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Original Contribution

SUPEROXIDE DISMUTASE MIMETICS ELEVATE SUPEROXIDE DISMUTASE ACTIVITY IN VIVO BUT DO NOT RETARD AGING IN THE NEMATODE *Caenorhabditis elegans*

MICHELLE KEANEY,[*,1] FILIP MATTHIJSSENS,[†,1] MARTYN SHARPE,[‡] JACQUES VANFLETEREN,[†] and DAVID GEMS*

*Department of Biology, University College London, London WC1E 6BT, UK; [†]Department of Biology, Ghent University, Ledeganckstraat 35, B-9000 Ghent, Belgium; and [‡]Miriam Marks Division of Neurochemistry, Institute of Neurology, Queen Square, London WC1N 3BG, UK

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Abstract—According to the oxidative damage theory a primary cause of aging is the accrual of molecular damage from reactive oxygen species (ROS), particularly superoxide and its derivatives. This predicts that treatments that reduce ROS levels should retard aging. Using the nematode *Caenorhabditis elegans*, we tested the effects on stress resistance and life span of treatment with EUK-8 and EUK-134, synthetic mimetics of the antioxidant enzyme superoxide dismutase (SOD), which neutralises superoxide. Treatment with SOD mimetics elevated in vivo SOD activity levels, particularly in mitochondria, where up to 5-fold increases in SOD activity were recorded. Treatment with exogenous SOD mimetics did not affect endogenous protein SOD levels. Where life span was reduced by the superoxide generators paraquat and plumbagin, EUK-8 treatment increased life span in a dose-dependent fashion. Yet in the absence of a superoxide generator, treatment with EUK-8 or EUK-134 did not increase life span, even at doses that were optimal for protection against pro-oxidants. Thus, an elevation of SOD activity levels sufficient to increase life span when it is limited by superoxide generators does not retard aging in the absence of superoxide generators. This suggests that *C. elegans* life span is not normally limited by levels of superoxide and its derivatives. © 2004 Elsevier Inc. All rights reserved.

Keywords—*Caenorhabditis elegans*, Superoxide dismutase mimetics, Aging, Life span, Survival, Free radicals, Paraquat

INTRODUCTION

One of the feats of biological homeostasis is the ability to withstand an atmosphere containing 21% oxygen, a highly reactive and potentially toxic gas [1]. It has been suggested that aging, the functional decline and increase in mortality with age seen in almost all metazoan species [2], is the result of accumulated molecular damage from reactive oxygen species (ROS) [3–5]. It has been estimated that some 1–3% of O_2 consumed by mitochondrial oxidative phosphorylation undergoes univalent reduction, forming the superoxide ($O_2^{\bullet -}$) radical [6]. This can be converted by the antioxidant enzyme superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2), which in turn may be converted into water and molecular oxygen by the enzyme catalase. It has been proposed that observed correlations between the rates of metabolism and aging reflect variation in levels of production of the highly reactive and toxic $O_2^{\bullet -}$ anion [7,8].

There is ample correlative evidence that indirectly supports the oxidative damage theory, such as observed increases, with increasing age, of molecular damage and ROS production, and correlations among animal species between life span and ROS production rates (reviewed in [8]). However, a more direct test of this theory is whether or not experimental interventions that alter ROS levels affect the rate of aging. Although it has been shown that treatments that increase oxidative stress promote molecular damage and reduce life span [8–10], ROS-induced pathology could shorten life span by a mechanism distinct from that of normal aging. By contrast, if reduc-

Address correspondence to: Dr. David Gems, Department of Biology, University College London, London WC1E 6BT, UK; Fax: +44 207 679 7096; E-mail: david.gems@ucl.ac.uk.

[1] These authors contributed equally to this work.

ing ROS and the damage that they cause leads to an increase in life span, then the oxidative damage theory must be correct. Reductions of intracellular ROS may, in principle, be achieved by increasing antioxidant defenses in the cell, either by dietary supplements of antioxidants or by overexpression of genes encoding antioxidant enzymes, such as SOD and catalase.

Over the years numerous studies of the effects of antioxidant administration on life span in a range of animal species have failed to provide clear support for the oxidative damage theory of aging (reviewed in [8]). Studies of overexpression of antioxidant enzymes on aging, largely conducted using *Drosophila*, have yielded mixed results [8]. Although a number of *Drosophila* studies have shown that overexpression of Cu/Zn SOD increases life span (see, e.g., [11–13]), it has been argued that the magnitude of this effect is linked to short life span in the control flies [5]. This raises the possibility that Cu/Zn SOD overexpression rescues the effects of deleterious genotypes or culture conditions, rather than retarding aging. This is supported by the weak effects on aging of overexpression of human Cu/Zn SOD in motor neurons of longer-lived *Drosophila* wild isolates [14]. In mice, constitutive overexpression of Cu/Zn SOD does not increase life span [15]. Moreover, mice heterozygous for loss of function of the Sod2 Mn SOD gene age normally, despite showing elevated levels of DNA damage [16].

To test the oxidative damage theory of aging we have employed the synthetic catalytic antioxidants EUK-8 and EUK-134, which have SOD activity in vitro and in vivo [17], and also slight catalase activity. We tested the effects of treatment with those compounds on SOD and catalase activity levels in vivo, resistance to oxidative stress, and life span in *C. elegans*. Our results show that treatment with such mimetics results in dose-dependent increases in SOD activity in vivo, particularly within mitochondria, and protection against two different superoxide generators. However, they have no ability to extend life span, contrary to a prior report.

METHODS

C. elegans stocks

The following strains were employed: N2 (wild type), LGI:GR1307 *daf-16(mgDf50)*, LGIV:NS3227 *daf-18(nr2037)*. The male stock of N2 provided by the *Caenorhabditis* Genetics Center (CGC) was used, as it represents the best estimate of a true wild type [18]. (Notes: (1) Hermaphrodites of N2 were used in this study. (2) Although the genetic background of the two mutant strains LGI:GR1307 *daf-16(mgDf50)* and LGIV:NS3227 *daf-18(nr2037)* is N2, it is possible that there are slight background differences from the CGC N2 male stock).

