Halving the selenophosphate synthetase gene dose confers hypersensitivity to oxidative stress in *Drosophila melanogaster*

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Received 5 November 2002; revised 29 November 2002; accepted 29 November 2002

First published online 11 December 2002

Edited by Barry Halliwell

Abstract Several lines of evidence indicate that selenoproteins mainly act as cellular antioxidants. Here, we test this idea comparing the sensitivity to oxidative stress (paraquat and hydrogen peroxide) between wild type and heterozygous flies for the selenophosphate synthetase *selD^{ptuf}* mutation. Whereas under normal laboratory conditions no difference in life span is observed, a significant decrease is seen in heterozygous flies treated with oxidant agents. In contrast, overexpression of the *selD* gene in motoneurons did not extend longevity. Our results strongly suggest that *selD* haploinsufficiency makes heterozygous flies more sensitive to oxidative stress and add further evidence to the role of selenoproteins as cellular antioxidants.

Key words: *selD^{ptuf}*; Selenoprotein; Oxidative stress; Life span

1. Introduction

Selenium (Se) is an essential dietary micronutrient of fundamental importance to health [1]. Most of the effects of Se are probably mediated by selenoproteins, which have this element covalently incorporated in the form of selenocysteine (Sec), the 21st amino acid. The majority of selenoproteins appear to have a role as antioxidants or catalyze oxidation–reduction reactions [1,2]. As a component of antioxidant enzymes, Se helps to protect cells from the harmful effects of reactive oxygen species (ROS). It is needed for proper function of the immune system, it is required for sperm motility and its deficiency may be linked to adverse mood states [1]. Evidence from prospective studies, intervention trials and studies on animal models has also suggested a strong inverse correlation between selenium intake and cancer incidence [3,4]. Nevertheless, the biological functions of Se are often inferred from epidemiological or cell culture studies pointing at a circumstantial relationship. In this work we have taken a genetic approach to assess the putative relationship between Se metabolism, oxidative stress and life span using the *selD^{ptuf}* mutation of *Drosophila*.

It has been postulated that an increase of macromolecular damage induced by ROS could be the central causal factor promoting the aging process [5]. Studies on oxidative stress and longevity often use molecular-genetic approaches in order to identify specific factors that may influence the rate of aging. The experiments carried out mainly in *Drosophila melanogaster* and *Caenorhabditis elegans* involve transgenic overexpression of antioxidant genes and induction of single loss of function gene mutations, but interpretation of such studies is quite controversial [6–8].

selD^{ptuf} is a null mutation affecting the gene encoding selenophosphate synthetase, a key enzyme of the selenoprotein biosynthesis pathway. Homozygous mutants die at third instar larvae and have extremely reduced and abnormal imaginal disks, with cells that accumulate ROS and enter apoptosis [9,10]. No selenoprotein synthesis is observed in those organisms [10]. Heterozygous flies are healthy and viable when kept under normal laboratory conditions. However, a downregulation of the Ras/mitogen-activated protein kinase (MAPK) pathway has been observed in transheterozygous combinations of *selD^{ptuf}* and activated members of this signaling pathway. Because a selenoprotein-independent increase in ROS caused by the catalase null allele *Cat^{n1}* also reduces Ras/MAPK signaling, increases in those free radicals may likely be responsible for this effect [11]. The read-out of our previous experiments strongly suggests that accumulation of ROS should be substantially different between heterozygous and wild type flies. However, changes in ROS might be subtle and biochemically difficult to detect since heterozygous flies are normal-appearing individuals. Therefore, it is necessary to confirm whether haploinsufficiency of *selD^{ptuf}* generates a background of oxidative stress sufficient to alter the efficiency of some cellular events, such as a reduction of the Ras/MAPK activity, without impairing the organism's viability. To that aim we measured the life span of flies on a highly oxidative diet, to test whether heterozygous *selD^{ptuf}* flies are more sensitive than wild type flies. We also overexpressed the *selD* gene specifically in motoneurons to assess the possible effects of increasing *selD* activity in longevity.

2. Materials and methods

2.1. Drosophila stocks

The *selD^{ptuf}* (*yw*; *l(2)k11320/CyO*) line was obtained from a collection of lethal mutants resulting from *PlacW* insertions on the second chromosome [9,12]. The viable revertant *selD^{rev}*, obtained by a precise excision of *PlacW* using the Δ2-3 transposase, was used as a control to minimize differences between genetic backgrounds. The transgenic line *UAS-selD* on chromosome 3 was generated in our laboratory [9] and its expression driven onto motoneurons using the *D42-GAL4* driver on the third chromosome [13] kindly provided by Dr. J.P. Phillips. In all experiments, only males were used because female life span is known to depend upon reproductive history [14]. All experiments were performed at 25°C, in constant humidity and light conditions.

