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Superoxide Dismutase Activities in Long-Lived *Drosophila melanogaster* Females: *chico*[1] Genotypes and Dietary Dilution

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Abstract
Superoxide dismutase (SOD) activities were determined for dietary dilution conditions that extend the life span of *Drosophila melanogaster*. The hypothesis motivating this research was that elevated SOD activity is associated with increased life span resulting from flies being held on a restricted diet. SOD activities were also measured for *chico*[1] which is a mutation in the insulin receptor substrate protein gene associated with life span extension. This allowed us to confirm the results of (Clancy et al. 2001) and extend the results by measuring CuZn SOD and Mn SOD activities in addition to the previously determined overall SOD activity. If the same form of SOD activity (CuZn SOD or Mn SOD) was elevated on the dilute diet that extends life span and in the long lived *chico*[1] homozygotes, then it would suggest that life span extension by dietary restriction and by insulin signaling mutations has a similar underlying mechanism. However, overall SOD activity, and CuZn SOD or Mn SOD activities did not differ among the diets tested. As observed previously (Clancy et al. 2001), overall SOD activity was elevated in *chico*[1] homozygotes compared to the heterozygote or wild type. Results from the present study indicate that elevated CuZn SOD activity, not Mn SOD, is the basis for the relatively high level of SOD activity in the *chico*[1] homozygotes.

Keywords: *Drosophila*, dietary restriction, caloric restriction, insulin signaling, superoxide dismutase (SOD), antioxidants, longevity

Abbreviations

BCA	Bicinchoninic acid
DTPA	Di-ethylenetriaminepentaacetic acid
NaCN	Sodium cyanide
NBT	Nitroblue tetrazolium
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TDB	Triethanolamine, diethanolamine–HCl

Introduction

Longevity is thought to be determined by two countervailing forces: (1) production of oxygen free radicals and corresponding damage to macromolecules opposed by (2) defense against oxidative damage or repair (Harman 1956; Hekimi et al. 2001). Superoxide dismutases (SODs) are key enzymes in the defense against oxidative damage. They convert the superoxide anion into hydrogen peroxide an oxidant can be converted by catalase into water. Transgenic over-expression of cytosolic CuZn SOD in *Drosophila melanogaster* has been shown to extend life span by 40–50% (Parkes et al. 1998; Sun and Tower 1999). Moreover, transgenic over-expression of mitochondrial MnSOD extends life span in this species (Sun et al. 2002). Relevant single gene mutations, those that affect life span and SOD activity, have been identified in species used as models for genetic studies (Martin et al. 1996). In the worm, *Caenorhabditis elegans*, *age-1* and *daf-2* insulin/IGF signaling mutations that extend lifespan also increase SOD activity as well as resistance to oxidative stress (Friedman and Johnson 1988; Kenyon et al. 1993; Larsen 1993; Vanfleteren and De Vreese 1996; Honda and Honda 1999; Wolkow et al. 2000). In *D. melanogaster*, mutations in the *InR* and *chico* genes, known to play an integral role in insulin-like growth factor signaling, both increase life span and overall SOD activity (Clancy et al. 2001; Tatar et al. 2001).

The mechanism underlying the extension of life span by caloric restriction is not known, but it has been suggested that caloric restriction might act by a mechanism similar to that of mutations that extend life span by decreased insulin signaling (Gems and Partridge 2001; Partridge and Gems 2002). Given that upregulation of SOD activity is associated with insulin signaling mutations, then upregulation of SOD activity might be expected under dietary reduction conditions that extend life span. In the present study, we tested the hypothesis that SOD activity is elevated when flies are exposed to dilute diet conditions. There was little effect of diet on SOD activity; exposure to dilute diets that extend life span did not increase overall SOD, CuZn SOD or Mn SOD activity. We confirmed the results reported in Clancy et al. (2001) by observing that overall SOD activity was elevated in *chico*^{1} homozygotes, and extended this work showing that this increase is due to an increase in CuZn SOD activity.

Materials and methods

SOD activities were measured using females because the effect of dietary dilution on life span has been observed to be more pronounced for females than for males (Chapman and

Partridge 1996). Virgin females were used to avoid the effect of courtship and mating, especially the impact male seminal proteins, on females. Two different methods were used to measure SOD enzyme activity on flies held on rich, normal or the most dilute diet. One assay generates a composite ("overall") measure of SOD activities as employed in Clancy et al. (2001). A different type of SOD assay allowed us to measure cytosolic SOD (CuZn SOD) and mitochondrial SOD (Mn SOD) after adult females were held on a diet. In addition, this assay allowed us to extend the results of Clancy et al. (2001).

