Enhanced catabolism of mitochondrial superoxide/hydrogen peroxide and aging in transgenic *Drosophila*

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The generation of superoxide anion radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) during mitochondrial respiration has been widely postulated to be causally linked to the aging process. The hypothesis that a specific enhancement of mitochondrial $O_2^{\cdot-}/H_2O_2$ catabolism would delay age-associated physiological changes and extend the lifespan was tested by simultaneous overexpression of MnSOD (manganese superoxide dismutase) and catalase, ectopically targeted to the mitochondrial matrix of transgenic *Drosophila melanogaster*. The increased activities of these antioxidative enzymes resulted in a decrease of mitochondrial H_2O_2 release and enhancement of free methionine content. The MnSOD/mitochondrial catalase transgenic flies displayed an enhanced resistance to experimental oxidative stress, induced by dietary H_2O_2

administration or by exposure to 100% ambient oxygen. However, the lifespan of the flies was decreased, by up to 43%, and this effect coincided with (i) an overall decrease in physical fitness, as measured by the speed of walking, and (ii) an age-related decrease in mitochondrial state 3 (ADP-stimulated) respiration. These findings support the notion that mitochondrial $O_2^{\cdot-}/H_2O_2$ production at physiological levels is essential for normal biological processes leading to the attainment of a normal lifespan.

Key words: aging, catalase, *Drosophila*, manganese superoxide dismutase, mitochondria, oxidative stress.

INTRODUCTION

The mechanisms that cause the deterioration of cellular functions during the aging process are at present poorly understood. The hypothesis that senescent changes are caused primarily by ROS (reactive oxygen species) and the accumulation of macromolecular oxidative damage generated by ROS, is supported by a considerable body of correlative evidence. For instance, the rates of production of superoxide anion radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) by mitochondria have been found to increase with age and are negatively correlated with the maximum lifespan of mammalian and insect species [1,2]. Steady-state amounts of oxidatively modified molecules increase as a function of age in virtually all the species that have been examined [2,3]. Furthermore, experimental regimens that prolong lifespan, such as caloric restriction in rodents and prevention of flying activity in insects, are associated with decreased rates of mitochondrial $O_2^{\cdot-}/H_2O_2$ generation and lower amounts of molecular oxidative damage [4].

It is estimated that, under normal physiological conditions, between 0.1 and 3% of the oxygen utilized by mitochondria is diverted to the generation of $O_2^{\cdot-}$ [5,6]. MnSOD (manganese superoxide dismutase), located in the mitochondrial matrix, converts $O_2^{\cdot-}$ into H_2O_2 , which in turn is degraded by mitochondrial peroxidases or diffuses into the cytosol [7]. The mitochondrial generation of $O_2^{\cdot-}/H_2O_2$ has important deleterious consequences. Indeed, increases in the rate of ROS production or decreases in the catalytic degradation of ROS, resulting from ablation of antioxidative defences, cause structural modifications of DNA, proteins and lipids, which are associated with detrimental effects on cellular functions [3,8]. Mitochondrial ROS generation is thus perceived to be an undesirable phenomenon, which is linked inextricably to oxygen consumption.

The main purpose of the present study was to determine whether or not an increase in the mitochondrial capacity to catabolize $O_2^{\cdot-}/H_2O_2$ would affect the progression of the aging process and the lifespan of *Drosophila melanogaster*. Specifically, it was hypothesized that an increase of ROS catabolism in mitochondria would result in an enhanced resistance to exogenous ROS, a postponement of age-related loss of physical fitness and extension of the lifespan. This hypothesis was tested by the simultaneous overexpression of MnSOD and ectopic catalase in the mitochondrial matrix of transgenic *Drosophila*. Flies containing one or two copies of both an MnSOD transgene and a mitochondrially targeted catalase transgene were generated, and the effects on lifespan, motor ability, mitochondrial respiration and resistance to experimental oxidative stress were determined. Contrary to predictions based on the hypothesis, the enhancement of mitochondrial antioxidative activities was associated with a decrease in lifespan.

MATERIALS AND METHODS

Transgenic flies

The generation and characterization of transgenic fly lines expressing catalase ectopically in the mitochondrial matrix have been described previously [9]. The transgene contains a 7 kb genomic fragment of *Drosophila* DNA, including the entire catalase coding sequence, with the putative mitochondrial presequence of the *D. melanogaster* OAT (ornithine aminotransferase) gene inserted upstream of the coding region. The generation and characterization of MnSOD transgenic lines, with inserts containing the genomic MnSOD gene of *Drosophila*, together with an approx. 7 kb upstream and 1.3 kb downstream sequence, have also been described previously [10].

Abbreviations used: MnSOD, manganese superoxide dismutase; OAT, ornithine aminotransferase; Dm2_{SOD/OAT}, flies containing one copy of the MnSOD transgene and one copy of the OAT-catalase transgene; Dm4_{SOD/OAT}, flies containing two copies of the MnSOD transgene and two copies of the OAT-catalase transgene; RCR, respiratory control ratio; ROS, reactive oxygen species.

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Table 1 Enzyme activities and lifespans of Dm2_{SOD/OAT} and Dm2_{Control} flies

Enzyme activities were determined at 10 days of age in whole body homogenates. Results are expressed as units/mg of protein and are means $\pm$ S.D.

