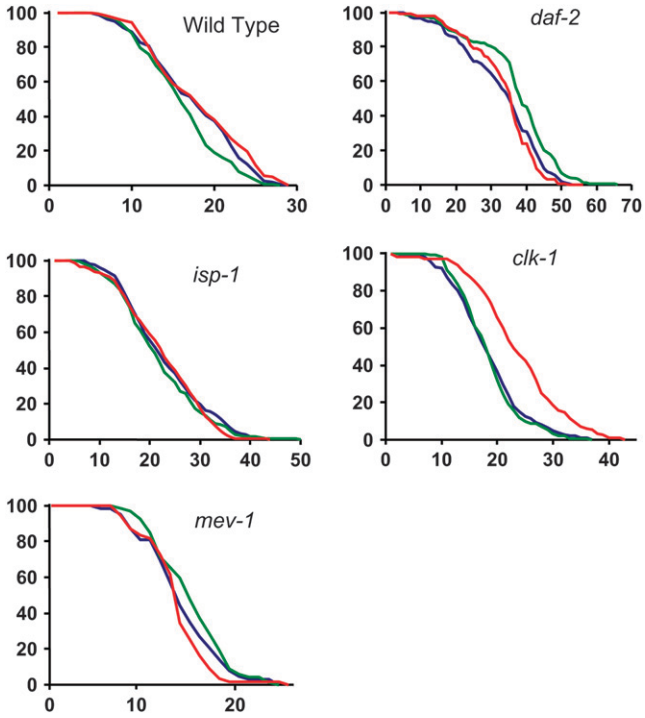

FIGURE 4.—Changes in life span of the wild type and different mutants after RNAi treatments against *sod-1* or *sod-2*. The *x*-axis is in days and the *y*-axis indicates the fraction of the worms in the sample that are still alive. Blue represents untreated worms, green represents worms treated by *sod-1(RNAi)*, and red represents worms treated by *sod-2(RNAi)*. The worms were checked every day. All statistical analyses are shown in Tables 1–4, and the results are discussed in detail in the text.

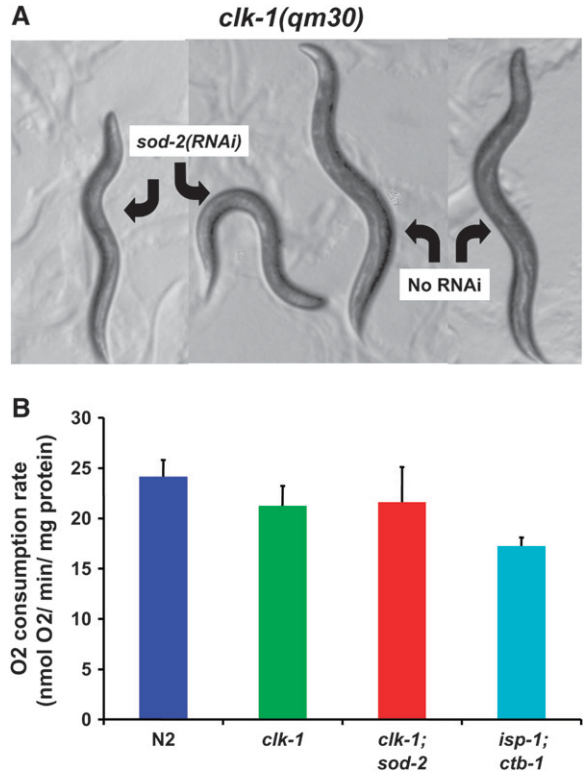

FIGURE 5.—RNAi treatment against *sod-2* alters the size and appearance of *clk-1* mutants but not their oxygen consumption rates normalized to protein content. (A) Four-day-old adult *clk-1(qm30)* mutants after treatment by *sod-2(RNAi)* or without treatment. (B) Oxygen consumption of *clk-1(qm30)* worms treated by *sod-2(RNAi)* compared to untreated wild-type worms, *clk-1(qm30)* worms, and *isp-1;ctb-1* worms ($n = 3$; error bars are SEM), which are known to have decreased oxygen consumption (FENG *et al.* 2001). *sod-2(RNAi)* treatment has no effect on *clk-1* oxygen consumption, although the effect of the treatment on growth rate, size, life span, and the localization of SOD-2 in the mitochondrial matrix suggests the existence of a deleterious effect on mitochondrial function.