Fly stocks and rearing conditions

To investigate the effect of diet on SOD activity, flies were reared from vials seeded with 75 eggs. Three diets, in which the proportion of sugar and yeast was altered, were used to hold adults before SOD assays. Holding adults on the most concentrated of these diets resulted in the shortest life span and those held on the most dilute diet had the longest life span (Chapman and Partridge 1996). Dextrose was present in varying proportions in the diets: 5% in the low diet, 10% in the normal diet and 15% in the high diet. Yeast percentage varied from 5% in the low diet, to 10% in the normal diet and 15% in the high diet. Virgin females in the present study were held on low, normal or high diet at 25°C under constant illumination at density of 70 flies/cage. The cages were made out of quart plastic containers with a grommet for the addition of fresh food vials every 3 days and a slit for removal of dead flies by aspiration. The same cages were used to assess the lifetime survival of female exposed to each dietary condition. The wild type Dahomey stock, described below, was used for the comparison of SOD activities after exposure of adult females to defined diluted diets.

Wildtype flies used to investigate the effect of different diets on SOD activity, and for comparison of *chico* genotypes, were reared from egg to adult on a diet described in Carlson et al. (1998) in which the proportion of sugar and protein is approximately that of the Normal diet described above. In the present study, the $chico^1$ genotypes and conditions used to compare SOD activity in genotypes are essentially those used in Clancy et al. (2001). Females were either Dahomey wildtype (+/+), or they had one copy of the $chico^1$ allele (–) as heterozygotes (+/–), or they were mutant homozygotes (–/–). As described in Clancy et al. (2001), the $chico^1$ allele is maintained as a balanced stock that has been backcrossed to the Dahomey outbred laboratory population.

Sample preparation and determination of SOD activity

Overall SOD activity was measured as described by Paoletti and Mocali (1990). Flies were homogenized in 10 mM Tris–HCl, pH 8, and 2.5 mM NaCl using a glass tissue grind pestle (Kontes/VWR) on ice. The homogenate was centrifuged at 2,500 × *g* for 5 min to remove debris. The supernatant was washed with the same buffer using a 10 kD spin column (Amicon YM10) to remove molecules smaller than 10 kDa that could potentially contribute to the observed activity in the SOD assays. The supernatant was concentrated to approximately 50 μl in a 10 kD spin column to remove small molecules with antireactive oxygen species (ROS) activity and the concentrate was diluted to 1.5 ml with homogenization buffer. This was repeated twice and the final volume was brought to 0.5 ml before being used in the assays. All steps during the sample preparation were carried out at 4°C. SOD

activity was measured as the sample inhibition of the oxidation of NADH by superoxide radicals that are generated chemically in the reaction mixture containing EDTA, $MnCl_2$ and mercaptoethanol. Oxidation of NADH was monitored spectroscopically at 340 nm at 25°C. The reaction mixture was prepared as follows: 800 μl TDB (100 mM Triethanolamine–100 mM diethanolamine–HCl), 40 μl NADH, 25 μl EDTA–$MnCl_2$, and 100 μl sample (or homogenization buffer for the control) was added to a cuvette and mixed thoroughly. This mixture served as a baseline and its absorbance was recorded at 340 nm for 5 min for NADH oxidation occurring in the mixture. The reaction was started with the addition of 100 μl of mercaptoethanol and the inhibition of NADH oxidation by SOD activity was followed at 340 nm for 20 min. Saturation curves were prepared for each sample to determine the amount of homogenate that resulted in 50% inhibition of NADH oxidation. Activity was calculated as described in Paoletti and Mocali (1990) and specific activities were reported. Five-day-old and 15-day-old flies used with this method and at least three repeat assays using homogenates from different generations of flies.