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0014-5793/02/$22.00
doi:10.1016/S0014-5793(02)03790-0

2.2. Life span measurements

Adult males (0–48 h old) were maintained in vials (10 flies/vial) containing standard medium. Flies were scored daily for survivorship and transferred to new vials every 3 days. For life span determination we generated isogenic strains for most of the genome of *selD^{ptuf}/CyO y^{+}* and *selD^{rev}/CyO y^{+}*. A crossing scheme employing a *w* stock carrying both *CyO* and *TM3* balancers was also devised to produce +/+; *D42-GAL4/UAS-selD* and +/+; *UAS-selD*/+ stocks to minimize variation in genetic background between stocks for the second and third chromosomes. For statistical analysis the mean life span of each strain was calculated as the time (in days) at which survival reached 50% of the starting population. Survival data were analyzed by stratified log rank tests, using the SURVIVAL application of the SPSS10.0 software package.

2.3. Stress treatments

Adult males (3–4 days old kept in standard medium) were transferred to vials with 2 ml of special medium containing 1% sucrose, 1.3% low melting agarose and the specified concentration of paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) or hydrogen peroxide (H_2O_2). To avoid loss of oxidative activity, both substances were added when the temperature of the medium was 45°C. Each vial contained 10 males and survival was scored every day without changing the medium.

3. Results

3.1. Life span determination of selD^{ptuf} heterozygous flies

Heterozygous *selD^{ptuf}* flies develop into normal-appearing perfectly viable adults able to mate and give progeny. They are therefore kept as a regular laboratory strain. To assess the behavior of heterozygous flies regarding viability we determined the life span of *selD^{ptuf}* flies as well as that of the *PlacW* revertant, *selD^{rev}*, used as a control. As expected, mean life span of flies with one wild type copy of the *selD* gene was not significantly different from control ones. The mean (50% mortality) life span for each genotype was as follows: *selD^{ptuf}/CyO y^{+}*, 40.00 ± 0.91 days ($n = 319$); *selD^{rev}/CyO y^{+}*, 38.88 ± 0.67 days ($n = 284$). After performing the log rank test, no significant differences were obtained between *selD^{ptuf}* and *selD^{rev}* flies ($P = 0.1291$).

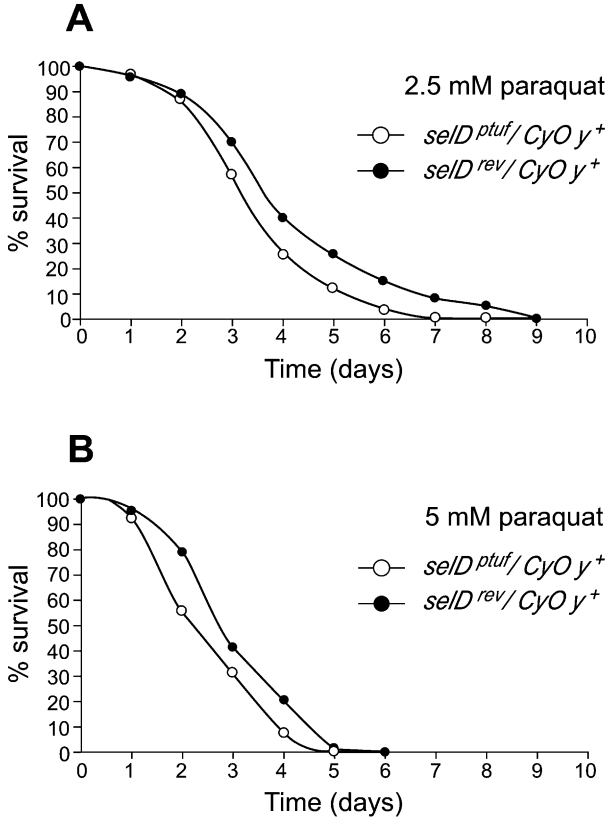

Fig. 1. Effect of different concentrations of paraquat on the longevity of *selD^{ptuf}/CyO y^{+}* and *selD^{rev}/CyO y^{+}* flies. *selD^{ptuf}* mutant in heterozygous condition is significantly more sensitive to paraquat oxidative treatment compared to its wild type revertant. A: 2.5 mM paraquat, $P = 0.0002$. B: 5 mM paraquat, $P = 0.0010$.