(Tables 1–4) and/or reduce survival on PQ (Figure 2) and/or increase oxidative damage (Figure 3). Thus, these reductions in detoxification should shorten the life spans of these mutants if their long life spans were due to low oxidative stress. To test this hypothesis, we have examined the effects of *sod* RNAi on the life spans of the mutant strains, expecting to observe a total or partial suppression of their increased life spans. Surprisingly, this was not observed. Instead, we observed the following (Figure 4): (1) an almost complete absence of effect of *sod-1(RNAi)* except for a minor life-span-shortening effect in the wild type and an equally minor, albeit statistically significant, life-span-lengthening effect in *daf-2* and (2) a life-span-lengthening effect of *sod-2(RNAi)* on *clk-1*.

The life-span-lengthening effect of *sod-2(RNAi)* on *clk-1* is accompanied by severely increased embryonic lethality and lengthening of post-embryonic development (Tables 1–4). In addition, the treated animals are significantly smaller than the untreated animals (Figure 5A). However, they show no reduction in oxygen consumption per milligram of protein (Figure 5B) and no elevation of the amount of oxidative damage per milligram of protein (Figure 3E). One possible explanation for the apparent paradox of a decrease in ROS detoxification resulting in an increase in life span is that the *sod-2* RNAi treatment of *clk-1* mutants damages their respiratory chain in some way. Indeed, the phenotype of these animals is similar to that observed when wild-type worms are treated by RNAi against subunits of respiratory chain complexes (DILLIN *et al.* 2002; LEE *et al.* 2003). However, this also suggests that the increased longevity is not due to low oxidative damage, as oxidative damage is not decreased.

Interestingly, the slight life-span-lengthening effect of *sod-1* RNAi on *daf-2* is correlated by a paradoxical decrease of carbonyl levels brought about by this treatment (Figure 3D). One hypothesis to explain this effect is that, in response to reduced SOD-1 levels, there is overexpression of other protective or detoxifying activities that we do not monitor here.

Effect of *sod* RNAi on life span of *mev-1*: In addition to testing the effect of *sod* RNAi on long-lived mutants,

