Free Rad. Res. Comms., Vol. 17, No. 1, pp. 73–85
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STRESS RESISTANCE OF *DROSOPHILA* TRANSGENIC FOR BOVINE CuZn SUPEROXIDE DISMUTASE

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(Received March 22, 1992; in revised form April 20, 1992)

Several oxidative and non-oxidative stresses were applied to two transgenic strains of *Drosophila melanogaster* (designated P(bSOD)5 and P(bSOD)11) that express superoxide dismutase (SOD) at elevated levels, and control strains that express normal SOD levels. Transgenic strain P(bSOD)5 exposed to paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride), a redox cycling agent that generates superoxide anion when metabolized *in vivo*, was significantly more resistant to this xenobiotic than control flies. When test flies were subjected to 100% oxygen for 20 min each day, the mean lifespan was 3.62 days for control strain 25, but 4.35 days for both transgenic strains. The mortality curves of all strains fed 1% H_2O_2 were similar, but the median lifespan of 72 h for controls and 64 h for transgenics suggests that the transgenic flies were slightly more sensitive to H_2O_2. The activity of catalase was the same for all strains. Using starvation resistance as a non-oxidative stress, flies maintained on water without any food had identical survival curves; for all strains, the median lifespan was 72 h. Throughout the lifespan, no statistically significant difference in physical activity was displayed for transgenic versus control flies. Collectively, these data suggest that the increased lifespan previously observed in SOD transgenics is specifically related to resistance to oxidative stresses.

KEY WORDS: Free radicals, transgenic *Drosophila*, superoxide dismutase, oxidative stress.

INTRODUCTION

A number of current studies are attempting to test the "free radical theory of aging" by examining the correlation between oxidative stress and age-dependent molecular damage.[1-3] This theory predicts that the chronic generation of unscavenged, highly reactive oxygen species from the respiratory chain is one of the main contributors to the progressive degeneration of the cell. The most unstable oxygen radicals are the superoxide and the hydroxyl; they are produced in the mitochondria as byproducts of normal oxygen metabolism and in other parts of the cell as the result of metabolism of certain xenobiotics and background radiation exposure.[2] By rapidly reacting with neighboring molecules to pair their single electron, they alter the redox status of various biotargets and damage their structure. Oxidative alterations usually include lipid peroxidation with associated changes in membrane characteristics, DNA

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mutations and enzyme inactivation. To counteract free radical toxicity, cells have developed sophisticated enzymatic and non-enzymatic antioxidant defense systems along with repair mechanisms. Four critical enzymes assume the first steps of the oxygen detoxification: the mitochondrial and cytoplasmic superoxide dismutases (SOD) convert the superoxide radical (O_2^-) to the less toxic hydrogen peroxide (H_2O_2). Downstream, the catalase (CAT) and glutathione peroxidase (GPX) are responsible for the breakdown of hydroperoxides to water. At the end of the scavenging process, two molecules of superoxide radical have been converted into one molecule of water.

Several publications have reported an age-related increase of oxidative stress in species with high metabolic rates, as measured by the decline of reduced glutathione content, and elevated rates of prooxidant generation, in old organisms.[4–9] Other investigators working with the rat or the frog could not detect such changes in old tissues, but this observation does not eliminate the possibility of an accumulation of free radical-induced damage throughout the life span.[10,11] Despite a wealth of data, it is still unclear whether variations in the activity rates of antioxidant enzymes,[12,13] or the fact that they are naturally present in suboptimal concentrations, are among the underlying causes of the increase of oxidative stress during aging.