Chemicals

EUK-8 and EUK-134 were prepared at the Institute of Neurology, London, by standard methods [19, 20]. EUK-8 was also obtained from Calbiochem, San Diego, CA, USA. All tests involving *C. elegans* used EUK-8 and EUK-134 prepared at the Institute of Neurology. EUK solid was stored in a desiccator at room temperature. Stock solutions were prepared using distilled water, sterilised using a 0.2-μm filter, and stored at 4°C.

Testing in vitro SOD and catalase activities of EUK-8 and EUK-134

SOD activity was measured using an assay involving the inhibition of superoxide-induced lucigenin chemiluminescence by SOD [21]. Duplicate 6.7 μl aliquots were taken from a sample dilution series and added to microtitre plate wells. Next, 20 μl aliquots of xanthine oxidase (XO) reagent (XO diluted in double-distilled water such that the blank reaction containing 6.7 μl water, 20 μl XO dilution, and 174 μl reaction mixture yielded approximately 1.2 × 105 counts/s) and 174 μl of reaction mixture (5.2 ml 0.1 M glycine, with or without 1 mM EDTA, adjusted to pH 9.0 with NaOH, 10 ml 0.108 mM xanthine, 2.1 ml 1 mM lucigenin, 1.2 ml water to give a total of 18.5 ml) was added quickly by using a multichannel pipet. Luminescence was measured for 0.1 s during the time span required for 25 consecutive plate measurements at 25°C using the Victor2 Multilabel Counter. One unit of SOD activity is defined as the amount of SOD able to reduce the luminescence intensity by 50%. The homogenate fraction (dilution) reducing luminescence by 50% was derived mathematically from plots of the luminescence intensities measured as a function of the homogenate fraction. Catalase was assayed as previously described [22].

EUK-8 and EUK-134 exhibited SOD activity in vitro as expected. According to Baudry et al., SOD activity of EUK-8 should correspond to about 0.12% of that of SOD protein on a molar basis [17]; we estimate values of 0.5–1% for EUK-8 and EUK-134, depending on the source of antioxidant (i.e., Institute of Neurology, or Calbiochem). The SOD activity of both EUK-8 and EUK-134 is completely inhibited by 1 mM EDTA.

Catalase activities were 0.60 and 0.50 U/mg in EUK-8 and EUK-134, respectively, comparable to an activity level of 0.48 U/mg in a commercial preparation of EUK-8.

Measurement of SOD and catalase activities in EUK-treated animals

Wild-type (N2) hermaphrodites were grown at 24°C on agar plates seeded with *Escherichia coli*. At stage L4/

young adult, the animals were transferred to S buffer [23] (20°C) to which different EUK-8 concentrations, 1×10^9 cells/ml frozen *E. coli*, were added, as was fluorodeoxyuridine (FUdR, 50 μM final concentration) to block reproduction. After 4 days, the animals were harvested and sampled for protein content and SOD and catalase activities. The cultures were washed using Percoll [24] and dense sucrose [23] and repeated washing steps in S buffer for at least half an hour, to ensure that there would be little residual EUK-8 in the medium or in the gut of the sampled animals. Reaction mixture without EDTA was used to measure total SOD activity (i.e., endogenous protein SOD, and added SOD mimetic). To measure protein SOD alone, EDTA was included in the reaction mixture.

Measurement of SOD activity in mitochondria

Mitochondrial fractions were prepared by rupturing the nematodes with a Polytron homogenizer (40 s at 14,000 rpm) in MSM buffer (220 mM mannitol, 10 mM sucrose, 5 mM Mops, pH 7.4, with KOH) containing 0.2% BSA. These homogenates were centrifuged twice in MSM buffer at 380*g* (5 min) to remove debris and intact worms. The supernatant was spun down at 4500*g* (5 min); this mitochondrial pellet was washed twice in MSM buffer without BSA (5 min, 4500*g*). Debris, pellets, and supernatants were pooled and centrifuged for 30 min at 14,000 rpm, and the supernatant was retained as a cytosolic fraction. Mitochondrial and cytosolic fractions were frozen, thawed, and treated with 0.8% CHAPS before SOD measurement. SOD assays were performed for three cultures treated with 0, 0.25, and 0.5 mM EUK-8, without or with 1 mM EDTA.

Effect of paraquat on cyanide-resistant oxygen consumption

Animals were raised from eggs (alkaline hypochlorite extraction) [23] synchronised to the L1 stage in S buffer. L1 hermaphrodites were transferred to agar plates seeded with *E. coli* at 24°C. At stage L4/young adult, the nematodes were transferred to liquid monoxenic medium, containing approximately 1×10^9 cells/ml *E. coli*. Fifty micromolar FUdR and 0 mM (control) or 2 mM paraquat was added to this medium and the animals were cultured 4 days at 20°C.

Before the oxygen consumption assay, the nematodes were washed on a Percoll gradient to remove dead animals and subsequently in sucrose to remove debris and bacteria. Finally, the nematodes were washed three times in S buffer and once in axenic medium (to protect against starvation). Oxygen consumption was measured using the respirometer from Strathkelvin (Glasgow, Scotland) as described previously [25]. Respiration was expressed as micromoles of O_2 consumed per hour per milliliter of nematode suspension. These measurements were run in 1.0 ml axenic medium containing approximately 10^3 nematodes for measurements without KCN and approximately 10×10^3 nematodes with KCN. Samples from the same nematode suspension were used each time for measuring respiration with or without 1 mM KCN. Cyanide was added to the samples directly before the oxygen consumption measurement, and cyanide-resistant respiration was calculated from the linear part of the plot, which appears less than a minute after KCN addition, after a shorter period of massive O_2 consumption, which represents the period in which KCN is not yet fully blocking normal respiration. Oxygen consumption in 1.0 ml cells containing axenic medium with or without 1 mM KCN was used as a blank measurement.

Comparing cyanide-resistant respiration (μmol O_2/ $h \cdot ml_{\text{nematode suspension}}$) with normal respiration (μmol O_2/ $h \cdot ml_{\text{nematode suspension}}$) yielded the final estimate of cyanide-resistant respiration.

Life span measurements

A new cultivar of *E. coli* strain OP50 was obtained from the *Caenorhabditis* Genetics Center prior to the commencement of these trials. Laboratory stocks of N2 CGCM were replaced every 6 months with a frozen stock. Ten millimolar aqueous stocks solution of EUK compounds were used to prepare a range of drug concentrations in a suspension of *E. coli* in S medium [23], to give a final concentration of 1×10^9 cells/ml. Treatment media were prepared within 48 h of transfer. After this time, unused media were discarded and fresh solutions made. *E. coli* was grown in minimal medium [23] and washed once with S medium, and the cell concentration estimated using a Helber bacterial counting chamber.