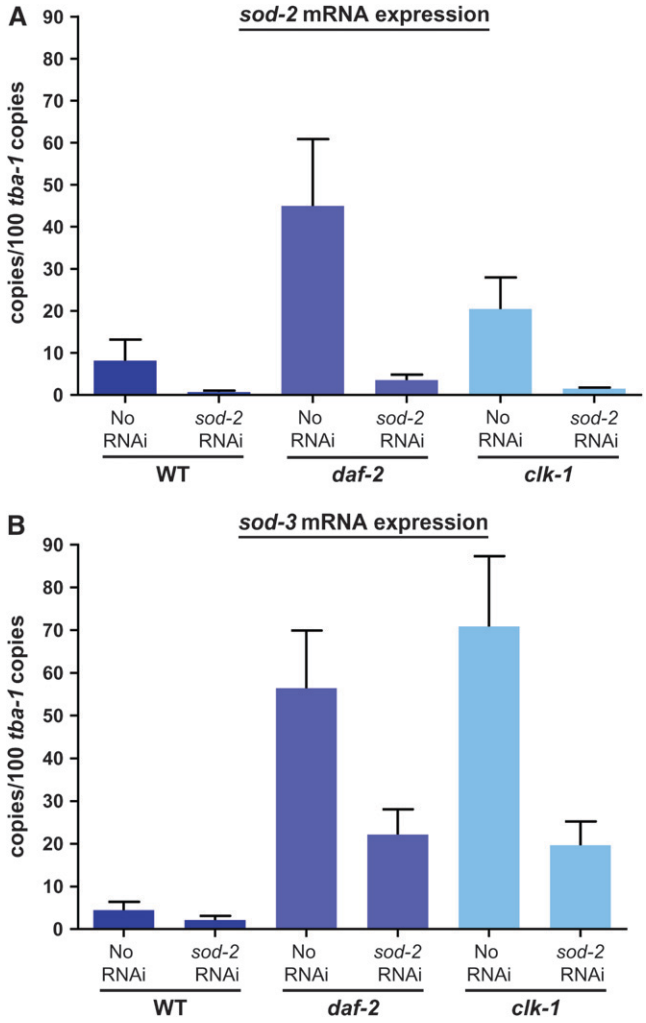

Figure 6.—mRNA levels by quantitative real-time PCR of *sod-2* and *sod-3* in the wild type as well as *daf-2* and *clk-1* mutants, with or without *sod-2* RNAi treatment ($n = 3$ repeats; error bars are SEM). (A) *sod-2* mRNA levels. (B) *sod-3* mRNA levels.

we tested the effect on the short-lived mutant *mev-1* (Figure 4E). *mev-1* encodes a subunit of the complex *II* of the respiratory chain and sustains increased oxidative stress (Ishii *et al.* 1998). We found that neither *sod-1(RNAi)* nor *sod-2(RNAi)* significantly altered the life span of the mutants. Yet we have shown that these treatments substantially increase oxidative stress as measured by carbonyl levels (Figure 3G). Although the short life of *mev-1* mutants has sometimes been hypothesized to result from increased oxidative stress, there is no immediate demonstration of this. In fact, recent data very strongly indicate that a large part of the effect of *mev-1* on life span is due to its effect on increasing programmed cell death through a direct effect on the expression of *ced-9* (Senoo-Matsuda *et al.* 2001, 2003). Our findings suggest that the degree by which *sod* RNAi treatment increases oxidative stress (Figure 3E) in this mutant does not shorten its life span. This is consistent with the absence of a life-span-shortening effect of *sod* RNAi treatments on the wild type and suggests that, as in the case of the long-lived mutants, the respiratory defect of *mev-1* (Senoo-Matsuda *et al.* 2001), rather than its increased oxidative stress, is the cause of its short life.

***sod* mRNA expression in *daf-2* and *clk-1* mutants:** The carbonyl levels of *daf-2* and *clk-1* mutants are unaffected by treatment with *sod-2*, although the same treatment increases carbonyl levels in the wild type. This could be due to an increase in the expression of *sod-3*, whose protein levels we cannot directly measure because of the absence of a SOD-3-specific antiserum. To determine whether changes in *sod-3* expression might be involved in this effect, we used quantitative RT–PCR to monitor the levels of *sod-2* and *sod-3* mRNAs after *sod-2* RNAi treatment of the wild-type and *daf-2* and *clk-1* mutants (Figure 6). Both *sod-2* and *sod-3* mRNA levels in untreated *daf-2* and *clk-1* mutants are elevated compared to the wild type. Treatment with *sod-2* RNAi reduces *sod-2* mRNA to very low levels in all three backgrounds (Figure 6A). The same treatment also reduces *sod-3* levels considerably in all backgrounds. This indicates that *sod-2* RNAi treatment also affects the level of *sod-3*, likely because of the sequence homology, which is consistent with the absence of MnSOD activity after *sod-2* treatment of the wild type (Figure 1A). Note, however, that an increased level of mRNA expression does not guarantee an increase in the level of protein present, as we do not observe an elevation of SOD-2 protein in *clk-1* mutants (Figure 1B). Thus, increased levels of *sod-2* and *-3* expression might or might not participate in the somewhat lower levels of protein carbonyls observed in *daf-2* mutants (Figure 3B). However, similar increases seem to have only a minimal effect on carbonyl levels in *clk-1* mutants (Figure 3B). Furthermore, *sod-2* RNAi has no effect on the life span of *daf-2* (Figure 4). Thus, as the total level of expression of mitochondrial *sod* genes is reduced by *sod-2* RNAi treatment (Figures 1 and 6), the levels observed without treatment are not necessary for the long life of the mutants. The situation is similar for *clk-1*, where the total level of *sod-2* and *-3* expression in response to *sod-2* RNAi treatment is lower than without treatment and thus cannot explain the absence of an increase in carbonyls in response to *sod-2* RNAi. Nor, of course, can it explain why *sod-2* RNAi prolongs the life span of *clk-1* (Figure 4).