<i>Drosophila</i> line	MnSOD activity	Catalase activity	Mean lifespan (days) (<i>n</i>)*	
			Experiment 1	Experiment 2
Dm2 _{Control} lines				
c23, c6	123 ± 8	300 ± 15	66.6 (99)	62.3 (98)
c8, c9	104 ± 13	302 ± 19	73.3 (93)	72.9 (99)
c3, c10	126 ± 5	264 ± 2	75.2 (95)	73.1 (102)
c2, c5	118 ± 4	268 ± 32	76.0 (101)	76.9 (99)
c22, c24	75 ± 6	287 ± 39	78.5 (100)	76.4 (101)
c24, c23	121 ± 8	272 ± 1	80.5 (99)	77.1 (96)
c21, c7	125 ± 11	313 ± 12	83.2 (100)	78.0 (98)
c5, c8	96 ± 7	253 ± 18	83.4 (98)	76.0 (100)
c7, c24	92 ± 1	286 ± 10	83.7 (100)	79.7 (102)
Mean ± S.D.	109 ± 18	283 ± 20	77.8 ± 5.7 (885)	74.7 ± 5.1 (895)
Dm2 _{SOD/OAT} lines				
MnSOD1, OAT-Cat1	168 ± 27	377 ± 12	62.4 (97)	62.8 (100)
MnSOD2, OAT-Cat2	168 ± 3	422 ± 8	64.7 (99)	64.2 (99)
MnSOD3, OAT-Cat1	213	458 ± 7	66.4 (97)	67.1 (100)
MnSOD4, OAT-Cat3	161 ± 18	287 ± 7	66.6 (97)	64.4 (98)
MnSOD5, OAT-Cat4	190 ± 20	387 ± 8	66.8 (97)	69.2 (99)
MnSOD1, OAT-Cat3	160 ± 10	338 ± 8	68.1 (93)	66.3 (100)
MnSOD6, OAT-Cat1	168 ± 8	435 ± 23	68.5 (99)	67.5 (99)
MnSOD7, OAT-Cat5	233 ± 39	322 ± 28	70.1 (97)	64.7 (97)
MnSOD8, OAT-Cat6	217 ± 16	420 ± 19	71.5 (99)	71.5 (97)
MnSOD7, OAT-Cat7	101 ± 19	561 ± 38	73.1 (96)	71.1 (93)
MnSOD6, OAT-Cat8	180 ± 6	341 ± 8	75.2 (95)	71.8 (100)
MnSOD9, OAT-Cat5	243 ± 86	312 ± 14	75.7 (96)	73.2 (102)
MnSOD8, OAT-Cat9	132 ± 15	359 ± 7	78.4 (99)	76.9 (91)
MnSOD8, OAT-Cat7	66 ± 2	505 ± 13	78.8 (97)	77.8 (96)
Mean ± S.D.	171 ± 49	395 ± 77	70.4 ± 5.1 (1358)	69.2 ± 4.7 (1371)

* *n* is the number of flies in each lifespan experiment.

For the present study, transgenic flies overexpressing both MnSOD and catalase in the mitochondrial matrix, designated Dm2_{SOD/OAT} (flies containing one copy of the MnSOD transgene

and one copy of the OAT-catalase transgene), were generated by crosses combining autosomal homologues containing the respective transgenes, permitting recombination between them. Progeny with the most darkly pigmented eyes were selected and outcrossed to strains possessing dominantly marked balancer chromosomes. Control lines (Dm2_{Control}) were constructed using a similar approach involving unmodified pCaSpeR vector transgenes. The individual transgenes recombined in each line are indicated in Table 1. In each case, the presence of both transgenes was confirmed by Southern blot analysis using digoxigenin-labelled probes complementary to either catalase or MnSOD gene sequences, or to the *white*⁺ marker gene sequence in the case of pCaSpeR vector control lines. Loss of either transgene in subsequent recombination events was prevented by maintaining the recombinant homologue over either the chromosome 2 balancer, *CyO*, or the chromosome 3 balancer, *TM3, Sb Ser*. In experiments involving Dm2_{SOD/OAT} and Dm2_{Control} lines, adult male flies, heterozygous with respect to the appropriate transgenes, were generated by outcrossing homozygous or balanced stocks with the parental *y w* strain.

Transgenic flies bearing two copies of the MnSOD transgene and two copies of the OAT-catalase transgene (Dm4_{SOD/OAT} flies) were obtained by direct crosses between male and virgin female flies from selected Dm2_{SOD/OAT} lines. For lifespan studies, a total of six Dm4_{SOD/OAT} transgene combinations and six controls (Dm4_{Control}) were generated. The genotypes of these flies are indicated in Table 2. The chromosome 2/chromosome 2 control and experimental groups were used for the measurements of H₂O₂ release, negative geotaxis, mitochondrial respiration, glutathione and methionine contents and resistance to experimental stress.

For all experiments, adult male flies were isolated using mild carbon dioxide anaesthesia and housed under constant light, 25 per vial, at 25°C. The flies were transferred into fresh vials containing standard media every second day, and every single day beyond 25–35 days of age. Flies used in biochemical assays and physiology experiments were provided with fresh vials approx. 2 h before beginning the experiments.