The second SOD assay (Mocket et al. 2002) permitted measurement of CuZn SOD or Mn SOD activity as components of overall SOD activity. For this procedure, flies were homogenized in 50 mM potassium phosphate buffer, pH 7.8. Debris was removed by centrifugation at 10,000 × *g* for 10 min. The supernatants were treated either with 5 mM sodium cyanide (NaCN) or with 2% SDS to measure Mn SOD and CuZn SOD activity, respectively. This assay utilizes a xanthine–xanthine oxidase system to generate superoxide radicals which reduce NBT to formazon. Formazon formation was followed spectroscopically at 560 nm at 25°C. The inhibition of formazon formation by the addition of samples (fly homogenates) was used to determine SOD activity. The reaction was conducted in 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM DTPA, 132 μg BSA, 1 unit catalase (to remove hydrogen peroxide from the reaction mixture), 60 μM NBT, 0.16 mM xanthine, 50 μM BCS, 0.0025 unit xanthine oxidase. For Mn SOD activity, 5 mM NaCN (final concentration) was added. The reaction was started by addition of xanthine oxidase. A reaction mixture without sample was used to determine the maximum uninhibited rate of formazon formation. Due to the fact that there was no difference between 5-day-old and 15-day-old flies for the first SOD assay, in the Mocket et al. (2002) SOD procedure we used one time point (7- day-old flies) as a source of homogenates for 3–4 replicate assays conducted using different samples of flies. For the second SOD procedure, only wildtype and homozygous mutant genotypes were compared.

Soluble protein concentration was determined by the BCA protein assay kit (Pierce Company) using the microplate version of the assay. Enzyme activities were calculated as specific activities meaning that they standardized by soluble protein concentration in the homogenates.

Results and discussion

Data analysis

Females were reared on low, normal and high food to assess their lifetime survival and to provide flies for assays (from almost identical conditions). The most dilute diet resulted in the longest life span and the most concentrated diet resulted in the shortest life span (fig.

1). The level of cumulative mortality was at or below 10% in all lines until approximately day 15. Thereafter, it declined steeply in the High and Normal diet. The onset of a rapid increase in mortality was delayed for most dilute diet (Low). Based on this data, flies for SOD assays were not sampled from time points at which substantial mortality had occurred. Survival at such ages could select for a genetically differentiated subset of the original population which is derived from the outbred, genetically heterogenous, Dahomey population. Such selection could affect SOD activities in flies held on different diets given the sharp differences in survival approximately after day 15.

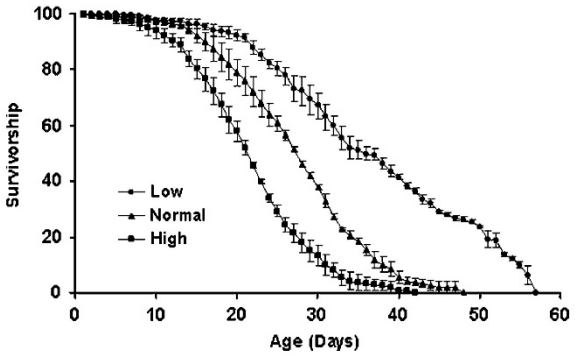

Figure 1. Lifetime survival of wild type Dahomey females held on different diets. Females were maintained in cages with low, normal, or high diet as described in the materials and methods. Mortality was recorded daily, and fresh food was provided every 3 days. Survival was longest on the dilute diet (low) and shortest on the most concentrated diet (high).

Overall SOD activity at either of two adult ages did not differ as a result of holding females on the different diets (fig. 2). There was no statistically significant effect of diet on overall SOD activity. The statistical analysis indicates that there were no significant main effects of diet ($P = 0.6162$) or age ($P = 0.1945$), or significant interaction between diet and age ($P = 0.4020$). Overall SOD activity was approximately the same among diets in 5-day-old females (fig. 2a). By day 15, the activity appeared to have increased on the High diet relative to the other diets (fig. 2b). Perhaps this increase is associated the onset of elevated mortality on this diet (fig. 2) which might be the case if that the survivors have.

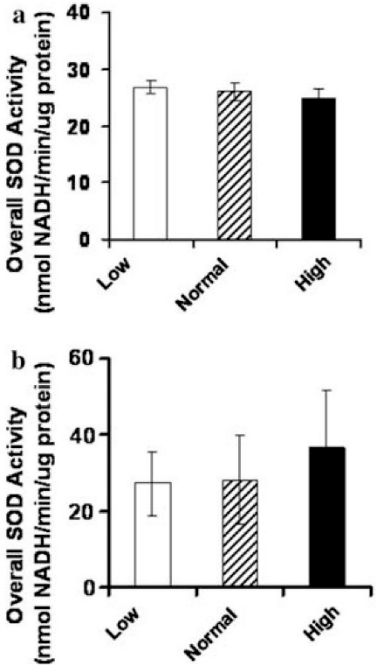

Figure 2. Overall SOD activities of 5-day-old and 15-day-old flies on diets that vary in nutrient concentration. Overall specific SOD activity was determined for flies kept on low, normal, and high diet at day 5 (a) of adult life and day 15 (b) using the method of Paoletti and Mocali (1990). Mean (SE) specific activities are presented for each diet and age. There was no significant difference between diet and age nor statistically significant interaction between diet and age.