Test animals were picked as fourth-stage larvae (L4s) from populations raised at 20°C on NGM agar plates seeded with *E. coli* [23]. In some trials, experimental animals were raised from eggs obtained by alkaline hypochlorite extraction of gravid hermaphrodites [23]. Eggs were placed into monoxenic culture. Once they had reached L4 stage (Day 0 of life span estimates), animals were placed in populations of 20 in 15 mm flat-bottom wells in 24-well Costar plates (Corning Inc., Corning NY, USA), each containing 500 μl of treatment medium. Worms were raised and maintained at 20°C. Mortality was scored at the time of transfer. Animals were scored as dead when they failed to move in response to agitation with a platinum wire.

Replication of previous study of the effects of EUK-8 and EUK-134 on life span

Animals were maintained in a suspension of *E. coli* in S medium [23], in multiwells. Animals were not exposed

to SOD mimetics during development; in the Melov et al. study, increased life span was seen in trials both with and without SOD mimetics present during development. The concentration of *E. coli* used is important, because this affects both fertility and life span in *C. elegans* [26]. Although the concentration of *E. coli* used in the Melov et al. study was not specified, we estimated it as that which gave the same level of fertility as in the prior study (Fig. 4A). Animals were transferred to fresh medium daily during the reproductive period, and every 2–3 days thereafter.

Fertility measurements

Single L4 wild-type (N2) hermaphrodites were placed in microtitre wells containing 75 μl monoxenic culture medium containing *E. coli* in a range of concentrations, and transferred to fresh wells every second day until the end of egg laying. After being pipetted onto agar plates seeded with *E. coli*, progeny were counted and removed using an aspirator.

Statistical analysis

Mortality data were subjected to Kaplan–Meier survival analysis using the JMP 5 statistical analysis program (SAS Institute Inc., Cary, NC, USA). The life spans of EUK compound-treated populations were compared with those of untreated controls using the log rank test.

RESULTS

EUK-8 and EUK-134 increase SOD levels in vivo

In this study we aimed to establish whether treatment with SOD mimetics can elevate SOD levels in vivo and increase resistance to superoxide generators and, if so, whether they can extend life span, as predicted by the oxidative damage theory of aging. We first verified that our SOD mimetic preparations exhibited the expected SOD and catalase activity levels in vitro. Enzymatic activities in vitro of EUK-8 and EUK-134 were comparable to those in previous reports (data not shown). In each case, measured SOD activity levels were 0.6% that of protein SOD, slightly lower than that of EUK-8 from a commercial source (1.0% of protein SOD, Calbiochem, San Diego, CA, USA).

We then tested whether treatment of wild-type adult *C. elegans* with EUK-8 or EUK-134 increased total SOD and catalase activity levels in vivo. EUK-8 or EUK-134 at 0.05 mM did not increase total SOD levels in whole homogenates from treated worms (p = .14 and .51, Student's t test), whereas 0.5 mM SOD mimetic increased total SOD activity (i.e., the sum of EUK SOD activity and endogenous protein SOD activity) by up to 90% (p = .01 and .02) (Figs. 1A, 1B). Such increases in SOD activity levels are comparable to the maximal increases seen in long-lived *age-1* and *daf-2* mutant adults: +146% and +106%, respectively [27,28]. EUK-8 and EUK-134 did not differ in their effects on total SOD levels in treated animals (p = .96, 0.5 mM). Neither EUK-8 or EUK-134 caused detectable increases in catalase levels in treated animals (data not shown).

SOD mimetic activity concentrated in mitochondria

According to the oxidative damage theory, the mitochondria are a major site of both superoxide production and oxidative damage [29]. Thus, if SOD mimetic treatment is to be a meaningful test of this theory, it is important that these compounds are capable of entering the mitochondria. We therefore examined SOD activity levels in mitochondria from EUK-8-treated *C. elegans*. Treatment with 0.25 and 0.5 mM EUK-8 resulted in 4- and 5-fold increases in SOD activity, respectively (p = .048 and .01) (Fig. 1C). By contrast, in the cytosolic fraction relatively small but significant increases in SOD activity were observed (p = .01 and .006 for 0.25 and 0.5 mM EUK-8, respectively) (Fig. 1C). Thus, EUK-8 is concentrated within mitochondria in treated nematodes, and relatively small increases in SOD activity occur in the cytosol.

Endogenous SOD levels unaffected by SOD mimetics

The addition of an exogenous source of SOD activity did not alter endogenous SOD activity. This could be established because EDTA inhibits the enzymatic activity of SOD mimetics but not protein SOD, presumably by chelating the Mn^{+} from EUK-8 and EUK-134. EDTA-treated extracts from SOD mimetic-treated nematodes showed no change in SOD activity (Fig. 1). Treatment with EDTA *per se* did not affect endogenous SOD activity levels.

Paraquat increases cyanide-resistant oxygen consumption

Treatment with SOD mimetics results in elevated SOD activity levels in vivo in *C. elegans*. But does this lead to a reduction in levels of superoxide? We tested this by establishing whether treatment with EUK-8 protects against a superoxide-generating compound. The bipyridyl herbicide paraquat (1,1′-dimethyl-4,4′-dipyridylium) is a redox cycling agent [1], which consumes oxygen and generates superoxide.