Table 2 Transgene combinations, enzyme activities and lifespans of Dm4_{SOD/OAT} and Dm4_{Control} flies

Enzyme activities were determined at 10 days of age in whole body homogenates except for mitochondrial catalase activity, which was determined at 2 days of age. Results are expressed as units/mg of protein and are means $\pm$ S.D.; n.d., not determined.

Chromosomes	Transgene combinations	MnSOD activity	Catalase activity		Mean lifespan (days) (<i>n</i>)*
			Total	Mitochondrial	
Control	Dm4 _{Control}				
Chr. 2/Chr. 2	c23, c6/c7, c24 (C1)	39 ± 2	283 ± 3	7 ± 23	77.2 (100)
	c22, c24/c6, c21 (C2)	56 ± 2	219 ± 11	11 ± 9	83.8 (100)
	c21, c7/c24, c23 (C3)	45 ± 1	257 ± 7	2 ± 9	86.2 (100)
Chr. 2; Chr. 3	c23, c6; c3, c10 (C4)	48 ± 3	259 ± 6	n.d.	70.7 (98)
	c22, c24; c2, c5 (C5)	47 ± 4	178 ± 4	n.d.	90.6 (99)
	c21, c7; c8, c9 (C6)	54 ± 11	226 ± 16	n.d.	89.3 (100)
Mean ± S.D.		48 ± 6	237 ± 37		83 ± 8 (597)
Experimental	Dm4 _{SOD/OCAT}				
Chr. 2/Chr. 2	M8, O6/M2, O2 (E1)	51 ± 3	659 ± 28	243 ± 19	50.9 (99)
	M9, O5/M7, O7 (E2)	95 ± 6	517 ± 25	194 ± 23	47.3 (99)
	M5, O4/M7, O5 (E3)	98 ± 4	449 ± 20	214 ± 5	59.8 (100)
Chr. 2; Chr. 3	M8, O6; M3, O1 (E4)	100 ± 7	588 ± 27	n.d.	65.6 (99)
	M9, O5; M6, O1 (E5)	78 ± 1	568 ± 7	n.d.	69.3 (100)
	M5, O4; M1, O3 (E6)	89 ± 6	428 ± 37	n.d.	66.3 (99)
Mean ± S.D.		85 ± 19	538 ± 88		60 ± 9 (596)

* *n* is the number of flies in the lifespan experiment.

Biochemistry

Whole body homogenates of flies and mitochondrial fractions were obtained as described in earlier reports [9,11]. Catalase activity was measured spectrophotometrically at 240 nm by recording the decomposition of H_2O_2 , as described previously [12,13]. One unit of catalase activity was defined as 1 μmol of H_2O_2 decomposed per min. This activity was fully (100%) inhibitable by preincubation of fly homogenates or mitochondria with 0.33 M 3-amino-1,2,4-triazole for 5 min at 30°C.

MnSOD activity was measured spectrophotometrically at 560 nm, by recording the cyanide-inhibitable reduction of Nitro Blue Tetrazolium [14,15]. One unit of SOD activity was defined as the amount that inhibited Nitro Blue Tetrazolium reduction half-maximally in a 1 ml reaction volume.

The amount of H_2O_2 released from purified flight muscle mitochondria of 2-day-old flies was quantified by a modification of the fluorimetric procedure of Hyslop and Sklar [16], based on the oxidation of *p*-hydroxyphenylacetate to a stable fluorescent compound ($\lambda_{\text{ex}} = 320$ nm and $\lambda_{\text{em}} = 400$ nm), as described previously [9].

The amounts of GSH, GSSG and methionine were measured by HPLC coupled with coulometric electrochemical detection, as described by Rebrin et al. [17].

Aconitase activity was measured at 30°C by a coupled assay in which the formation of β -NADPH was followed spectrophotometrically at 340 nm, using citrate as a substrate, essentially as described in [18].

Physiology

Mitochondrial respiration was measured polarographically at 28°C, using a Clark-type oxygen electrode connected to a computer-operated Oxygraph control unit (Hansatech Instruments, Norfolk, U.K.). Fly mitochondrial protein (30–60 μg) obtained from isolated thoraces was added to the respiration buffer (120 mM KCl, 5 mM potassium phosphate, 3 mM Hepes, 1 mM EGTA, 1 mM MgCl_2 and 0.2% BSA, pH 7.2) in a 1.0 ml reaction chamber, followed by the addition of NAD^+ -linked substrate (10 mM pyruvate + 10 mM proline) and subsequently 100 μM ADP. After recovery of state 4 respiration (ADP-depleted), a second addition of ADP (400 μM) was made and was used to calculate the rate of state 3 respiration. All measurements were performed in quadruplicate, within 1 h after the isolation of mitochondria.

In negative geotaxis experiments, the speed of walking was measured after placing individual flies in disposable plastic pipettes at 25°C, as described previously [19].

Resistance to exogenous H_2O_2 and hyperoxia

Exposure to dietary H_2O_2 or 100% oxygen was initiated 15 days post-eclosion. Exposure to H_2O_2 was performed in standard polystyrene vials in which the regular food medium was replaced by a strip of filter paper saturated with a 1% sucrose solution containing 0.25, 0.5 or 1.0% H_2O_2 . Flies exposed to the sucrose solution alone served as controls. For the hyperoxia experiment, a steady flow of 100% oxygen was bubbled through water and passed through a sealed Plexiglass® chamber, containing flies in standard polystyrene vials, under a low positive pressure.

