Extension of Life-Span with Superoxide Dismutase/Catalase Mimetics

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We tested the theory that reactive oxygen species cause aging. We augmented the natural antioxidant systems of *Caenorhabditis elegans* with small synthetic superoxide dismutase/catalase mimetics. Treatment of wild-type worms increased their mean life-span by a mean of 44 percent, and treatment of prematurely aging worms resulted in normalization of their life-span (a 67 percent increase). It appears that oxidative stress is a major determinant of life-span and that it can be counteracted by pharmacological intervention.

Reactive oxygen species (ROS) primarily arise as by-products of normal metabolic activities and are thought to influence the etiology of age-related diseases (*1*, *2*). If ROS contribute to aging, then the aging process would be slowed if either the production of ROS were reduced or the endogenous antioxidant or repair activities were increased. Indeed, genetic mutations and manipulations that confer resistance to oxidative stress also cause extended life-span (*3*–*8*); however, dietary antioxidant supplements have been shown to have very limited effects on longevity (*9*).

We were prompted to test the oxygen radical theory of aging by the development of synthetic catalytic compounds that ameliorate oxidative stress in several disease models (*10*–*15*) and partially rescue mice that are mutant for mitochondrial superoxide dismutase (SOD) (*16*). We tested the effect of two mimetics, EUK-8 and EUK-134, on life-span in *Caenorhabditis elegans*. In vitro, these compounds exhibit both SOD- and catalase-like activities (they are SOD/catalase mimetics). EUK-134 is an analog of EUK-8, with increased catalase activity and equivalent SOD activity (*13*).

We treated synchronous adult hermaphrodite populations of worms in S medium (*17*) with various concentrations of mimetics and maintained those populations concurrently with control populations of untreated wild-type worms and, in some experiments, of long-lived *age-1(hx546)* mutant worms (*18*). The adult worms were transferred each day to new medium during the worms' reproductive period and every 3 days thereafter. In every experiment, treatment of wild-type worms with SOD/catalase mimetics significantly increased mean and maximum life-span (n = 14 comparisons, Table 1). Treatment of wild-type worms with 0.05 mM EUK-134 resulted in an increase in mean life-span of 54% ($P < 0.0001$; Fig. 1, A and B, and Table 1). Higher concentrations of EUK-134 and various concentrations of EUK-8 also extended life-span ($P < 0.0001$; Table 1). No overall dose response was observed. These compounds probably enter the worm through ingestion alone. As the worms age, they feed less, and consequently there is likely to be an increasing limitation on the amount of drug taken up.

We took a genetic approach to determining the mode of action of the mimetics. We examined the effects of EUK-134 on the life-span of a mutant worm strain that exhibits accelerated aging. Mutation of the *mev-1* gene, encoding the cytochrome b subunit of succinate dehydrogenase (complex II) of the electron transport chain, results in an elevated accumulation of oxidative damage during aging, an increased sensitivity to oxygen, and a life-span shortened by 37% ($P < 0.0001$; Fig. 1C) (*19*, *20*). Treatment with 0.5 mM EUK-134 restored a normal life-span to the *mev-1(kn1)* mutants by increasing their life-span by 67% ($P < 0.0001$; Fig. 1C). These results are consistent with amelioration of an endogenous and chronic oxidative stress.

Genetic manipulation of the life-span is generally associated with pleiotropic effects on life history traits such as reduced fertility and altered growth rate (*21*). Consequently, we tested whether the SOD/catalase mimetics affected

Table 1. Effect of SOD/catalase mimetics on wild-type *C. elegans* life-span. Five experiments are shown. N^+ is the number of worms after censoring. wt, wild type.

Strain	Treatment [mM]	Mean survival	Maximum life-span	N^+	P value*
Experiment 1					
wt	–	21 ± 6	39	70	
wt	EUK-8 [0.05]	28 ± 11	44	42	0.0001
wt	EUK-8 [0.5]	29 ± 10	57	51	0.0001
wt	EUK-8 [5]	27 ± 6	54	63	<0.0001
wt	EUK-8 [10]	27 ± 11	58	64	<0.0001
wt	EUK-134 [0.05]	27 ± 10	59	60	<0.0001
wt	EUK-134 [0.5]	29 ± 11	58	66	<0.0001
wt	EUK-134 [5]	32 ± 10	53	69	<0.0001
wt	EUK-134 [10]	32 ± 8	51	66	<0.0001
Experiment 2					
wt	–	24 ± 7	60	96	
wt	EUK-8 [5]	32 ± 13	59	68	<0.0001
wt	EUK-134 [5]	34 ± 13	67	68	<0.0001
wt	EUK-134 [0.05]	53 ± 14	69	46	<0.0001
age-1 (hx546)	–	46 ± 14	70	40	<0.0001
Experiment 3					
wt	–	24 ± 6	35	24	
wt	EUK-134 [0.5]	37 ± 12	62	27	<0.0001
age-1 (hx546)	–	41 ± 17	70	30	<0.0001
Experiment 4					
wt	–	24 ± 4	34	33	
wt	EUK 134 [0.05]	31 ± 10	56	32	0.0002
age-1 (hx546)	–	38 ± 14	63	30	<0.0001
Experiment 5					
wt	–	26 ± 6	36	17	
wt	EUK 134 [0.05]	36 ± 10	48	16	0.0013
age-1 (hx546)	–	39 ± 9	61	15	<0.0001