If superoxide causes aging, as the oxidative damage theory suggests, then treatment with paraquat should result in accelerated aging and reduced life span. We tested the effect of paraquat on *C. elegans* life span over a range of doses. Although at high concentrations, paraquat is highly toxic (data not shown), 2 mM paraquat causes a moderate (46–64%) reduction in median life

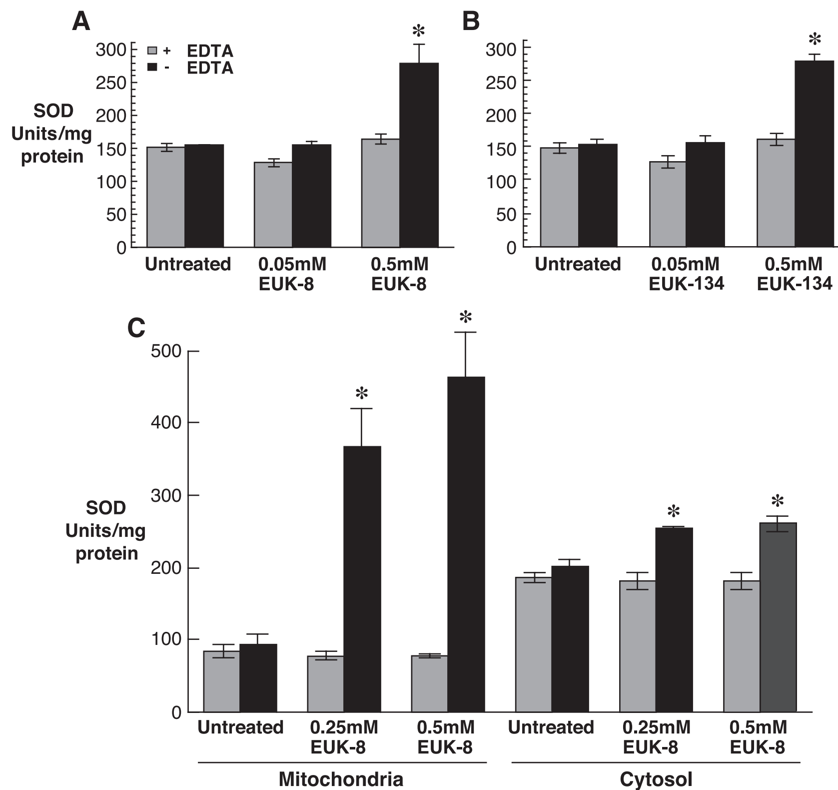

Fig. 1. Treatment with SOD mimetics increases SOD activity levels in vivo. (A, B) Whole lysates. (A) EUK-8. (B) EUK-134. (C) Effects of treatment with EUK-8 on SOD levels in mitochondria and cytosol. EDTA (1 mM) in the assay medium abolishes mimetic SOD activity but not that of protein SOD. Addition of SOD mimetics does not affect protein SOD activity levels. $*p < .05$ that SOD activity level is the same as in non-SOD mimetic-treated nematodes.

span, which may or may not reflect accelerated aging (Fig. 2A, Table 1).

Does 2 mM paraquat generate superoxide in *C. elegans*? The production of superoxide by redox cycling compounds consumes oxygen, and this can be detected as an increase in cyanide-resistant oxygen consumption [30]. We found that 2 mM paraquat caused a 77% increase in cyanide-resistant oxygen consumption (Fig. 3), consistent with action as a superoxide generator in *C. elegans*. It was previously reported that 1 mM paraquat does not increase cyanide-resistant oxygen consumption in *C. elegans* [30].

SOD mimetics extend life span in paraquat- and plumbagin-treated nematodes

Although it is unclear whether superoxide levels limit life span during normal aging, they presumably do in animals treated with 2 mM paraquat. By testing the effect of EUK-8 on life span in paraquat-treated populations, we asked the question: Where a superoxide generator limits life span, is EUK-8 capable of extending life span? We found that EUK-8 does indeed protect against the life-shortening effects of paraquat, in a dose-dependent fashion (Fig. 2B, Table 1), although it was not able to restore life span to normal; possibly this reflects a second component of paraquat toxicity. As EUK-8 concentration was increased, maximal protection occurred at 0.25 mM (Table 1); increasing the EUK-8 dose from 0.25 to 0.5 mM did not further rescue paraquat-induced life shortening in three of four trials. Five millimolar EUK-8 reduced life span, in the presence and absence of paraquat (Table 1). These results are consistent with recently reported protective effects of SOD mimetics against the lethal effect of high doses of paraquat [31].

To confirm that SOD mimetics protect against superoxide generators generally, rather than paraquat in particular, we tested the capacity of EUK-8 to protect against life shortening induced by the naphthoquinone redox cycling compound plumbagin, which was previously shown to increase cyanide-resistant oxygen consumption in *C. elegans* [30]. EUK-8 extended life span in populations exposed to 50 μM plumbagin in a manner similar to that seen in paraquat-treated populations. EUK-8 at 0.25 mM increased mean life span of plumbagin-treated *C. elegans* by 40%, from 12.4 ± 0.3 to 17.3 ± 0.5 days ($p < .0001$, log rank test; number of deaths scored: plumbagin alone, 82; plumbagin + EUK-