Statistical analysis

The significance of differences in enzymatic activities or H_2O_2 release from mitochondria was assessed by unpaired, two-sided Student's *t* tests, using SYSTAT 10 software (Systat Software, Richmond, CA, U.S.A.). The mean lifespans of entire cohorts

of flies from each line or transgene combination were also compared using unpaired, two-sided *t* tests. The use of group means in the analysis of survivorship data was deemed necessary because treating individual flies with identical genotypes as independent samples may overestimate the extent of replication in the experiment [20]. This possibility arises to the extent that the effects on survivorship are influenced by the sites of insertion of the transgenes in the genome.

The significance of differences in mean survival times during exposure to exogenous H_2O_2 was assessed by two-way ANOVA. The factors in the analysis were group (experimental versus control) and H_2O_2 dosage. Similarly, differences in resistance to 100% oxygen exposure were compared with fly group and replicate experiment as factors. For the analysis of glutathione and methionine content and differences in walking speed, a nested ANOVA was performed with age and group as factors, and individual transgene combinations nested within group type. Nesting was used because only three transgene combinations were examined per group, with multiple replicate measurements per combination. Mitochondrial respiration rates were compared without nesting, because only a single transgene combination from each group was examined.

RESULTS

Simultaneous overexpression of MnSOD and ectopic expression of catalase in mitochondria

Mitochondrial overexpression of MnSOD with simultaneous expression of ectopic, mitochondrially targeted catalase was achieved in *D. melanogaster* by recombination of autosomal homologues containing MnSOD and OAT-catalase transgenes, as described in the Materials and methods section. A total of 27 $\text{Dm2}_{\text{SOD/OAT}}$ lines were generated, containing a single MnSOD transgene and a single OAT-catalase transgene, each at one of ten unique insertion sites. Similarly, 24 $\text{Dm2}_{\text{Control}}$ lines were obtained, containing two *pCaSpeR* vector insertions, each at one of 13 unique insertion sites. For subsequent lifespan studies and measurement of resistance to experimentally elevated ROS concentrations, nine $\text{Dm2}_{\text{Control}}$ lines and 14 $\text{Dm2}_{\text{SOD/OAT}}$ lines were selected on the basis of maximum overexpression of one or both enzymes at the whole body level and minimum transgene redundancy. As compared with $\text{Dm2}_{\text{Control}}$ lines, the MnSOD and catalase activities of these $\text{Dm2}_{\text{SOD/OAT}}$ lines were increased by an average of 57 and 40% respectively (Table 1).

Further overexpression of MnSOD and mitochondrial catalase was achieved by generating transgenic flies with two MnSOD and two OAT-catalase inserts in the genome. A total of six $\text{Dm4}_{\text{SOD/OAT}}$ combinations and six $\text{Dm4}_{\text{Control}}$ combinations were generated by crosses among $\text{Dm2}_{\text{SOD/OAT}}$ and $\text{Dm2}_{\text{Control}}$ lines (Table 2). Enzyme activities of MnSOD and catalase in whole body homogenates of $\text{Dm4}_{\text{SOD/OAT}}$ flies were 77 and 126% higher, on average, than the respective activities in $\text{Dm4}_{\text{Control}}$ flies ($P < 0.001$, Table 2). Mitochondrial catalase activity ranged from 194 to 243 units/mg of protein in $\text{Dm4}_{\text{SOD/OAT}}$ flies, but no activity was detected in $\text{Dm4}_{\text{Control}}$ mitochondria (Table 2). This manipulation of enzymatic antioxidant activities was associated with a 66% slower rate of release of H_2O_2 from the mitochondria of $\text{Dm4}_{\text{SOD/OAT}}$ versus $\text{Dm4}_{\text{Control}}$ flies ($P < 0.0005$, Figure 1).

Curtailement of lifespan

To determine whether or not the enhanced capacity for catalytic removal of mitochondrial ROS influenced longevity under standard, unstressed conditions, lifespan studies were performed

Figure 1 Cumulative H_2O_2 release

Results are presented as percentage of $\text{Dm4}_{\text{Control}}$ H_2O_2 accumulation at 3 min for thoracic mitochondria obtained from 2-day-old $\text{Dm4}_{\text{Control}}$ flies (C1–C3, open symbols) and $\text{Dm4}_{\text{SOD/OCAT}}$ flies (E1–E3, closed symbols). $P < 0.0005$ for cumulative H_2O_2 release from 3 to 8 min.