CuZn SOD or Mn SOD activities did not differ among sets of females held on Low, Normal and High diets (fig. 3). At day 7 of adult life there was no statistically significant difference in CuZn SOD activity ($P = 0.6833$) nor Mn SOD activity ($P = 0.6604$) (fig. 3a, b, respectively).

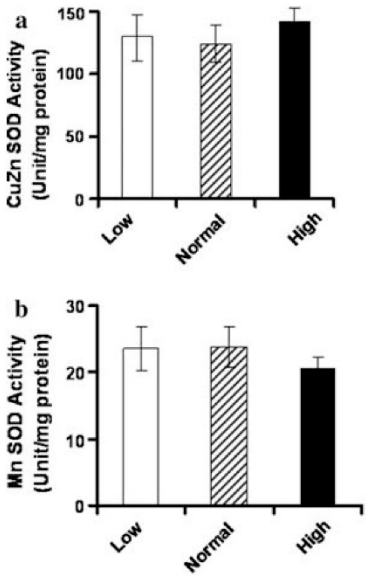

Figure 3. CuZn SOD and Mn SOD specific activities of females held on diets that differ in nutrition concentration. CuZn SOD (a) and Mn SOD (b) specific activities were determined by the method of Mockett et al. (2002) on a dilute diet (low), twice the amount of sugar and yeast (normal) and a rich diet that had three times the dilute diet concentration of sugar and yeast (high). There was no statistically significant Cu Zn SOD nor Mn SOD activity differences among the three diets.

There was approximately a two-fold elevation in homozygous *chico*^{1} females compared to the heterozygote and wild type (fig. 4). The statistical analysis indicates that there is a difference in specific activity among the three genotypes ($P = 0.0370$). Moreover, *chico*^{1} was significantly different than the heterozygote ($P = 0.0272$) and wildtype (0.0215), whereas the latter two genotypes do not differ in activity ($P = 0.7287$).

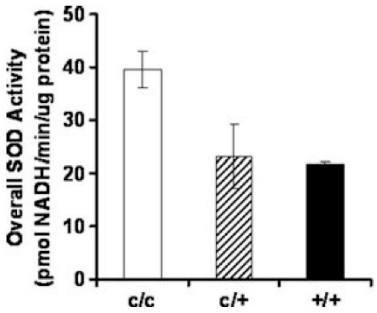

Figure 4. Overall SOD activities in an insulin substrate protein gene mutation homozygote (c/c), heterozygote (c/+) and wild type (+/+). Mean (SE) specific net enzyme activities of 7-day-old females are presented. Homozygous *chico*^{1} exhibited higher specific activity than the heterozygote or wildtype, whereas the latter two genotypes did not differ in activity.

CuZn SOD activity was elevated in homozygous *chico*[1] females compared to wildtype, but Mn SOD activity did not differ (fig. 5). There is a statistically significant difference between in CuZn SOD activity (P = 0.0504). Elevated levels of the cytosolic form of the enzyme apparently account for the difference observed in overall activity for these two genotypes (fig. 4). The activity of Mn SOD in homozygous *chico*[1] females was not higher than in wild type females (P = 0.4635).

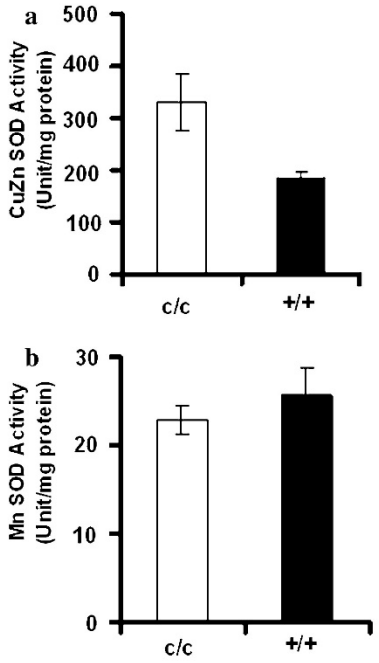

Figure 5. CuZn SOD, Mn SOD activities for genotypes (homozygous mutant *chico*[1] and wild type). There was a statically significant difference between genotypes in CuZn SOD specific activity; *chico*[1] homozygotes had approximately two-fold higher in activity (a). The specific activity of Mn SOD *chico*[1] homozygote females did not differ from that in wild type Dahomey females (b).