*P value log rank as compared with untreated wild-type worms.

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such traits. Treatment of wild-type worms with mimetics did not significantly alter either hermaphrodite self-fertility or body size measured at two stages during development (Fig. 2, A through F). These results suggest that the mimetic-associated life-span increase is not due to a nonspecific reduction in metabolism. This is consistent with the observation that the treated worms did not appear moribund until close to the end of the extended life-span.

We demonstrated that treatment of adult hermaphrodite worms with SOD/catalase mimetics causes a large increase in life-span. In addition, these compounds rescue the life-span defect of the *mev-1(kn1)* mutation. We propose that EUK-8 and EUK-134 extend life-span by augmenting natural antioxidant defenses without having any overt effects on other traits. These results suggest that endogenous oxidative stress is a major determinant of the rate of aging.

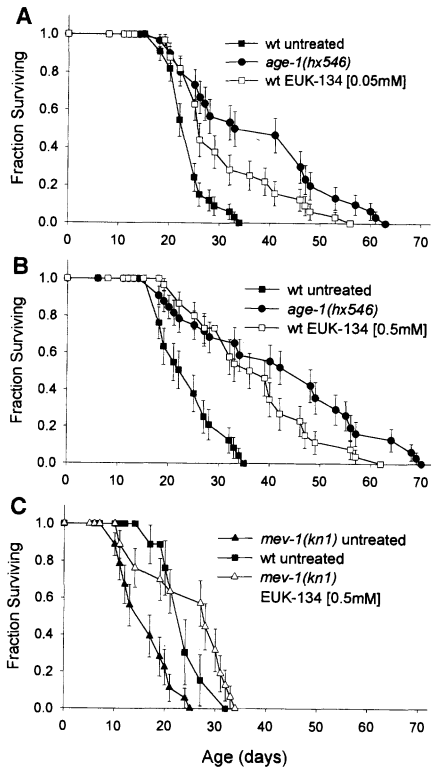

Fig. 1. Kaplan-Meier survival curve (±SE) of wild-type (wt) and *mev-1(kn1)* adult worms treated with SOD/catalase mimetics. Synchronously aging hermaphrodite worms were cultured in S medium with *Escherichia coli* as a food source (*17*). Worms were scored as dead when they failed to respond to repeated touching with a platinum wire pick. (**A**) Mean life-span (±SEM) in days of strain N2 (wild-type) = 24 ± 1 (solid squares); of strain TJ1052 [*age-1(hx546)*] = 38 ± 2 (circles); and of strain N2 (wild-type) treated with 0.05 mM EUK-134 = 31 ± 3 (open squares). (**B**) Mean life-span (±SEM) in days of strain N2 (wild-type) = 24 ± 1 (squares); of strain TJ1052 [*age-1(hx546)*] = 41 ± 3 (circles); and of strain N2 (wild-type) treated with 0.5 mM EUK-134 = 37 ± 2 (open squares). (**C**) Mean life-span (±SEM) in days of strain N2 (wild-type) = 24 ± 2 (squares), n = 7 worms; of strain *mev-1(kn1)* = 15 ± 1 (solid triangles), n = 19 worms; and of strain *mev-1(kn1)* treated with 0.5 mM EUK-134 = 25 ± 2, n = 16 worms. Very similar results were obtained in independent experiments.