DISCUSSION

Alterations in the regulation of SOD expression: We have found increased expression of *sod-1*, *-2*, and *-3* in several long-lived strains, but these changes did not correlate with life span, growth rate, embryonic lethality, or brood size as measured here (Tables 1–4 and Figure 4) or as published previously (Wong *et al.* 1995; Lakowski and Hekimi 1996; Feng *et al.* 2001). Furthermore,

sod(RNAi) is equally effective in reducing SOD expression to background levels in all strains (data not shown), and we have not found a correlation between the level of *sod*'s and the sensitivity of the growth rate or of the life span to *sod(RNAi)*.

Testing the ROS toxicity theory of aging: From the point of view of evolutionary theory, it is a reasonable hypothesis to speculate that aging is due to the accumulation of unrepaired damage, as there is an expected trade-off between reproduction and the costs associated with somatic maintenance (Hekimi *et al.* 2001b). Toxicity from the ROS produced in mitochondria is an obvious candidate for an important source of damage in this process (Sohal 2002; Droge 2003). In particular, aging appears to be strongly correlated with increases in oxidative stress and oxidative damage accumulation (Beckman and Ames 1998), in particular in the mitochondria (Van Remmen and Richardson 2001). Yet a causal role for ROS toxicity in bringing about aging remains a fundamentally unproven hypothesis.

To test this hypothesis, we have been analyzing mutant strains of *C. elegans* that display an increased life span. Beyond the initial hypothesis of an involvement of ROS in life-span determination, there were numerous reasons to suppose that the phenotypes of these strains, including their life span, are due to altered ROS metabolism. One of the main sources of cellular superoxide production is believed to be the bifurcating transfer of electrons from ubiquinone to the "Rieske" iron–sulfur protein and the cytochrome *b* of complex *III* of the mitochondrial respiratory chain (Raha and Robinson 2000). Two of the three long-lived mutants that we are analyzing are directly involved in this process: *isp-1 (qm150)* is a point mutation in the iron–sulfur protein (Feng *et al.* 2001), and *clk-1* affects the biosynthesis of ubiquinone (Miyadera *et al.* 2001). The third gene, *daf-2*, encodes an insulin-receptor-like kinase (Kimura *et al.* 1997) and affects a variety of processes in worms, including their resistance to oxidative stress (Honda and Honda 2002). The one short-lived mutant that we have also been studying, *mev-1*, is a point mutation in a subunit of mitochondrial complex *II* (Ishii *et al.* 1998).

Lack of support for the ROS toxicity theory of aging: Strikingly, although several of the mutant strains tested have increased SOD levels (Figure 1B) and although the RNAi treatments are effective (Figure 1A), frequently appear deleterious (Tables 1–4 and Figure 2), and can be shown to increase measurable oxidative damage to proteins in the wild-type and mutant backgrounds (Figure 3), the RNAi treatments do not shorten the increased life span of the long-lived mutants (Figure 4) or worsen the short life span of *mev-1* (Figure 4). Furthermore, we observe some paradoxical effects; for example, *sod-1(RNAi)* against *daf-2* and *sod-2(RNAi)* against *clk-1* increase life span. Thus, our results provide no substantial support for the hypothesis that the increased life span of most of the mutants that we have examined is due to low levels of ROS damage. Our results are consistent with observations using pharmacological SOD mimetics that were capable of protecting from experimental increases in oxidative stress, but were not capable of positively affecting wild-type life span in *C. elegans* (Keaney *et al.* 2004).