Figure 2 Lifespans at 25°C

(A) Pooled survivorship curves of nine $\text{Dm2}_{\text{Control}}$ and 14 $\text{Dm2}_{\text{SOD/OCAT}}$ transgenic lines. Approximately 100 flies were used for each line. Results are shown for experiment 1 ($P = 0.004$) and are representative of two independent experiments. Mean lifespans of the individual lines are presented in Table 1. (B) Pooled survivorship curves of six $\text{Dm4}_{\text{Control}}$ and six $\text{Dm4}_{\text{SOD/OCAT}}$ combinations. Approximately 100 flies were used for each combination. Results are shown for experiment 1 ($P = 0.0007$) and are representative of two independent experiments. Mean lifespans of the individual combinations are indicated in Table 2.

at 25°C for both the $\text{Dm2}_{\text{SOD/OCAT}}$ and the $\text{Dm4}_{\text{SOD/OCAT}}$ flies. In two independent experiments, using 14 $\text{Dm2}_{\text{SOD/OCAT}}$ lines, the average lifespan was not increased as predicted, but was decreased by 9.5% ($P = 0.004$) and 7.4% ($P = 0.007$) in comparison with nine $\text{Dm2}_{\text{Control}}$ lines (Table 1 and Figure 2A). The lifespans of the flies with two MnSOD and two OAT-catalase inserts in the genome were shortened even more dramatically. In two independent experiments, the lifespans of $\text{Dm4}_{\text{SOD/OCAT}}$ flies were decreased by 27–28% on average (to 60–63 days, versus 83–

Figure 3 Maximum walking speed

Individual flies were confined in 5 ml graduated pipettes and gently tapped to the base, and the maximum height reached by each fly after 10 s was recorded. The maximum walking speed (cm/s) was determined on the basis of the greatest height attained in three trials, using 15 different flies for each of three $\text{Dm4}_{\text{Control}}$ groups (C1–C3) and three $\text{Dm4}_{\text{SOD/OCAT}}$ groups (E1–E3), at each age. Results are pooled data and are expressed as means $\pm$ S.E.M. for the three groups of $\text{Dm4}_{\text{Control}}$ flies (white bars) and $\text{Dm4}_{\text{SOD/OCAT}}$ flies (black bars). The $\text{Dm4}_{\text{SOD/OCAT}}$ flies were significantly slower than the control flies ($P < 0.0005$) and the walking speed decreased with advancing age ($P < 0.0005$).

86 days for $\text{Dm4}_{\text{Control}}$ flies, $P < 0.002$), and by as much as 43% for combination E2 (Table 2 and Figure 2B). Similarly, at 30°C, at which temperature the metabolic rate of *Drosophila* is increased, the average lifespan of $\text{Dm4}_{\text{SOD/OCAT}}$ flies was 20–22% shorter than the lifespan of $\text{Dm4}_{\text{Control}}$ flies (24–25 days versus 31 days, $P < 0.03$).

Decreased motor performance

The speed of walking, a marker of physical fitness, was measured in $\text{Dm4}_{\text{SOD/OCAT}}$ flies. The speed of walking decreased significantly as a function of age ($P < 0.0005$, Figure 3) and the $\text{Dm4}_{\text{SOD/OCAT}}$ flies had a slower walking speed than the $\text{Dm4}_{\text{Control}}$ flies ($P < 0.0005$). At 40 days of age, the speed of walking of the $\text{Dm4}_{\text{SOD/OCAT}}$ flies, averaged among three groups with different transgene combinations, was 60% lower than that of the $\text{Dm4}_{\text{Control}}$ flies.

Age-related decrease in mitochondrial respiration

The mitochondrial respiration rates of $\text{Dm4}_{\text{SOD/OCAT}}$ and $\text{Dm4}_{\text{Control}}$ flies were measured at different ages, in order to test the hypothesis that impaired mitochondrial respiratory function could be responsible for the decrease in lifespan. Among young adult flies 10 days of age, there were no differences in the state 3 (ADP-stimulated) or state 4 (ADP-depleted) respiration rates, RCR (respiratory control ratio; ratio of state 3 rate/state 4 rate) or in the ADP:O ratio (the number of ADP molecules consumed per oxygen atom during state 3 respiration in the presence of 400 μM ADP) (Table 3). Additionally, there was no difference in the activity of aconitase, a tricarboxylic acid cycle enzyme that is particularly susceptible to oxidative inactivation [21]. However, the state 3 respiration rates of $\text{Dm4}_{\text{SOD/OCAT}}$ flies were, respectively, 15 and 21% lower than the $\text{Dm4}_{\text{Control}}$ rates at 20 and 40 days of age (Figure 4A). Overall, there were significant differences between fly types ($P = 0.01$) and age groups ($P = 0.001$), and a significant interaction between fly type and age ($P = 0.02$). State 4 respiration did not differ significantly between fly types or among age groups (Figure 4B).

Table 3 Mitochondrial function of Dm4_{SOD/OCAT} and Dm4_{Control} flies

Mitochondrial respiration rates (states 3 and 4) were determined using pyruvate + proline as substrates and flies 10–15 days of age. Rates are expressed as nmol O₂ · min⁻¹ · mg of protein⁻¹, and are means ± S.D. for four measurements. The RCR is the ratio of state 3 rate/state 4 rate, and the ADP:O ratio is the number of ADP molecules per oxygen atom during state 3 respiration. Results from a representative experiment are shown; the experiment was repeated independently three times (pooled results are presented in Figure 4). Aconitase activity is expressed as mM β-NADPH · min⁻¹ · mg of protein⁻¹ and values are means ± S.D. for three independent experiments, each involving two different preparations, with four measurements per preparation.