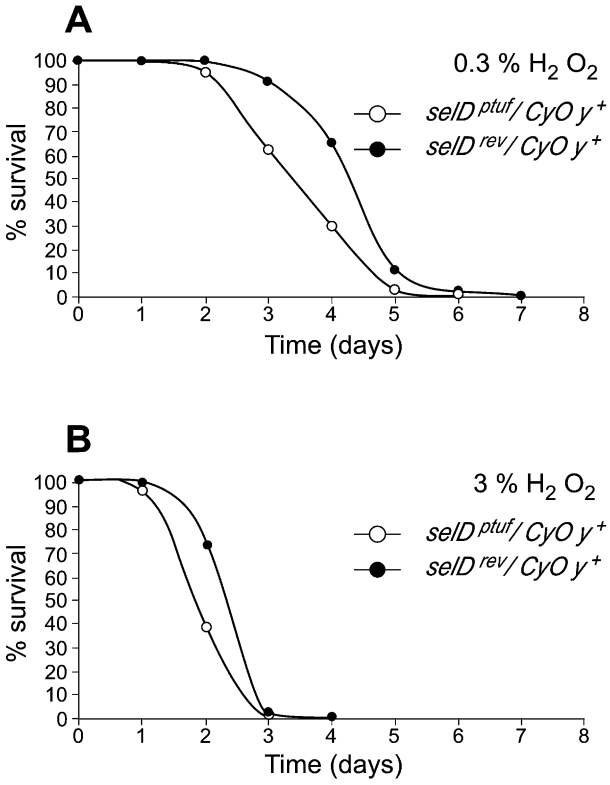

Fig. 2. Effect of different concentrations of hydrogen peroxide on the longevity of *selD^{ptuf}/CyO y^{+}* and *selD^{rev}/CyO y^{+}* flies. *selD^{ptuf}* mutant in heterozygous condition is significantly more sensitive to H_2O_2 oxidative treatment compared to its wild type revertant. A: 0.3% H_2O_2, $P < 0.001$. B: 3% H_2O_2, $P < 0.001$.

3.2. Sensitivity to paraquat and hydrogen peroxide toxicity

The sensitivity of *selD^{ptuf}/CyO y^{+}* and *selD^{rev}/CyO y^{+}* to enhanced production of ROS was tested feeding adult *Drosophila* with aqueous paraquat or H_2O_2 added to culture medium containing only sucrose as a nutrient. Such treatments likely expose flies to concentrations of ROS above the tolerance level of the fly's endogenous protective mechanisms. To minimize the effects of lower nutrient intake, animals were kept in standard medium for 3–4 days before the start of the experiment. Under these conditions, lack of one functional copy of *selD* conferred hypersensitivity to paraquat (Fig. 1). At 2.5 mM, mean life spans for each genotype were as follows: *selD^{ptuf}/CyO y^{+}*, 3.88 ± 0.11 days ($n = 150$) and *selD^{rev}/CyO y^{+}*, 4.55 ± 0.18 days ($n = 110$). At 5 mM, mean life spans were: *selD^{ptuf}/CyO y^{+}*, 2.91 ± 0.10 days ($n = 110$) and *selD^{rev}/CyO y^{+}*, 3.43 ± 0.11 days ($n = 100$). After performing the log rank test, the difference between both strains was significant at 2.5 mM ($P = 0.0002$) and 5 mM ($P = 0.0010$) paraquat concentrations. No differences were observed between both strains when lower (1 mM, not reaching the toxicity threshold) or

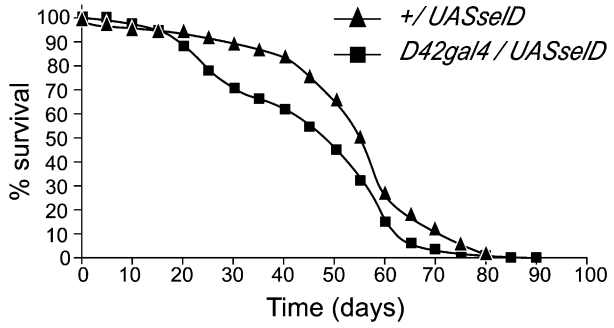

Fig. 3. Longevity determination of flies overexpressing *selD* in motoneurons using the specific driver *D42-GAL4*. Overexpression of *selD* in motoneurons significantly reduces life span compared to the control flies ($P < 0.001$).

higher (10 mM, highly toxic) doses of paraquat were used (data not shown).

A lower resistance of the $selD^{ptuf}$ genotype compared to the $selD^{rev}$ genotype was also observed when flies were exposed to different concentrations of hydrogen peroxide (Fig. 2). At 0.3% H_2O_2, mean life spans were: $selD^{ptuf}/CyO$ y^+, 3.90 ± 0.08 days ($n = 124$) and $selD^{rev}/CyO$ y^+, 4.70 ± 0.09 days ($n = 90$). At 3% concentration mean life spans were: $selD^{ptuf}/CyO$ y^+, 2.36 ± 0.05 days ($n = 150$) and $selD^{rev}/CyO$ y^+, 2.75 ± 0.05 days ($n = 110$). The difference between $selD^{ptuf}$ and $selD^{rev}$ flies was significant at 0.3% ($P < 0.001$) and 3% ($P < 0.001$) H_2O_2 concentrations.