One approach to this question is the development of transgenic animals with elevated levels of one or more of the antioxidant enzymes. Presently, several models with elevated SOD levels have been obtained. They also reflect the recent interest given to antioxidant therapy through high SOD levels,[14] but results are controversial regarding the biological consequences of such an increase. Contradictory conclusions with respect to resistance to oxidative stress have been reported for transgenic organisms[15–17] or transfected cells.[18–21] In some cases, both the beneficial and the detrimental aspects of elevated SOD have been described within the same model: the overproduction of human CuZn SOD in transfected cells decreases paraquat mediated cytotoxicity and paradoxically enhances lipid peroxidation.[22] Transgenic mice with elevated levels of CuZn SOD are resistant to pulmonary oxygen toxicity,[23] but these animals also display some of the abnormalities found in Down's syndrome patients, such as diminution of blood serotonin,[24] abnormalities in neuromuscular junctions,[25] and diminished prostaglandin synthesis.[26] Expression of bovine SOD in *Drosophila* augments resistance to oxidative stresses, but has been associated with death of imagoes in some strains with high SOD activity.[17] In transgenic plants, a moderate increase of Mn SOD either in chloroplast or in mitochondria is detrimental during dark incubations with paraquat, but with maximal Mn SOD expression, this effect is completely reversed.[27]

An important clue to understanding the SOD antioxidant properties has emerged from studies focused on preventing myocardial reperfusion injury by administrating Mn or CuZn SOD. They report bell-shaped dose response curves, with a consistent loss of protection at high doses of SOD, and are directing research toward a definition of the optimal dose for SOD.[28,29]

Our laboratory has generated transgenic *Drosophila* with elevated levels of SOD by introducing the bovine CuZn SOD cDNA into their genome.[17] In the present study, we report on the resistance of the transgenic files to acute oxidative stresses that generate the superoxide anion, among other free radicals, and act upstream of the dismutation reaction.

MATERIALS AND METHODS

Drosophila *Strains and Cultures*

Flies transgenic for the bovine SOD were generated by microinjecting *Drosophila* embryos with *p*-elements containing the bovine CuZn SOD cDNA under the control of the *Drosophila* actin 5C gene promoter. These strains are described in detail elsewhere.[17] Populations were developed and raised at 25°C in half-pint bottles containing Formula 4–24 instant *Drosophila* medium (Carolina Biological Supply Co., Burlington, NC) under a 12 h light–12 h dark cycle.

Paraquat and Hydrogen Peroxide Toxicity Studies

To determine sensitivity of flies to the xenobiotic, groups of 30 males 1–5 days old were placed in 35 ml glass vials with a 3 × 3 cm piece of Whatman ®1 filter paper wetted with a 15% sucrose solution containing either 20 mM paraquat or 1% H_2O_2, made fresh daily. H_2O_2 gradually deteriorates under these conditions, hence the filter paper is wetted twice daily with freshly made solution stored at 4°C. As previously described,[17] the number of dead flies was counted at 16, 24, 42 and 48 h for the paraquat study and 24, 48 and 72 h for the H_2O_2 study. These data were derived from more than 700 flies.

Hyperoxia

For determination of transgenic and control flies sensitivity to hyperoxia, groups of approximately 100 male adult flies, 1–5 days old, were placed in 1000 ml sealed conical side arm flasks containing instant *Drosophila* medium. Flasks were then flushed for 20 min with 100% medical grade oxygen each day and the flasks were sealed. The number of dead flies was counted each day following the exposure period. These data were derived from more than 2000 flies.

Heat Shock

Heat shock was applied by transferring 20 males to 35 ml thin-walled glass vials (containing filter paper moistened with 15% sucrose) and placing them in a 39.5°C incubator for 2.5 h. The flies were then allowed to recover at 25°C for a 24 h period, at the end of which the number of dead was counted. These data were derived from more than 700 flies.

Evaluation of Resistance to Starvation

Groups of 30 males 1–5 days old were transferred from vials containing instant *Drosophila* medium to vials containing filter paper moistened with deionized water only. The filters were rewetted each day, and the number of dead flies was counted every 24 h for a period of 4–6 days. These data were derived from more than 700 flies.