	Control (C3)	Experimental (E1)
Respiration rate (nmol of O ₂ · min ⁻¹ · mg of protein ⁻¹)		
State 3	633.2 ± 13	631.5 ± 44
State 4	58.8 ± 3	71.5 ± 7
RCR	10.8 ± 1	8.9 ± 1
ADP:O ratio	3.1	3.0
Aconitase activity (mM β-NADPH · min ⁻¹ · mg of protein ⁻¹)	0.50 ± 0.07	0.53 ± 0.04

Figure 4 Mitochondrial respiration as a function of age

Mitochondrial respiration rates (states 3 and 4) of Dm4_{Control} C3 and Dm4_{SOD/OCAT} E1 flies were measured using pyruvate + proline as substrates, and expressed as nmol of O₂ · min⁻¹ · mg of protein⁻¹. Results are means ± S.D. for three independent experiments at each age. (A) State 3 respiration (in the presence of excess substrate and ADP) was the same in both groups at 10 days of age, but it was lower in experimental versus control flies at 20 and 40 days of age. (B) State 4 respiration (in the presence of excess substrate after exhaustion of ADP) was well maintained at all ages tested.

Effects on GSH/GSSG ratio and content of free methionine

The ratio between GSH and GSSG is considered to be an indicator of the overall redox state [22], which is defined as the ratio of the concentrations of oxidizing and reducing equivalents. Free methionine is a precursor in cysteine biosynthesis, and it may also act as an antioxidant via reversible oxidation to methionine sulfoxide [23]. To determine whether the level of oxidative stress and the redox state of the organism as a whole were affected by the manipulation of mitochondrial O₂⁻/H₂O₂ catabolism, GSH,

Figure 5 Methionine content

Results are presented as means ± S.D. of the data for three Dm4_{Control} (C1–C3; □) and three Dm4_{SOD/OCAT} combinations (E1–E3, ■) and are expressed as nmol/ml of initial preparation [5 % (w/v) homogenates in 5 % (w/v) metaphosphoric acid]. Results for each combination, at each age, were obtained from four independent preparations of 50 flies each. The methionine content was significantly higher in Dm4_{SOD/OCAT} flies than in Dm4_{Control} flies ($P < 0.0005$) and decreased as a function of age ($P < 0.0005$).

Figure 6 Resistance to H₂O₂ exposure

H₂O₂ was administered to control flies (□) and experimental flies (■) in 1 % sucrose solutions. Survival times (mean ± S.D.) were pooled for three Dm4_{Control} groups (C1–C3) versus three Dm4_{SOD/OCAT} groups (E1–E3) ($P < 0.001$).

GSSG and methionine were quantified in whole body homogenates of 10- to 40-day-old Dm4_{SOD/OCAT} and Dm4_{Control} flies. The GSH and GSSG contents and the GSH/GSSG ratio were not significantly different between groups (results not shown), but the concentration of free methionine was significantly higher in Dm4_{SOD/OCAT} flies versus control flies ($P < 0.0005$, Figure 5) and decreased as a function of age ($P < 0.0005$). This higher level of free methionine was also observed in enriched mitochondrial fractions obtained from thoracic homogenates (results not shown).

Increased protection against acute oxidative stress

To investigate whether the specific enhancement of mitochondrial catalytic O₂⁻/H₂O₂ removal would result in protection against acute oxidative stress, flies were exposed to various concentrations of exogenous H₂O₂. The survival times of the Dm2_{SOD/OCAT} flies, fed solutions of 0.25, 0.5 and 1.0 % exogenous H₂O₂ in 1 % sucrose, were increased by 33, 30 and 22 % respectively when compared with Dm2_{Control} flies ($P < 0.0005$). Similarly, Dm4_{SOD/OCAT} flies exposed to 0.25, 0.5 and 1.0 % H₂O₂ exhibited increases in survival times of 35, 43 and 14 % respectively when compared with Dm4_{Control} flies ($P < 0.0005$, Figure 6).

The transgenic flies were also exposed to 100 % oxygen, which causes an increase in mitochondrial ROS production [24]. In two independent experiments involving 9–10 Dm2_{Control} lines and 13–14 Dm2_{SOD/OAT} lines, the average survival times of the Dm2_{SOD/OAT} lines were 12–15 % greater than those of the controls ($P = 0.02$, results not shown). However, the Dm4_{SOD/OAT} flies were not significantly more resistant to hyperoxia than the Dm4_{Control} flies.

DISCUSSION

Results of the present study indicate that an experimental increase in mitochondrial $O_2^{\cdot-}/H_2O_2$ removal capacity, induced by simultaneous overexpression of MnSOD and ectopic expression of catalase in the mitochondria of transgenic *D. melanogaster*, caused a decrease in the rate of mitochondrial H_2O_2 release and an enhancement of resistance to acute oxidative stress, induced by exposure to exogenous H_2O_2 . However, the lifespan was significantly shortened, both under standard conditions (25 °C) and at an elevated ambient temperature (30 °C), the walking speed was decreased and the active state of mitochondrial respiration (state 3) was impaired at older ages.