3.3. Overexpression of $selD^{ptuf}$ does not extend life span

To determine the effects of *selD* overexpression on longevity, the *D42-GAL4* and *UAS-selD* transgenes were introduced into flies with normal $selD^{+/+}$ genetic background. Increased *selD* activity in motoneurons did not extend life span; on the contrary, it reduced longevity (Fig. 3). Mean life spans and sample sizes for each genotype were: +/+, *UAS-selD*/+, 50.97 ± 0.91 days ($n = 343$) and +/+, *D42-GAL4*/*UAS-selD*, 42.21 ± 0.93 days ($n = 352$). A significant decrease ($P < 0.001$) in life span was observed in flies overexpressing the selenophosphate synthetase compared to control flies.

4. Discussion

The main conclusion of our study is that the heterozygous condition of *selD* is more sensitive than wild type to oxidative stress conditions. Heterozygosity may lead to a less efficient biosynthesis of selenoproteins due to a limitation on selenium monophosphate availability. Because selenoproteins are involved in redox balance reactions, it is fair to assume that heterozygous flies have higher rates of ROS accumulation than wild type controls. Therefore, we propose that *selD* heterozygous flies accumulate ROS to levels not enough to impair cell viability, but sufficient to be detected in sensitized genetic backgrounds such as the Ras/MAPK signaling pathway [11]. It has been suggested that synthesis of selenoproteins in *Drosophila* may be driven by a selenophosphate synthetase other than *selD*, selenophosphate synthetase 2 (Sps2) [15,16]. However, the phenotypes observed in $selD^{ptuf}$ mutant animals, the lack of Se^{75}-labeled bands in mutant larval extracts [9,10], and the fact that Sps2 is itself a selenoprotein [16] back the key role of *selD* in the pathway.

The beneficial effects of Se on organisms could potentially be divested by a dietary selenium deficiency or impairing its metabolism (i.e. selenoprotein biosynthesis). Recently it has been shown that dietary selenium deficiency shortens while supplementation normalizes *Drosophila* life span [17]. This is consistent with reports on the deleterious effects of low Se intake on several aspects of human and animal health [1,18]. The reduced *selD* activity of heterozygous flies is sufficient for normal life, provided the animals grow in regular yeast-based medium that contains enough Se traces. Le Bourg [6] has suggested that antioxidant enzymes could be mainly considered stress enzymes, which would act as shields if necessary though are not essential for everyday life. Similarly, the haploinsufficiency of *selD* only becomes evident under oxidative stress conditions.

The production of oxidants, together with the ability to respond to oxidative stress, is intricately connected to aging and life span ([19] and references therein). ROS produced during normal metabolism cause damage to macromolecules that, if not repaired, places the organism at risk [5]. Intracellular defense systems that protect cells from ROS-induced damage include glutathione peroxidase (GPX), glutathione reductase (GR), thioredoxin reductase (TrxR), superoxide dismutase (SOD) and catalase (Cat) [20]. *D. melanogaster* flies lack GPX and recently it has been found that the single GR homolog specifies TrxR activity, which compensates for the absence of a true GR system for recycling GSH [21,22]. Although only three selenoproteins have been identified so far in the *Drosophila* genome [17,23] it is not possible at this moment to correlate the antioxidant activity with a particular protein. As the list of selenoproteins is increasing in higher organisms, this might also be the case in *Drosophila*. However, since SOD, Cat and TrxR are normal and functional in the flies used for this study, our results indicate that the burden of ROS metabolism in *Drosophila* is also shared by a defense system that includes selenoproteins.

The effects on longevity of numerous studies overexpressing antioxidant enzymes, such as Cat and SOD, have been controversial because life spans increase in some but not in others [6–8]. Following a report showing extended life span in *Drosophila* expressing human SOD1 in motoneurons [13], we tested the effects of *selD* overexpression in such cells. As expected, because *selD* is just one member of the complex machinery needed to synthesize selenoproteins, higher amounts of *selD* do not extend life span. The reduction observed could be explained by accumulation of toxic intermediaries (maybe selenophosphate) due to *selD* overexpression. Further experiments overexpressing specific *Drosophila* selenoproteins together with elements of the biosynthesis pathway may be a better way to test the contribution of selenoproteins' antioxidant function to prevent aging and extend life span.

Acknowledgements: We thank J. Baguñà, L. Serra, H. Tricoire and E. Sanchez-Herreros for insightful discussions and suggestions. This work was supported by Grant PB96-1253 from Dirección General de Investigación Científica y Técnica (DGICYT), by Grant 01/0906 from FIS, Ministerio de Sanidad y Consumo (to M.C.) and by a fellowship from CIRIT, Generalitat de Catalunya (to M.M.). The experiments reported in this paper were carried out according to the current laws of Spain concerning experimental manipulation.

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