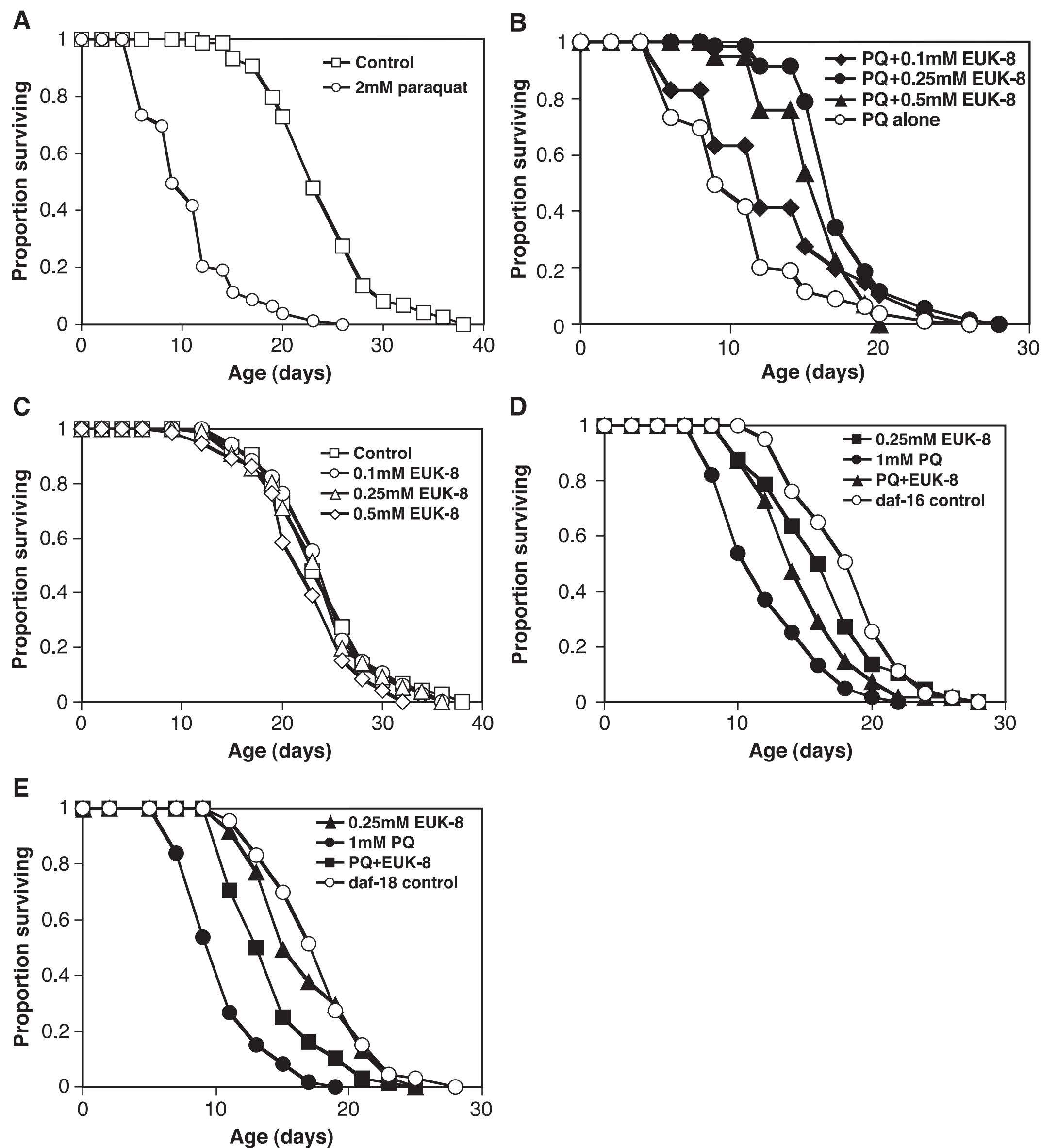

Fig. 2. EUK-8 extends life span in paraquat-treated nematodes. (A) Life-shortening effect of 2 mM paraquat (PQ). (B) Dose-dependent increase in life span by EUK-8 in paraquat-treated *C. elegans*. (C) The same doses of EUK-8 do not increase life span in the absence of paraquat. (D, E) EUK-8 rescues the life-shortening effect of paraquat in hormesis-defective *daf-16* and *daf-18* mutants. One millimolar paraquat was used in trials involving *daf-16* and *daf-18*, as these strains exhibit elevated sensitivity to paraquat toxicity.

8, 60). Similar results were obtained using 0.5 mM EUK-8 (data not shown).

0.25 mM EUK-8 provides optimal protection against paraquat, but does not retard aging

We have shown that in animals in which life span is limited by superoxide generators, administration of EUK-8 increases life span. Therefore, if superoxide levels limit life span in animals cultured under nonstress conditions (i.e., if the oxidative damage theory is correct), SOD mimetics should increase their life span too. However, treatment with EUK-8 did not increase life span, but instead significantly decreased it at 0.5 mM in all trials (Fig. 2C; Table 1). We also tested the effect of EUK-8 doses across a range from that producing no effect on life span (0.1 mM, 0.25 mM) to that that shorten life span (0.5 mM), and saw no increase in life span. Thus, it is unlikely that we have missed a narrow dose window at which life extension occurs, such as that described in *Drosophila* for 4-phenylbutyrate [32]. It is striking that 0.25 mM EUK-8, which provides maximal protection against paraquat (Fig. 2B), has no effect on normal aging (Fig. 2C).

Table 1. Effect of EUK-8 on Life Span in the Presence and Absence of Paraquat

Strain	Paraquat (mM)	EUK-8 (mM)	Mean life span ± SE	Median life span	% Difference from EUK-8 (median)	*N* (censors)	*p* (log-rank)[a]
Trial 1							
N2	2.0	0	9.2 ± 0.3	9	—	79 (21)	—
N2	2.0	0.05	9.6 ± 0.4	9	0	76 (23)	.1097
N2	2.0	0.1	9.4 ± 0.4	9	0	79 (21)	.1785
N2	2.0	0.25	10.9 ± 0.4	11	+22	90 (10)	<.0001
N2	2.0	0.5	11.4 ± 0.3	11	+22	91 (9)	<.0001
N2	2.0	1	11.8 ± 0.3	13	+44	89 (11)	<.0001
N2	2.0	5	3.3 ± 0.1	4	−55	101 (0)	<.0001
Trial 2							
N2 1×10^9 cells/ml	0	0	22.3 ± 0.6	24	—	80 (15)	—
N2	0	0.1	22.6 ± 0.6	24	0	86 (14)	.6448
N2	0	0.25	20.8 ± 0.6	21	−12.5	88 (12)	.1848
N2	0	0.5	19.2 ± 0.6	18	−25	87 (13)	.0002
N2	2.0	0	13.5 ± 0.7	13	—	95 (5)	—
N2	2.0	0.1	10.9 ± 0.4	9	−31	79 (9)	.0014
N2	2.0	0.25	13.7 ± 0.7	13	−0	72 (8)	.7225
N2	2.0	0.5	13.9 ± 0.5	15	+15	58 (7)	.9616
Trial 3							
N2 1×10^9 cells/ml	0	0	23.3 ± 0.7	25	—	76 (24)	—
N2	0	0.1	23.3 ± 0.6	23	−8	85 (15)	.8043
N2	0	0.25	22.1 ± 0.6	23	−8	23 (17)	.0812
N2	0	0.5	20.4 ± 0.5	19	−24	21 (17)	.0003
N2	2.0	0	10.2 ± 0.4	9	—	11 (1)	—
N2	2.0	0.1	10.8 ± 0.5	9	0	11 (2)	.2586
N2	2.0	0.25	14.5 ± 0.4	13	+44	15 (13)	<.0001
N2	2.0	0.5	14.4 ± 0.5	13	+44	15 (10)	<.0001
daf-16(mgDf50)	0	0	18.6 ± 0.5	20	—	20 (37)	—
daf-16(mgDf50)	0	0.25	16.8 ± 0.5	18	−10	66 (34)	.0533
daf-16(mgDf50)	1.0	0	12.4 ± 0.5	12	—	63 (37)	—
daf-16(mgDf50)	1.0	0.25	15.3 ± 0.5	14	+17	55 (41)	.0002
daf-18(nr2037)	0	0	18.0 ± 0.5	19	—	66 (38)	—
daf-18(nr2037)	0	0.25	17.0 ± 0.5	15	−21	61 (39)	.2510
daf-18(nr2037)	1.0	0	10.8 ± 0.4	11	—	63 (37)	—
daf-18(nr2037)	1.0	0.25	14.5 ± 0.4	13	+18	68 (32)	<.0001
Trial 4							
N2	0	0	24.3 ± 0.6	23	—	73 (29)	—
N2	0	0.1	24.6 ± 0.6	26	+13	67 (32)	.9909
N2	0	0.25	24.0 ± 0.6	26	+13	76 (32)	.7100
N2	0	0.5	22.5 ± 0.6	23	0	72 (30)	.0384
N2	2.0	0	10.8 ± 0.5	9	—	79 (20)	—
N2	2.0	0.1	13.1 ± 0.6	12	+33	87 (19)	.0022
N2	2.0	0.25	17.7 ± 0.4	17	+88	70 (27)	<.0001
N2	2.0	0.5	15.7 ± 0.4	17	+88	58 (24)	<.0001
daf-16(mgDf50)	0	0	17.7 ± 0.5	19	—	79 (20)	—
daf-16(mgDf50)	0	0.25	16.1 ± 0.6	15	−20	67 (33)	.0344
daf-16(mgDf50)	1.0	0	11.9 ± 0.4	12	—	80 (19)	—
daf-16(mgDf50)	1.0	0.25	16.8 ± 0.7	17	+42	79 (20)	.0001
daf-18(nr2037)	0	0	15.8 ± 0.5	17	—	40 (30)	—
daf-18(nr2037)	0	0.25	15.3 ± 0.6	17	0	39 (25)	.8962
daf-18(nr2037)	1.0	0	11.8 ± 0.5	12	—	68 (29)	—
daf-18(nr2037)	1.0	0.25	15.7 ± 0.5	15	+25	68 (27)	.0001