Studies on the effects of overexpression of antioxidative enzymes in transgenic *Drosophila* have given rise to two seemingly contradictory interpretations. One group of studies has shown that the lifespan is extended, particularly when the overexpression of SOD is restricted to the adult phase of the life cycle [25] or to motor neurons [26]. These results are consistent with the traditional free radical hypothesis of aging [27], which postulates that oxidative damage resulting from ROS production is a major causal factor in the aging process. Indeed, the accrual of such damage is correlated with rates of ROS production and aging in animals [28,29]. However, a second set of studies has shown that overexpression of genes encoding antioxidative enzymes, using native *cis*-regulatory sequences, does not affect the aging process if long-lived fly strains are used as controls [30,31]. In fact, small decreases in lifespans can be observed in the survivorship data of flies overexpressing single copies of either genomic MnSOD or ectopic, mitochondrially targeted catalase transgenes [10,32], or of some individual long-lived lines in which overexpression of Cu–Zn SOD was induced only during adult life [33]. The seeming discrepancies among these findings may be explained either in terms of differences in the intracellular sites of the overexpressed enzymes or of the lifespans of the control flies employed in the various studies. A negative correlation has indeed been noted previously between the control lifespan and the degree of lifespan extension associated with overexpression of several antioxidant enzymes [31,34]. On this basis, the augmentation of antioxidants may be postulated to compensate for the adverse effects of specific genetic backgrounds and environmental conditions, which may compromise the lifespan of flies for reasons unrelated to the normal aging process.

In the present study, flies in long-lived backgrounds were used to test the hypotheses that (i) the length of life is positively correlated with levels of antioxidative defences and negatively correlated with rates of ROS production, and (ii) the simultaneous overexpression of multiple antioxidants in mitochondria, a major site of ROS production in *Drosophila*, would have a particularly strong impact on lifespan. To test these hypotheses, flies were generated containing one or two copies of both genomic MnSOD and OAT-catalase transgenes, resulting in significantly enhanced MnSOD and mitochondrial catalase activities. In contrast with the effects of either transgene alone [10,32] or the simultaneous overexpression of cytosolic SOD/catalase [36], there was a con-

sistent and highly significant decrease in longevity. In addition, the decrease in lifespan was much more pronounced in flies containing two copies versus one copy of both the MnSOD and the OAT-catalase transgenes. These findings illustrate the importance of the subcellular site of the overexpressed enzymes and demonstrate (i) that some removal of mitochondrial ROS can be tolerated before longevity is affected and (ii) that the increased capacity for removal of both mitochondrial $O_2^{\cdot-}$ and H_2O_2 is more harmful than for either $O_2^{\cdot-}$ or H_2O_2 alone.

Direct measurement of H_2O_2 production by mitochondria from Dm4_{SOD/OAT} flies confirmed that the increase in SOD and catalase activities and decrease in lifespan coincided with a substantial curtailment of H_2O_2 release. However, under the assay conditions used in the present study, H_2O_2 release was also virtually abolished in OAT-catalase flies [32]. The dramatic differences in lifespan effects and minor differences in rates of H_2O_2 release between OAT-catalase and Dm4_{SOD/OAT} flies could be accounted for by differential effects on $O_2^{\cdot-}$ levels, resulting from the additional overexpression of MnSOD. Alternatively, differences in the amount of mitochondrial catalase activity could be primarily responsible for the decrease in lifespan. Indeed, the levels of catalase overexpression in OAT-catalase, Dm2_{SOD/OAT} and Dm4_{SOD/OAT} flies averaged 35, 40 and 126 % respectively, whereas the average decreases in lifespan, in relation to the respective controls, were 2–6, 7–9 and 27–28 %. These results suggest that the levels of catalase activity required to cause a large decrease of lifespan are higher than those needed to abolish H_2O_2 release by isolated mitochondria. This differential effect could arise, notwithstanding the complete abolition of H_2O_2 release by OAT-catalase mitochondria under *in vitro* assay conditions, if the additional mitochondrial catalase in Dm4_{SOD/OAT} flies quenches H_2O_2 release that might otherwise occur *in vivo*.

An alternative possibility is that the mere presence of excessive amounts of the overexpressed proteins in the matrix could cause mitochondrial dysfunction, independent of the levels of enzymatic activity. In theory, this idea could be tested by overexpressing an inert, mutant form of the ectopic catalase in *Drosophila* mitochondria. In practice, however, a protein of this type could be misfolded, and consequently more susceptible either to proteolysis or aggregation, resulting in an artifact of the type it was intended to exclude. Accordingly, a control experiment of this type was not performed in the present study. However, the plausibility of the hypothesis that the lifespan was decreased as an artifactual effect, caused by the physical presence of the ectopic protein, was decreased by evidence obtained from both structural and functional studies. First, mitochondrial respiratory activity was indistinguishable between Dm4_{SOD/OAT} and Dm4_{Control} flies after 10 days of adult life. Instead, the impairment of state 3 respiration, specifically at older ages, and the decrease in locomotor activity, suggested that the Dm4_{SOD/OAT} flies were aging more quickly than the controls. Secondly, there was no obvious difference in the profile of protein expression between Dm2_{Control} and Dm2_{SOD/OAT} flies (results not shown). Collectively, these findings suggest that the ectopic catalase was not sufficiently abundant to displace or compromise the activities of other mitochondrial proteins, or to prevent their import or assembly into electron-transport complexes, as would be predicted if the mitochondrial protein translocation pores were jammed.