Measurements of Vitality

Two-day-old adult males of each strain were randomly distributed into groups of 50 per 0.5 pint (*ca.* 0.24 l) bottle and transferred three times weekly into new bottles

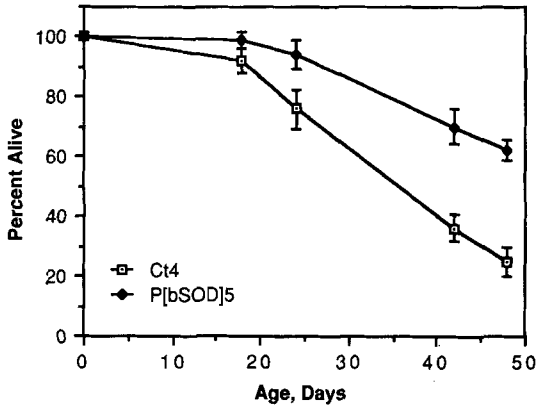

FIGURE 1 Survival curves for populations of bSOD transgenics and control flies that were continuously fed a solution containing 20 mM paraquat. The error bars are the standard error of the mean of over 700 flies.

containing instant medium. Vitality was determined as described previously.[30] Transgenic and control flies were transferred weekly at the bottom of a 500 ml graduated cylinder, and the number of flies passing the 100 ml mark in 10 s was recorded. Experiments started with 7-day-old *Drosophila* and lasted throughout their lifespan. These data were derived from more than 600 flies.

Catalase Activity

Crude extracts were prepared from *Drosophila* as follows: 25 males were homogenized in 250 μl of 20 mM Tris-acetate, pH 7.8, 0.1% TX-100, 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged at 11 000 $\times$ *g* for 15 min at 4°C, and the collected supernatant was used for determination of the CAT activity by the spectrophotometric method of Clairborne.[31] The protein content of each fraction was determined by the Bio-Rad method (Bio-Rad Laboratories, Richmond, CA).

RESULTS

SOD Transgenics are Resistant to Paraquat and Hyperoxia

Exposure to paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride, methyl viologen) and hyperoxia are known to generate superoxide radical anions, the substrate of the dismutation reaction, via redox cycling *in vivo*.[32-34]

In a first set of experiments, flies were fed a sucrose solution containing paraquat at 20 mM final concentration. The number of dead flies was recorded at 16, 24, 42 and 48 h. As profiled in Figure 1, compared to control flies, transgenic *Drosophila* with elevated levels of CuZn SOD are significantly more resistant to paraquat. At all time points, the transgenic strain P(bSOD)5 is more resistant than the control flies to the xenobiotic administration of the prooxidant. The resistance interval between the two strains steadily increases with time and goes from 1.1 times at 15 h to 1.3 times at 24 h, to 2.0 times at 42 h, to 2.6 times at 48 h. We previously reported that at 48 h, control

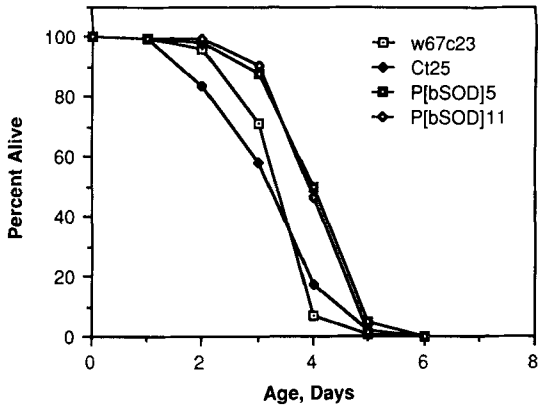

FIGURE 2 Survival curves of populations of transgenic and control flies that have been exposed to 100% oxygen atmosphere for specified times each day.

flies are more sensitive than transgenic flies at any paraquat concentration within 5–40 mM concentration range.[17]