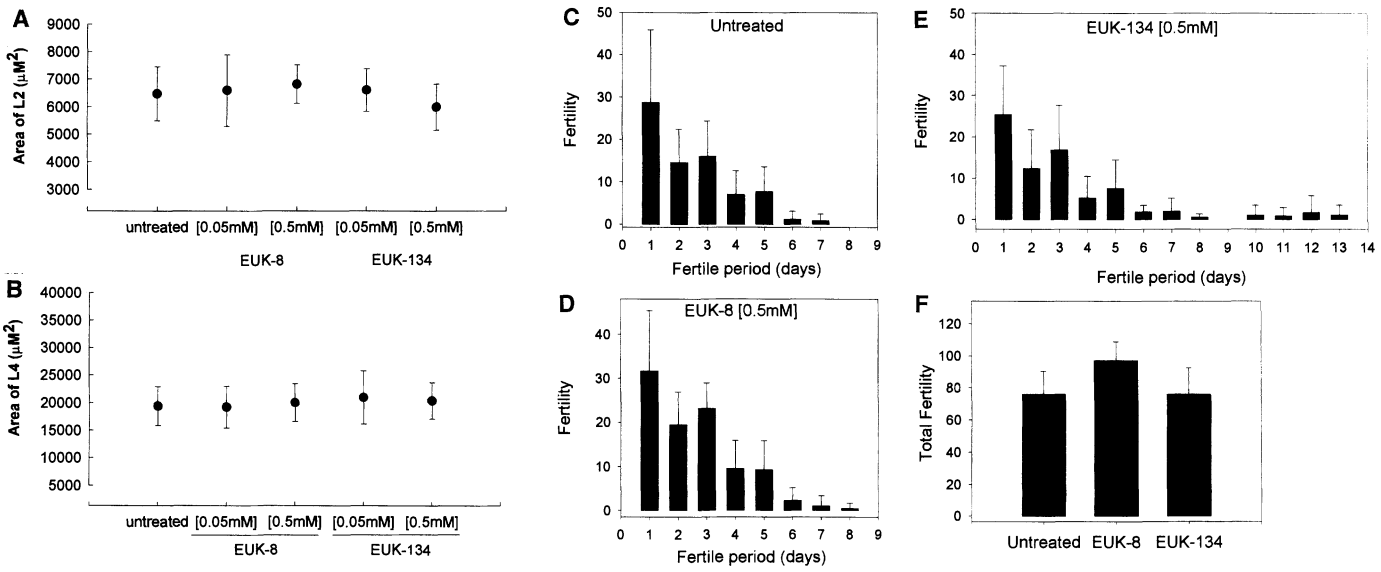

Fig. 2. Growth and fertility of wild-type worms treated with SOD/catalase mimetics. (**A**) Mean area (±SD) of second-stage larvae (L2) worms (*22*) cultured under the conditions indicated. Untreated, n = 27 worms; treated with 0.05 mM EUK-8, n = 13 worms; treated with 0.5 mM EUK-8, n = 20 worms; treated with 0.05 mM EUK-134, n = 12 worms; treated with 0.5 mM EUK-134, n = 12 worms. No significant differences in area were detected. (**B**) Mean area (±SD) of fourth-stage larvae (L4) worms cultured under the conditions indicated. Untreated, n = 20 worms; treated with 0.05 mM EUK-8, n = 22 worms; treated with 0.5 mM EUK-8, n = 15 worms; treated with 0.05 mM EUK-134, n = 11 worms; treated with 0.5 mM EUK-134, n = 23 worms. No significant differences in area were detected. (**C**) Daily fertility (±SD) of untreated wild-type worms (*23*). (**D**) Daily fertility (±SD) of wild-type worms treated from hatching with 0.5 mM EUK-8. (**E**) Daily fertility (±SD) of wild-type worms treated from hatching with 0.5 mM EUK-134. (**F**) Total fertilities (±SD) for untreated, EUK-8–treated, and EUK-134–treated worms. Untreated worms exhibited a mean total fertility (±SEM) of 76 ± 14 offspring (n = 6 worms). Worms treated with EUK-8 had a mean total fertility of 97 ± 12 offspring (n = 6 worms). Worms treated with EUK-134 had a mean fertility of 76 ± 16 offspring (n = 6 worms). Comparisons were made of total fertilities and for each day throughout the fertile period, but no significant differences were observed between treatments (as assessed with Student's t test).

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22. Worms were transferred in 10 μl of medium onto a glass slide and then sealed under a glass coverslip. The area of image defined by the worm outline was measured from digitized images made with Scion Image (Scion, Frederick, MD). The grayscale image was transformed with a Hat 17×17 convolution, and automatic edge detection was used to define perimeter length and area after calibration with a stage micrometer. Measurement error was ±69.8 μm² (1 SD) for second-stage larvae (L2) and ±158.9 μm² for fourth-stage larvae (L4).
23. Worms were individually cultured for each treatment group. Worms were transferred to new culture wells daily during the fertile period. Eggs laid in each well were allowed to hatch and were cultured until the worms had reached adulthood.
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Responses of Vomeronasal Neurons to Natural Stimuli

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The vomeronasal organ (VNO) of mammals plays an essential role in the detection of pheromones. We obtained simultaneous recordings of action potentials from large subsets of VNO neurons. These cells responded to components of urine by increasing their firing rate. This chemosensory activation required phospholipase C function. Unlike most other sensory neurons, VNO neurons did not adapt under prolonged stimulus exposure. The full time course of the VNO spiking response is captured by a simple quantitative model of ligand binding. Many individual VNO neurons were strongly selective for either male or female mouse urine, with the effective concentrations differing as much as a thousandfold. These results establish a framework for understanding sensory coding in the vomeronasal system.