[a] Probability of being the same as treatment without EUK-8.

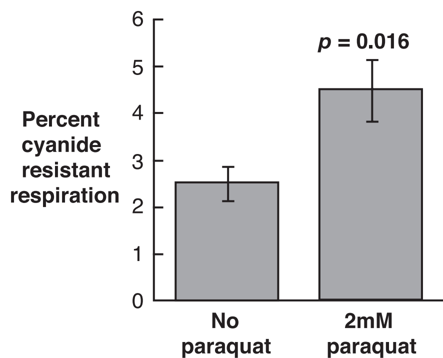

Fig. 3. Two millimolar paraquat increases cyanide-resistant oxygen consumption in *C. elegans*.

Evidence that EUK-8 rescue of life shortening by paraquat is independent of induced stress resistance

It remains possible that ROS do limit life span in the absence of superoxide generators, but that SOD mimetics are more effective at protecting against ROS in the presence of superoxide generators. This might be the case given that treatment with nonlethal stressors can result in hormesis—induction of endogenous defenses against stress [33]. For example, heat shock induces increased expression of chaperonin genes, increased thermotolerance, and extended life span [34,35]. It is therefore possible that only in the context of a superoxide-induced hormetic response does EUK-8 have sufficient potency against ROS to extend life span.

Induction of stress resistance and increased life span as the result of brief exposure to shock are suppressed by mutations in *daf-16* and *daf-18* [36], genes acting in the insulin/IGF-1 signalling pathway (reviewed in [37]). We therefore tested the effect of the null mutations *daf-16(mgDf50)* and *daf-18(nr2037)* on life extension by EUK-8 in paraquat-treated populations, reasoning that if EUK-8 provided better protection against ROS in the presence of a hormetic response, it should provide weaker protection against paraquat in these mutants. However, EUK-8 provided protection against paraquat in these mutants as in the wild type (Figs. 2D and 2E, Table 1). In addition, we tested the effects of 2 mM paraquat on endogenous SOD and catalase activity levels in wild-type animals. No induction of SOD or catalase activity levels by paraquat was observed (data not shown), consistent with earlier observations [38,39]. The effect of EUK-8 on life span in paraquat-treated *daf-16* and *daf-18* mutants suggests that life span extension by EUK-8 with but not without paraquat does not reflect hormetic enhancement of the effect of EUK-8 by paraquat.

EUK-8 and EUK-134 do not retard aging

It was previously reported that treatment with either EUK-8 or EUK-134 significantly extended life span in *C. elegans* in a dose-independent manner (at 0.05, 0.5, and 5 mM), by on average 44% [40]. These results differ from those reported here and in our earlier study, where