General discussion

In the present study, we have shown that dietary dilution conditions that extend life span do not affect SOD activities in females held on the different diets. We also show that the overall level of SOD activity in *chico*[1] homozygotes is approximately two fold higher than in the heterozygote and wildtype which confirms the result reported in Clancy et al. (2001). The suggestion is that insulin signaling and dietary restriction do not result in life span extension by the same mechanisms given that SOD activities were not similarly affected in the long-lived insulin signaling mutation and dietary conditions that extend life span. Finally, we show that the increase in overall SOD activity reported in Clancy et al. (2001) was due to an increase in CuZn SOD activity in homozygous *chico*[1] females.

Approximately the same level of SOD activity was observed on each of the three diets tested irrespective of the effect of diet on female life span. Thus, we reject the hypothesis that SOD activity plays a role life span extension of *D. melanogaster* by dietary dilution. *chico*^{1} homozygotes exhibited higher levels of overall SOD activity than heterozygous or wild type females as was observed in Clancy et al. (2001). In the present study, *chico*^{1} homozygotes exhibited relatively high activity for CuZn SOD compared to wild type, but no increase was observed for MnSOD activity when these genotypes were compared. These results suggest that the increase in overall SOD activity associated with *D. melanogaster* insulin signaling mutations (Clancy et al. 2001; Tatar et al. 2001) is due to an elevated level of CuZn SOD activity.

Biochemical studies on the effect of caloric restriction on the activity of antioxidant enzymes, including SOD activity, have been conducted using rodents. Caloric restriction was found to increase catalase and glutathione peroxidase activity in rats, along with a slight increase in glutathione S-transferase activity, but there was no change in cytosolic (CuZn) SOD activity (Kim et al. 1996). However, Rao et al. (1990) observed that caloric restriction can attenuate the age dependent decrease of SOD activity. In general, caloric restriction has been observed to increase, decrease, or have no effect on antioxidant enzyme activity in rodents (Koizumi et al. 1987; Laganiere and Yu 1989; Rao et al. 1990; Feuers et al. 1993; Luthala et al. 1994).

Insulin signaling mutations that extend life span typically exhibit elevated SOD activity as a pleotropic effect. *C. elegans age-1* mutations have relatively high basal (uninduced by exposure to oxidants) levels of SOD and catalase activities and are resistant to oxidative stress (Larsen 1993; Vanfleren 1993; Vanfleteren and De Vreese 1996). *daf-2* mutants have relatively high overall SOD activity (Vanfleteren and De Vreese 1995) and higher *sod-3* gene (Mn SOD) activity than wildtype (Honda and Honda 1999). *C. elegans* Mn SOD is regulated by the transcription factor at the base of the insulin signaling pathway (Ogg et al. 1997; Kops et al. 2002). In *Saccharomyces cerevisiae*, an insulin signaling-like mutation (*sch9*) regulates cell life span and through its effect on Mn SOD (Fabrizio et al. 2001, 2003). In *Mus musculus*, a mutation in the insulin-like growth factor type 1 receptor gene was not viable as a homozygote, but the heterozygote exhibited a longer life span and survived longer when an oxidant (paraquat) was injected into mice (Holzenberger et al. 2003). In the present study, Mn SOD activity was not elevated in *chico*^{1} homozygous mutant females compared to wildtype females. It might be relevant that there is no predicted Daf16 binding site binding site upstream of the Mn SOD gene in both *C. elegans* and *D. melanogaster* (Lee et al. 2003); the corresponding promoter is a regulator of Mn SOD in worms. CuZn SOD activity is typically much higher than Mn SOD activity in invertebrates and vertebrates and in the present study there is approximately an order of magnitude greater CuZn SOD activity than Mn SOD. Based on this consideration, it was perhaps to be expected that increased CuZn SOD activity was responsible for the elevation of overall SOD activity in the present study and as described in Clancy et al. 2001). This result highlights a topic for future research which would be to determine if the CuZn SOD gene is regulated by the Drosophila ortholog of *daf 16* gene, the key transcription factor at the base of the insulin signaling cascade.

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