The second set of experiments is summarized in Figure 2 and Table I. It involved hyperoxygenation of the various *Drosophila* strains, and the results are similar to those obtained in the paraquat study. Several groups of 100 flies each were exposed to 20 min of pure oxygen every 24 h, and the number of dead flies was recorded at the end of each day. Again, a significant difference in the resistance to oxidative stress is observed between the transgenic and control flies. Under hyperoxia, the mean survival is 3.62 days for control 25 and 4.35 days for P(bSOD)5. The other transgenic strain studied, P(bSOD)11, behaved exactly like P(bSOD)5 with a mean survival calculated at 4.35 days. The original recipient strain Df(1)w67c23,y, used for microinjection had a mean survival of 3.75 days.

SOD Transgenics are Resistant to Heat Shock

When groups of 25 flies are exposed to the elevated temperature of 39.5°C for 2.5 h, and allowed to recover at 24°C (at which *Drosophila* strains are routinely cultured) for 24 h, insects that carry the extra copy of the CuZn SOD gene and constitutively express the protein at relatively high amounts are more resistant to heat-induced mortality than controls. Results presented in Table II indicate that both transgenics P(bSOD)5 and P(bSOD)11 withstand hyperthermia better than control strains Ct10 and Ct25. Although these results are not statistically significant for every comparison,

TABLE I
Mean lifespan for transgenic and control strains exposed to a 100% oxygen atmosphere

Strains	Mean age, days	Standard deviation
Df(1)w67c23,y	3.75	0.67
Ct25	3.60	1.01
P(bSOD)5	4.41	0.82
P(bSOD)11	4.32	0.76

TABLE II
Percent of population that died by 2.5 h following exposure to 39.5°C

Strains	Mean	Standard deviation
Ct10	40.0	7.5
Ct25	29.8	6.0
P(bSOD)5	19.4	4.3
P(bSOD)11	30.0	3.5
Controls[a]	35.8	5.1
Transgenics[a]	25.2	2.8

[a]Controls are the combined values of Ct10 and Ct25. Transgenics are the combined values of P(bSOD)5 and P(bSOD)11.

Student *T* test for these data generated the following *p* values:

Ct10 vs Ct25 = 0.33; Ct10 vs P(bSOD)5 = 0.01;
Ct10 vs P(bSOD)11 = 0.17;
Ct25 vs P(bSOD)5 = 0.19; Ct25 vs P(bSOD)11 = 0.97;
Ct10 + Ct25 vs P(bSOD)5 + P(bSOD)11 = 0.05.

the trend is clear that transgenics are more resistant than control strains to heat shock. Moreover, when the data are combined for all controls and all transgenics, the results are statistically significant.

SOD Transgenics are not Resistant to Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a product of the dismutation reaction and the substrate for catalase which converts it to water and molecular oxygen. H_2O_2 is very toxic when administered to *Drosophila*. Through the Fenton reaction, H_2O_2 can initiate the formation of the highly reactive hydroxyl radical.[35] Flies are fed a sucrose solution containing 1% H_2O_2, and the number of dead flies is determined at regular time intervals. Figure 3 shows no significant difference between the survival rates of the various strains, at 24 and 48 h. But it should be noted that at 72 h, compared to

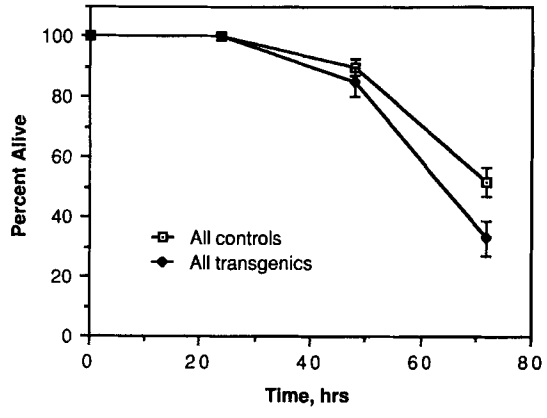

FIGURE 3 Survival curves of bSOD transgenics and control flies that were continuously fed a solution containing 1% hydrogen peroxide. The error bars are the standard error of the mean of over 700 flies.