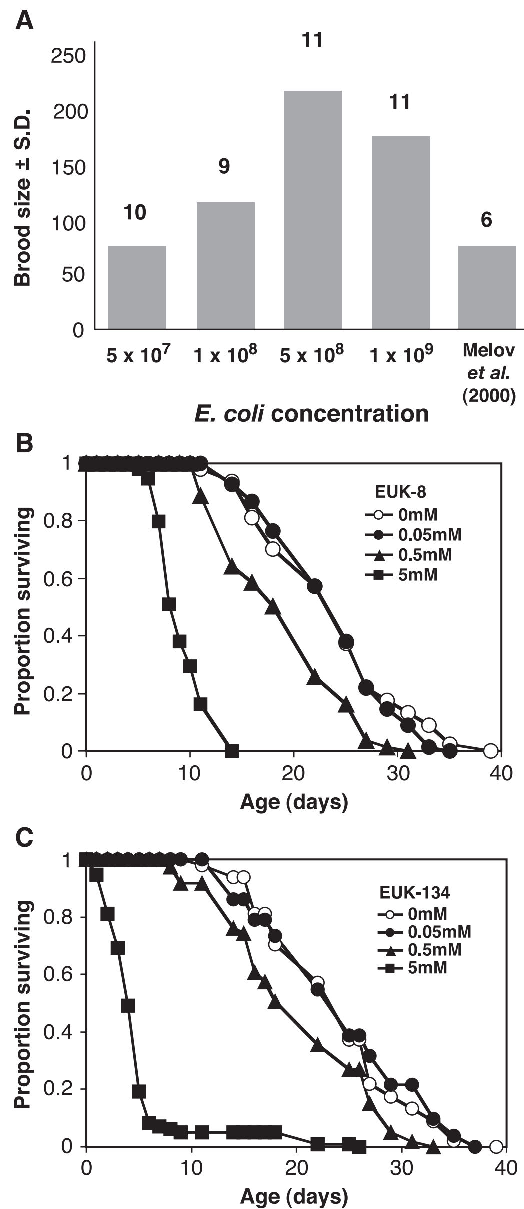

Fig. 4. Treatment with EUK-8 and EUK-134 results in dose-dependent reduction of life span. (A) Effect of bacterial concentration on brood size in wild-type *C. elegans*. This was measured to estimate the concentration of *E. coli* used in the Melov et al. study, where life span and brood size per nematode of 76 ± 14 (SD) was measured in an unknown *E. coli* concentration. In our trials, culture in 5×10^7 cells/ml gave a brood size of 75.3 ± 21. We therefore tested the effects on life span of EUK-8 and EUK-134 at two *E. coli* concentrations closely flanking this concentration: 2.5×10^7 cells/ml and 7.5×10^7 cells/ml. Numbers above bars: numbers of broods counted. (B, C) Effects of EUK-8 and EUK-134 on *C. elegans* survival. Here SOD mimetics were administered as previously described, using an *E. coli* concentration of 7.5×10^7 cells /ml. Similar results were obtained using 2.5×10^7 and 1×10^9 cells /ml (Table 2) [40].

Table 2. Treatment with EUK-8 and EUK-134 Does Not Increase Nematode Life Span

E. coli concentration	EUK-8 or EUK-134 (mM)	Mean life span ± SE	Median life span	% Difference from EUK-8 (median)	*N* (censors)	*p* (log-rank)[a]
EUK-8 Trial 1						
1×10^9	0	26.0 ± 1.0	25	—	80 (20)	—
1×10^9	0.05	25.2 ± 0.9	25	0	95 (5)	0.0625
1×10^9	0.5	20.1 ± 0.5	22	−22	48 (54)	<0.0001
1×10^9	5	7.7 ± 0.3	8	−68	36 (57)	<0.0001
2.5×10^7	0	24.0 ± 1.0	27	—	65 (34)	—
2.5×10^7	0.05	24.1 ± 0.7	27	0	84 (17)	0.2175
2.5×10^7	0.5	24.7 ± 0.8	27	0	46 (59)	0.1203
2.5×10^7	5	6.6 ± 0.1	7	−74	68 (32)	<0.0001
7.5×10^7	0	19.0 ± 0.3	25	—	86 (14)	—
7.5×10^7	0.05	20.1 ± 0.5	25	0	92 (5)	0.5888
7.5×10^7	0.5	19.3 ± 0.6	22	−22	80 (20)	<0.0001
7.5×10^7	5	9.4 ± 0.3	9	−64	95 (5)	<0.0001
EUK-8 Trial 2						
1×10^9	0	25.5 ± 1.1	24	—	44 (51)	—
1×10^9	0.05	25.4 ± 0.9	24	0	60 (40)	0.6513
1×10^9	0.5	18.9 ± 0.6	18	−25	92 (7)	<0.0001
1×10^9	5	5.3 ± 0.3	5	−79	100 (0)	<0.0001
2.5×10^7	0	25.9 ± 1.2	28	—	42 (58)	—
2.5×10^7	0.05	26.6 ± 1.1	28	0	54 (44)	0.7271
2.5×10^7	0.5	21.9 ± 0.6	22	−11	62 (39)	0.0510
2.5×10^7	5	4.6 ± 0.1	5	−82	89 (0)	<0.0001
7.5×10^7	0	25.9 ± 0.9	24	—	94 (4)	—
7.5×10^7	0.05	25.2 ± 1.1	26	+8	53 (47)	0.1648
7.5×10^7	0.5	19.8 ± 0.8	20	−17	40 (57)	<0.0001
7.5×10^7	5	4.5 ± 0.1	5	−79	100 (0)	<0.0001
EUK-134 Trial 1						
1×10^9	0	26.0 ± 1.1	25	—	62 (39)	—
1×10^9	0.05	23.8 ± 0.1	25	0	65 (33)	0.4405
1×10^9	0.5	18.1 ± 0.7	22	−12	58 (41)	0.0011
1×10^9	5	2.9 ± 0.7	3	−88	100 (0)	<0.0001
2.5×10^7	0	24.0 ± 1.3	27	—	48 (54)	—
2.5×10^7	0.05	24.4 ± 0.6	25	−7	28 (80)	0.0265
2.5×10^7	0.5	22.3 ± 1.0	25	−7	18 (82)	0.0075
2.5×10^7	5	10.0 ± 0.9	14	−48	94 (1)	<0.0001
7.5×10^7	0	18.2 ± 0.8	25	—	46 (59)	—
7.5×10^7	0.05	18.1 ± 0.4	25	0	66 (33)	0.1648
7.5×10^7	0.5	20.3 ± 1.0	22	−12	61 (39)	<0.0001
7.5×10^7	5	5.1 ± 0.8	4	−84	109 (2)	<0.0001
EUK-134 Trial 2						
1×10^9	0	25.5 ± 1.1	24	—	44 (51)	—
1×10^9	0.05	21.5 ± 0.8	20	−17	86 (13)	0.0100
1×10^9	0.5	15.1 ± 0.8	18	−25	101 (1)	<0.0001
1×10^9	5	1.7 ± 0.1	1	−96	98 (0)	<0.0001
2.5×10^7	0	25.9 ± 1.2	28	—	42 (58)	—
2.5×10^7	0.05	25.5 ± 1.0	26	−7	45 (55)	0.7271
2.5×10^7	0.5	23.0 ± 0.8	24	−14	88 (12)	0.0510
2.5×10^7	5	2.2 ± 0.1	2	−93	79 (24)	<0.0001
7.5×10^7	0	25.9 ± 0.9	24	—	94 (4)	—
7.5×10^7	0.05	24.1 ± 0.9	24	0	90 (5)	0.1648
7.5×10^7	0.5	19.9 ± 0.6	20	−17	90 (9)	<0.0001
7.5×10^7	5	1.4 ± 0.1	1	−96	100 (0)	<0.0001

[a] Probability of being the same as treatment without EUK-8 or EUK-134.

no increase in life span but instead a dose-dependent reduction in fecundity and life span, was seen [41]. However, in our previous preliminary report, SOD mimetics were administered under conditions similar but not identical to those in the Melov et al. study, and only EUK-8 was tested. We therefore retested the effect of both compounds on life span under conditions exactly as described by these authors [40]. Because the concentration of the bacterial food source (*E. coli*) used in the prior study was not specified [40], we performed tests at three concentrations of *E. coli*: 2.5×10^7 and 7.5×10^7 cells/ml, because 5×10^7 cells/ml resulted in fertility levels similar to those in the prior study (Fig. 4A), and 1×10^9 cells/ml, which is optimal for *C. elegans* fertility. No increase in life span was observed at any *E. coli* concentration with either compound: rather a dose-dependent decrease in life span was seen (Figs. 4B and 4C, Table 2). We conclude that administration of EUK-8 and EUK-134 as described by Melov et al. [40] does not retard aging in *C. elegan*s.