Our findings about the role that oxidative stress might or might not play in the long life span of *daf-2* mutants are not conclusive. Indeed, *daf-2* mutants are unaffected by our treatments except in a paradoxical fashion: *sod-1* RNAi decreases oxidative damage and increases life span. Thus, we have not succeeded in uncoupling low oxidative stress from the increased life span of *daf-2* mutants.

A superoxide-independent role for electron transport in life-span determination: Electron transport in the mitochondrial respiratory chain generates superoxide at sites where electrons are transferred from prosthetic groups to ubiquinone and vice versa (Raha and Robinson 2000). Several of the mutants that we have examined (*clk-1*, *isp-1*, and *mev-1*) are closely involved in the process of electron transport (Ishii *et al.* 1998; Feng *et al.* 2001; Miyadera *et al.* 2001). Thus, it is likely that these mutants have altered ROS metabolism (Feng *et al.* 2001; Senoo-Matsuda *et al.* 2001; Shibata *et al.* 2003), as indicated by the various effects of SOD knockdowns on the mutant phenotypes of the long-lived mutants (Tables 1–4). Yet our results indicate that it is not a reduction of oxidative damage that is responsible for the observed increased life span. Our results therefore suggest that electron transport has a role in life-span determination that is independent of its role in the production of oxidative damage, as has been suggested previously on different grounds (Dillin *et al.* 2002; Hekimi and Guarente 2003). Our findings of the dramatic effect of *sod-2(RNAi)* on *clk-1* (Figure 5) can also be interpreted in this light. That is, that the reduction of the activity of the mitochondrial matrix SOD-2 in the *clk-1* background, with its altered quinone profile, damages the respiratory chain in a way that we do not yet understand, but that is favorable for life span. In fact, the phenotype of *clk-1* mutants treated with *sod-2(RNAi)* is highly reminiscent of that obtained by knocking down subunits of mitochondrial complexes (Dillin *et al.* 2002; Lee *et al.* 2003), which also prolongs life span.

Oxidative stress and aging: The possibility that the oxidative stress theory is not generally valid has been considered by others (Koc *et al.* 2004; Kujoth *et al.* 2005), but remains surprising (Hekimi and Guarente 2003). However, most evidence about oxidative stress and aging points to a general correlation and not necessarily to a causal relationship. A scientific theory has to be falsifiable to be meaningful. Thus, although a single study cannot address at once all the observations that previously appeared to be consistent with the theory, sound experimental results that are inconsistent with a theory represent an important challenge to that theory.

For example, although for technical reasons we could examine only oxidative damage to proteins, but not to other macromolecules, no part of the oxidative stress theory of aging suggests that damage to proteins does not matter in bringing about aging or suggests how an increase of oxidative stress that is sufficient to damage proteins could fail to damage other macromolecules.

More generally, it is of interest that our results—which suggest that oxidative stress from mitochondrial respiration is likely not causal in aging in the mutants that we have examined—do not imply that oxidative stress does not play a role in producing the aged phenotype in these mutants or in any other organism. In fact, a sharp increase of oxidative damage with age is well documented in many organisms and tissues. For example, oxidative damage to DNA (Hamilton *et al.* 2001), measured as an increase in 8-hydroxguanosine, as well as oxidative damage to proteins (Yasuda *et al.* 1999; Levine and Stadtman 2001), measured as an increase in carbonyls, increase dramatically with age. Interestingly, the increase in carbonyls is most marked later in life, at a time when in fact the organisms are already aged (Levine and Stadtman 2001), which further implies that the increase itself cannot be causal in aging.

The higher oxidative damage of the aged phenotype suggests that aged organisms have higher level of ROS. This is supported by the finding that in Drosophila transgenic overexpression of superoxide dismutase can increase life span (Sun *et al.* 2002), especially in short-lived strains (Orr and Sohal 2003). However, this again does not indicate that oxidative stress is the cause of aging but only that oxidative damage is deleterious and that relieving it in part through a genetic or pharmacological intervention might improve the health of aged organisms. In summary, while our observations suggest that oxidative stress is not the cause of aging, observations of high oxidative stress and damage in old organisms, on the other hand, are consistent with the notion that increased oxidative stress is a consequence of aging.

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