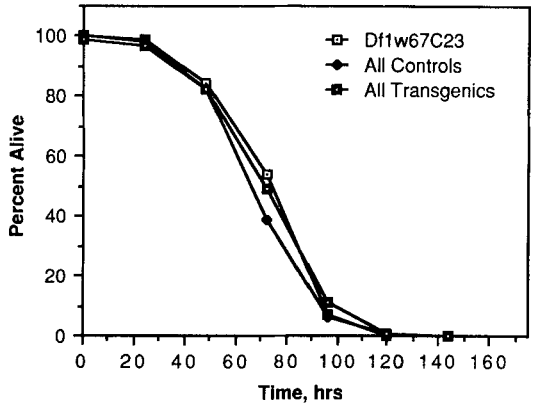

FIGURE 4 Survival curves of bSOD transgenics and control flies that were maintained on water in the absence of any food.

control flies, the transgenic strains seem to be slightly more sensitive (1.6 times) to hydrogen peroxide treatment.

Mortality curves of Starving SOD Transgenic and Control Flies are Identical

We have studied the effect of starvation on the survival rates of the transgenic and control flies. In our laboratory and in absence of other forms of stress, *Drosophila* that are only given deionized water invariably die within 5 days. Survivors are counted each day for 5 days and survival curves are established. As can be seen in Figure 4, SOD transgenic and control flies show no difference in starvation resistance. Results obtained with the recipient strain Df(1)w67c23,y were similar. The mean lifespans plus standard deviations for these experiments are 82.6 + 20.7 for Df(1)w67c23,y; 75.6 + 18.8 for controls and 81.5 + 23.6 for transgenics.

Activity Levels of SOD Transgenics and Control Flies are Identical

Vitality of the insects was assessed by their ability to pass a 100 ml graduation mark in an upright graduated cylinder within 10 s.[30] Data were collected once a week throughout the lifespan, starting with flies 7 days of age. At the end of the study, and with flies 50 days old and over, the activity is reported to be null even though some movement can be seen, as long as the "100" mark is not reached. Figure 5 displays the percentages of active flies plotted against their age. The initial differences between the strains, apparent during the first two weeks of life, are not meaningful, as indicated by statistical analysis of the numbers. They may be due to the difficulty for the human eye to precisely record fast-moving *Drosophila* of young age. There was no significant difference in activity levels between control and transgenic flies.

Catalase Enzymatic Activity is Unaffected by CuZn SOD Overexpression in SOD Transgenics

Catalase acts downstream of the superoxide dismutase, and both proteins work in concert to protect cells against oxidative damage. Glutathione peroxidase, GPX, an

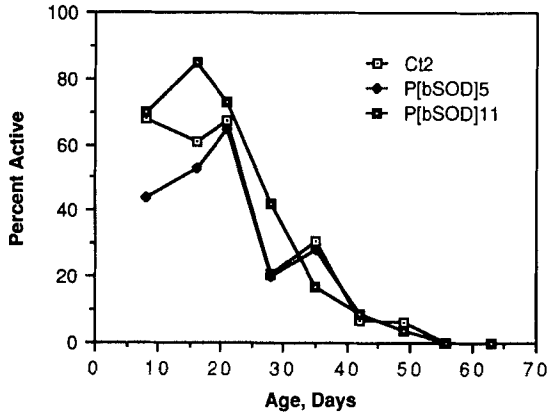

FIGURE 5 Percent of physically active bSOD transgenics and control flies for specified time points throughout their lifespan.

enzyme that also scavenges peroxides has not been demonstrated in *Drosophila*. Although it has been shown that GPX is in *Musca domestica* after the flies are supplemented with selenium, a role for GPX in scavenging hydroperoxides in *Drosophila* has not been established.[52] We investigated the potential variations of the CAT activity that may be affected by elevated levels of the CuZn SOD in SOD transgenics. Spectrophotometric evaluation of the CAT activity in P(bSOD)5 and Ctr4 is presented in Table III. No difference was observed between transgenics and controls.