Recent studies indicate that ectopic expression of catalase in mammalian mitochondria has positive effects on lifespan and stress resistance. Specifically, overexpression of MnSOD and mitochondrial targeting of catalase had additive, protective effects against glucose-deprivation-induced cytotoxicity and GSSG accumulation in human PC-3 cells [37]. Very recently, Schriener et al. [38] reported that mice expressing ectopic human catalase

at high levels in heart and skeletal muscle mitochondria live significantly longer than controls. These findings illustrate that the presence of ectopic catalase within mitochondria does not have adverse effects on lifespan in all species, suggesting either that any artifactual mitochondrial dysfunction caused by the presence of ectopic catalase is minor (not sufficient to offset the life-extending effect in mice) or that it is species-specific. The reasons for the contrasting effects of mitochondrial catalase in mice and flies are unknown, but they might imply that flies are more sensitive to perturbation of physiological levels of H_2O_2 production. Such a sensitivity could involve either the cellular signalling or bactericidal activities of H_2O_2 , or some additional, unknown role. In either case, it appears that the rapid rate of mitochondrial $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$ production in *Drosophila* could account for the rapid accrual of oxidative damage and short lifespan of this species, but the physiological roles of ROS interfere with tests of this hypothesis involving a direct decrease in $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$ production.

The decreased lifespans of flies overexpressing MnSOD and mitochondrial catalase suggest that a basal level of mitochondrial ROS production is essential for the attainment of a normal length lifespan. In support of this view, the level of H_2O_2 affects the expression of at least 80 different genes or proteins, including numerous components of the mitogen-activated protein kinase and nuclear factor κB signalling pathways [39]. ROS may also modulate other signal-transduction pathways [40]. Accordingly, a spatially and quantitatively controlled generation of H_2O_2 is believed to have beneficial, physiological roles. In contrast, ROS also have adverse effects, including lipid, protein and DNA oxidative damage initiated by $\cdot\text{OH}$, the product of metal-catalysed scission of H_2O_2 [41,42].

This apparent duality of effects of $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$ provides a plausible explanation for the decrease in lifespan coinciding with increased resistance to experimental oxidative stress in flies overexpressing MnSOD and OAT-catalase. Supraphysiological concentrations of $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$, induced by H_2O_2 feeding or exposure to normobaric hyperoxia, likely accelerate oxidative damage and possibly interfere with signal transduction mediated by $\text{O}_2^{\cdot-}$ and H_2O_2 . Overexpression of MnSOD and OAT-catalase would be expected to attenuate these adverse effects. However, when the overexpressed enzymes decrease $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$ below threshold levels, the resulting interference with cellular signalling presumably outweighs any benefit resulting from the slower accrual of oxidative damage. Other reports from this laboratory have shown that either thioredoxin reductase gene overexpression [19] or genetic selection for delayed reproduction [43] also had opposite effects on lifespan and resistance to hyperoxia. These findings are incompatible with the idea that there is a simple relationship between the rate of aging and resistance to experimental oxidative stress. Thus notwithstanding the partial overlap of patterns of gene expression induced by aging and hyperoxia [44], increased resistance to experimental oxidative stress is not a reliable predictor of extended lifespan under normal conditions, and it could not account for the differences in lifespan observed in this study.

Perturbation of the glutathione or thioredoxin redox state and redox-sensitive signalling pathways is one plausible mechanism by which alteration of the $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$ steady state could shorten the lifespan of flies. However, the differences in GSH and GSSG concentrations between MnSOD/OAT-catalase transgenic flies and controls in the present study were not significant. Thus among the various possible factors which could explain the decreased lifespan of the flies, those dependent on changes in the total GSH and GSSG content may be excluded. In contrast, the increased amounts of free methionine at all ages in $\text{Dm4}_{\text{SOD/OAT}}$ flies are indicative of a biochemical impact of increased mitochondrial

$\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$ catabolism at the organismal level. H_2O_2 has been shown to oxidize methionine to methionine sulfoxide, and this reversible oxidation is believed to regulate protein function [45] and to provide an antioxidative defence mechanism [23]. Similarly, the observed decrease in free methionine content as a function of age is consistent with increased H_2O_2 production in older flies [2]. A possible role for methionine metabolism in the aging process is supported by a direct relationship between methionine sulfoxide reductase activity and lifespan in flies [47] as well as mice [48], and by the observation that Fischer 344 rats live longer when fed a methionine-restricted diet [49]. However, the shorter life expectancy of flies with either increased or decreased methionine content ($\text{Dm4}_{\text{SOD/OAT}}$ transgenics and aged flies respectively) argues against a direct causal relationship between free methionine content and life expectancy. Instead, the results suggest that methionine content may serve as a useful biomarker of the level of oxidative stress in *Drosophila*.

In conclusion, the results of the present study demonstrate that enhancement of the capacity for mitochondrial $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$ catabolism leads to progressive decreases in the longevity of flies. Although the underlying mechanisms remain to be determined, the present findings do establish that the rate of mitochondrial H_2O_2 release is not simply and negatively related to the lifespan. Although this finding is seemingly at odds with the oxidative stress hypothesis of aging and with recent results obtained using transgenic mice [38], it remains possible that rates of ROS release are directly proportional to the rate of accumulation of oxidatively damaged macromolecules, which in turn govern the rate of aging under normal conditions. However, decreasing $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$ levels artificially could shorten the lifespan of flies by interfering with various signal-transduction pathways, thereby masking any beneficial effect on the underlying rate of aging. Thus mitochondrial ROS generation, at normal physiological levels, may be necessary for the attainment of a full-length lifespan in *Drosophila*.

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