DISCUSSION

We have shown that treatment of *C. elegans* with SOD mimetics increases SOD activity levels in vivo, and that this leads to protection against the toxicity of two superoxide generators. However, treatment with SOD mimetics alone does not increase life span, even at a dose optimally protective against paraquat.

These results are consistent with dose-dependent toxicity observed in EUK-8- and EUK-134-treated houseflies [42] and *Drosophila melanogaster* (M. West and L. Partridge, personal communication). In houseflies, the dose dependence of toxicity is very similar to that seen here: 0.05 mM EUK-8 has little effect on life span, 0.5 mM reduces it slightly, and 5 mM reduces it substantially [42]. The reason for the discrepancy with the earlier *C. elegans* study [34] remains unclear. It is unlikely to be due to compound batch differences: although we prepared our own EUK-8 and EUK-134 for this study (because these compounds are not licensed for sale in the UK), we have shown that they elevate SOD activity levels in *C. elegans*. Moreover, in our earlier, preliminary report [41], EUK-8 from the same supplier as used in the Melov et al. [40] study was tested, with the same effects as seen here. No extension in life span has ever been seen in SOD mimetic-treated nematodes in the London or Ghent laboratory.

Although other findings have implied a role for ROS in *C. elegans* aging, none provide direct proof. For example, long-lived insulin/IGF-1 signalling (IIS) mutants, such as those associated with the genes *age-1* and *daf-2*, exhibit elevated levels of SOD and catalase [27,28,43]. Yet expression of many genes is altered in IIS mutants: recent DNA microarray studies have identified hundreds of genes for which expression is altered in *daf-2* mutants [44, 45]. Moreover, inactivation of the heat shock factor gene *hsf-1* suppresses the *daf-2* life extension trait without blocking overexpression of antioxidant genes such as *sod-3*, which encodes a Mn SOD [46].

An important finding is that culture in 1% oxygen increases *C. elegans* life span (+24%) relative to normoxic controls [10]. Yet it is not clear by what mechanism hypoxia increases life span. More generally, the observation that oxidative damage levels increase with age does not prove that oxidative damage causes aging, as many (if not all) forms of pathology are accompanied by increases in oxidative damage [1].

SOD mimetics increase SOD activity levels in vivo in *C. elegans*, particularly in mitochondria, and increase life span in populations treated with superoxide generators, yet not in nonstressed populations. Taken together, these results suggest that under nonstress culture conditions, *C. elegans* life span is not limited by superoxide and its derivatives. However, they do not entirely disprove the oxidative damage theory of aging. For example, it cannot be excluded that the sites of action of paraquat toxicity and of aging-associated ROS damage are different, such that EUK-8 can rescue the former, but not the latter. In *Drosophila* there is evidence that oxidative stress exerted by paraquat occurs largely in the cytosol and not in the mitochondria [47]. In *C. elegans*, EUK-8 protects against paraquat toxicity, despite evidence that the former compound is highly concentrated in the mitochondria. This suggests that EUK-8 acts as a superoxide scavenger in both cytosolic and mitochondrial compartments of the cell.

A standard explanation for why elevated SOD levels do not retard aging is that they lead to increased levels of hydrogen peroxide production; thus, increases in both SOD and catalase activity may be required to retard aging [48]. Yet this is unlikely to account for the failure of EUK-8 to extend life span in the present case, because if the capacity of EUK-8 to extend life span where limited by superoxide levels required elevated catalase levels, then EUK-8 would not be able to extend life span in paraquat-treated *C. elegans*. Moreover, there is little empirical evidence that the protective capacity of increased SOD is limited by catalase.

It was recently shown that overexpression of glutathione reductase or mitochondrially targeted catalase in *Drosophila* increases resistance to oxidative stress, but does not increase life span [49,50]. The authors of these studies proposed two hypotheses for how these findings could still be consistent with the oxidative damage theory. First, it was argued that the lower the level of ROS production, the more difficult it might be for antioxidant defenses to neutralise it. This could be

because rare oxidants will usually encounter and damage a target molecule before they encounter an antioxidant [49]. Perhaps this is true, but if so it would mean that the increased antioxidant defenses seen in a number of long-lived mutants are unlikely to contribute to this phenotype. Second, it was proposed that under optimal conditions, ROS levels are maintained at a low, optimal "set point." Reducing levels below this point might be deleterious, as ROS may function, e.g., in intracellular signalling or immune defense [50]. However, given that 0.25 mM EUK-8 gives optimal protection against paraquat, yet does not affect normal aging, this interpretation seems unlikely, unless it is true that lower levels of pro-oxidant are increasingly difficult to remove.

Another possible reason why elevation of antioxidant defenses may not retard aging is that exogenous or transgene-expressed antioxidants may cause compensatory reductions in endogenous antioxidant levels [5]. However, it is unlikely that such compensatory effects may account for the failure of EUK-8 and EUK-134 to extend life span, for the following reasons. First, treatment with SOD mimetics increases overall SOD activity in vivo, and does not depress endogenous SOD or catalase levels (Fig. 1, data not shown). Second, treatment with EUK-8 clearly can increase protection against superoxide sufficiently to extend life span where superoxide levels are elevated (Fig. 2, Table 1). However, we cannot rule out the possibility that paraquat treatment induces changes that enhance EUK-8 activity in vivo, in a *daf-16*- and *daf-18*-independent fashion. Nonetheless, the most parsimonious explanation for the failure of SOD mimetics to retard aging in *C. elegans* is that superoxide plays at most a minor role in limiting life span during normal aging in this organism.

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