DISCUSSION

The engineering of transgenic *Drosophila* in our laboratory with elevated levels of the CuZn superoxide dismutase allowed us to test the efficiency of this free radical scavenging enzyme against certain forms of acute oxidative damage.

Our results suggest that elevated levels of SOD may confer a clear advantage to organisms exposed to an accumulation of superoxide anions. The treatment of flies with paraquat and hyperoxia generates non-physiological, high concentrations of O_2^- within the cells. Since the primary enzymatic scavengers of O_2^- are the superoxide dismutases, we expected and observed a higher survival rate with the strains over-producing the CuZn SOD, after paraquat or oxygen exposure. These data are consistent with recent reports on the effects of similar oxidative stresses in other SOD

TABLE III
Table showing catalase activity levels of transgenic strain P(bSOD)5 and control strain 4 as determined by the spectrophotometric method of Clairborne.[31] Data are the mean of four separate experiments for each strain

Strains	Abs/s/μg protein
Ct4	3.31×10^{-4}
P(bSOD)5	3.36×10^{-4}

transgenic models. Bowler *et al.*[27] observed that in *Nicotiana tabacum*, MnSOD expression (controlled by the cauliflower mosaic virus promoter), with concomitant accumulation of MnSOD in the mitochondria, protects against superoxide induced damage. In old bolting leaves of transgenic plants, light and dark mediated paraquat toxicity is markedly diminished, as measured by a decrease in lipid peroxidation dependent membrane deterioration and pheophytin formation.[27] In transgenic mice with elevated levels of the human CuZn SOD, survival of young heterozygotes breathing 99% oxygen at 630 torr atmospheric pressure, is 2–3 times that of control value.[23] The fact that increased SOD activity counteracts the cytotoxicity of hyperoxia in animal models should be relevant for the research that aims at preventing bronchopulmonary displasia in neonates. SOD treatment of infants and adults under intensive respiratory therapy might gain more consideration after isolation and approval of adequate pharmaceutical forms of human SOD.[14]

Not surprisingly, we also observed that elevated levels of SOD confer some resistance to heat stress. After 2.5 h at 39.5°C, the number of survivors for transgenic flies P(bSOD)5 is twice that of control flies. Several publications have reported that hyperthermic and oxidative stresses involve common mechanisms, such as the intracellular release of free radicals within the cells.[36] In cell cultures, a similar set of well characterized heat-shock proteins can be induced both by heat and H_2O_2,[37–39] or by nicotine/heat and nicotine/ethanol treatments.[40] Increased enzymatic and non-enzymatic antioxidant defenses have also been associated with resistance to hyperthermia.[41–44] More recently, our laboratory has established that the electrophoretic properties of CuZn SOD and CAT in non-denaturing polyacrylamide gels were altered by heat stress, in normal *Drosophila* of different ages.[45]

Starvation, or removal of essential nutrients (glucose, amino acids, vitamins) that are required to maintain basic biological processes (DNA, RNA, protein synthesis and function) was employed to test the specificity of the oxidative stress resistance of the SOD transgenics. Elevated levels of SOD did not sustain starving transgenic flies longer than the controls. Starvation and diet restriction have different impacts on the cell physiology, the latter might involve subtle molecular mechanisms. A recent publication on energy intake restriction and antioxidant defense describes a significant shortening of the age-related SOD specific activity decline, from 37% to 17%, in livers of diet-restricted rats.[46] The remaining 17% decrease of SOD, unaffected by diet restriction, could still be a factor limiting the lifespan. Similar observations were reported for CAT and GPX activities. It is conceivable that a combination of constitutive overproduction of antioxidant enzymes, and life compatible measures that reduce their age dependent activity decline, may be a way to stabilize the free radicals enzymatic defenses throughout the lifespan.