Pheromones of mammals induce complex behaviors and neuroendocrine changes, such as the choice of a mate, territorial defense, the female estrous cycle, and onset of puberty (*1, 2*). It has been argued that pheromones are detected primarily by the VNO (*2, 3*). The identification of a large number of putative pheromone receptor genes, grouped into two divergent gene families, suggests that the population of sensory neurons is highly heterogeneous (*4–7*). Individual glomeruli of the accessory olfactory bulb collect projections from multiple types of VNO receptor neurons, and therefore the sensory code is likely to involve patterns of activity across the receptor population (*8, 9*). We reasoned that such a distributed population code should be observed by simultaneously recording the activity of a large number of VNO neurons in response to natural stimuli. This type of approach might reveal how sex, social dominance, or individual identity are represented by activity patterns in the VNO.

We recorded the action potentials of VNO neurons using a flat array of 61 extracellular electrodes (*10*). Even in the absence of stimulus, VNO neurons were spontaneously active (*11*), most of them firing intermittent bursts of spikes (Fig. 1A). When interpreting sensory responses, this pattern of maintained activity poses a hazard: A spontaneous burst may synchronize with the stimulus by chance and may be mistaken for a response. Such chance events may have confounded previous studies (*12–14*). We overcame this difficulty by delivering stimuli repeatedly, under precise temporal control (*15*). Of 221 neurons recorded in five preparations, 84 responded reproducibly to dilute urine by increasing their firing rate (see, for example, Fig. 1B). The sensitivity varied considerably across neurons, and the effective urine concentration (relative to undiluted urine) sufficient to elicit a response ranged from <0.0001 to 0.01. In no case did we observe a reproducible stimulus-induced inhibition (*13, 14*).

In addition to pheromones, mouse urine contains urea and potassium ions, which could potentially cause neurons to fire by direct membrane depolarization. Three lines of evidence establish that, instead, a specific chemosensory pathway underlies these responses. First, "artificial urine," containing the most abundant ionic and organic components of urine (*10*), did not affect firing, even at a relative concentration of 0.1 (*16*). Second, in any given VNO, some neurons were far more sensitive to female than to male mouse urine, whereas other neurons displayed an opposite selectivity (discussed further below). Third, responses to urine, but not to potassium ions, depend on a signal transduction cascade: 50 mM potassium excited the neurons, but the kinetics of the response differed sharply from that to urine (Fig. 2A). The onset of the urine response was delayed relative to the potassium response (by 0.33 ± 0.18 s, mean ± SD; $P < 10^{-7}$, if one assumes a gaussian distribution), and it also lasted considerably longer (by 3.1 ± 2.5 s, measuring the difference in exponential decay times; $P < 10^{-5}$). Presumably, potassium ions act directly to depolarize the membrane, whereas dilute urine achieves this only through a slower sensory transduction mechanism. A similar response delay occurs when odorants are presented to dissociated neurons of the main olfactory epithelium (*17*).

To obtain direct evidence for a signal transduction cascade, we applied pharmacological agents to the neuroepithelium. An inhibitor of phospholipase C, 10 μM U-73122 (*18, 19*), blocked spiking responses to urine but not to potassium (Fig. 2B). A nearly inactive structural analog, U-73343 (*18*), had no measurable effect on the response to urine. An inhibitor of phosphodiesterase, 500 μM isobutyl methylxanthine (IBMX), also had no effect on firing activity (44 cells). These results indicate that the response of VNO neurons to urine components involves the specific activation of an intracellular signal transduction pathway. Moreover, they identify phospholipase C–β (PLC-β) as a key element of the cascade and also confirm that cyclic nucleotides are not essential (*20, 21*). Molecular similarity has been found between the signaling pathways of mammalian VNO neurons and *Drosophila* photoreceptors, including specific expression of ion channels of the TRP family (*22*). Requirement for PLC-β function in the VNO parallels the involvement of the NorpA protein in the *Drosophila* eye (*23*) and provides additional support for similarity between the two pathways.

Having established the specificity of these VNO responses, we proceeded to a quantitative analysis of sensory coding. In most sensory systems (*24, 25*), a sustained stimulus causes the primary receptor cells to adapt by altering their sensitivity. For example, olfactory receptor cells change their dose-response relation within seconds (*26*). In contrast, we found little or no adaptation in

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