The physical activity levels of SOD transgenic and control flies, as measured by their ability to fly or crawl the 100 ml position of a graduated cylinder in a given time, decreases dramatically with age. However, the relative abilities of SOD transgenic and control flies do not differ significantly from each other. Vitality of *Drosophila* has been shown to decline at similar rates with age.[30] In these studies, it was shown that activity was correlated with metabolic rate (O_2 consumption), thus suggesting that the O_2 consumption is not significantly altered in our transgenic flies.

H_2O_2-mediated cell injury occurs at different target sites within the cell, but it is still unclear what constitutes the initial molecular events in the cascade that eventually leads to cell death. DNA is highly sensitive to oxidative stresses, and H_2O_2 creates strand breaks.[47] Its extensive repair leads to the NAD and ATP depletion known to

be associated with cell lysis.[48] H_2O_2 activation of the hexose monophosphate shunt and glutathione redox cycling also participate in the depletion of intracellular ATP. Other critical damage includes inhibition of the glycolytic pathway due to direct inactivation of enzymes[49] and various alterations of the cell morphology at the cytoskeleton and plasma membrane levels.[50] We have verified that SOD activity does not change during the course of our experimental H_2O_2 stress. Both control and SOD transgenic *Drosophila* were analyzed for their SOD content on non-denaturing polyacrylamide gels, after 72 h on a diet consisting of sucrose only, or 1% H_2O_2 in sucrose. No noticeable change was detected in the activity of either isozyme (Mn or CuZn containing SOD), and in the transgenic flies, both the bovine and the *Drosophila* forms were functional (data not shown). These findings correlate with a study showing that H_2O_2 administration does not affect SOD and CAT activities in 15 days old *Musca domestica*.[51] On the other hand, a recent gel analysis in our laboratory of CAT activity from 4 days old, and 24 days old flies, which were fed 1% and 3% H_2O_2, showed a shift of activity from the usual slow electromorph (unstressed flies) toward a faster electromorph. This shift is not associated with a variation in total CAT activity.[45] It is not clear whether these electrophoretic shifts contribute to physiological alterations. In Figure 3, it is evident that at 48 h, SOD transgenics are slightly more sensitive to 1% H_2O_2 compared to the controls, and the gap increases at 72 h. The specific mechanisms involved in H_2O_2 hypersensitivity are not known. One possibility would be if, in cells producing high SOD levels, the equilibrium of the dismutation reaction were to shift toward an accumulation of the product. In this case, SOD transgenics would be confronted with above normal amounts of endogenous H_2O_2. If put on a diet containing H_2O_2, the intracellular toxicity limit might be reached more rapidly in these organisms compared to control flies. As presented in Table III, SOD transgenics do not upregulate the catalase enzyme. These results imply that a coordinated increase in the functionally coupled SOD and CAT enzymes is needed to address the subtle, long-term effects of toxic oxygen species on cellular function and integrity.

From the present analysis it is clear that SOD transgenic *Drosophila* are better protected against oxidative stresses incriminating O_2^-, while stresses that interfere downstream of the dismutation reaction affect SOD transgenic and control flies in a similar way. Tolerance of transgenic *Drosophila* to other exogenous sources of free radicals, such as UV radiations or pollutants, remains to be investigated. Depending upon the nature of the free radical generated by the stress, it should be possible to predict whether high synthesis of CuZn SOD in organisms would inhance their resistance to such adverse environmental conditions.

Acknowledgements

This research was supported in part from a grant to I. Reveillaud from the American Federation of Aging Research, and to R. Park from the Glenn Foundation. We thank Z. Herman for statistical analysis of data, and D. Read for helping with the manuscript.

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