Review article

Oxidative damage, aging and anti-aging strategies

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Received 28 February 2005; accepted in revised form 4 April 2005

Key words: antioxidant system, *Drosophila*, methionine sulfoxide reductases, oxidative stress theory of aging, protein oxidation, ROS

Abstract

The last two decades brought remarkable insight into the nature of normal aging in multicellular organisms. However, we are still far away from realizing extension of maximum lifespan in humans. An important modulator of lifespan is oxidative damage induced by reactive species, such as reactive oxygen species (ROS). Studies from yeast, *Caenorhabditis* and *Drosophila* primarily focused on (1) reduced generation or (2) elimination of ROS but have two principal shortcomings: (1) dietary restriction and single gene mutations are often associated with physiological impairments and (2) overexpression of components of the antioxidant system extend lifetime only under stress-induced conditions. Recent results from *Drosophila* indicate the involvement of an endogenous repair and elimination system for oxidatively damaged proteins in the process of aging. This system includes methionine sulfoxide reductase A (MSRA) and the carbonyl reductase Sniffer, the protein-ubiquitin ligase Parkin and the chaperone Hsp22. In this review we summarize different anti-aging strategies and discuss a synergistic interaction between protection against free radicals and specific repair/elimination of oxidative damage in lifespan extension primarily using the model system *Drosophila*. To achieve lifespan extension, available experiments are often methodically grouped into (1) caloric restriction, (2) single gene mutation, and (3) overexpression of genes. Here we summarize different strategies by a more causal classification: (1) prevention of ROS generation, (2) reducing free ROS level, and (3) repair and elimination of ROS-damaged proteins.

Abbreviations: CR – caloric restriction; met-O – methionine sulfoxide; MSRA/B – methionine sulfoxide reductases A/B; ROS – reactive oxygen species

Introduction

“Age is not a particularly interesting subject. Anyone can get old. All you have to do is live long enough.” (Groucho Marx)

Changes in cellular redox status have been postulated to contribute to aging and lifespan determina-

tion (free radical theory of aging: Harman 1956). A wide variety of efforts have been undertaken to delay senescence by manipulation of the overall oxidative stress level. A simple internet-search using keywords such as ‘oxidative stress’ and ‘lifespan’ will lead to a bewildering array of commercial ‘anti-oxidant’ and ‘anti-aging’ products, and even ‘Anti-Aging-Hospitals’ are meanwhile inaugurated. Unfortunately, most

of their rejuvenating promises are based on blind faith or even quackeries. Even though the causal relationship between oxidative damage and aging/lifespan has not been firmly established, numerous studies have indeed confirmed that increased levels of reactive oxygen species (ROS) are positively correlated with an overall increase in oxidatively modified protein level, functional impairment of oxidized proteins and subsequent cellular and organismal decline. Therefore, minimizing macromolecular damage induced by ROS may be a key not only for slowing aging but also for combating age-related diseases, such as some neurodegenerative diseases and cardiovascular diseases.

Homeostasis of ROS involves maintaining a fine balance between their production and elimination. Reactive oxygen radicals are generated as inevitable by-products of normal aerobic metabolism in mitochondria, but are also produced at other cellular loci by various oxido-reductases, such as NADPH oxidase at the plasma membrane. Because of their small size and high mobility coupled with their high reactivity, ROS readily oxidize cellular macromolecules and thereby change the overall cellular redox state. Regulated oxidation of specific molecules may mediate intracellular signal transduction (Finkel 2000, Stadtman and Levine 2002) and transient variations of ROS may be vital for normal cell function such as immune defense. However, a variety of cellular components may easily undergo uncontrolled oxidation. This oxyradical attack, probably an inevitable 'side effect' of oxygen consumption, contributes to age-related oxidative damage.

In addition to DNA and fatty acids, proteins represent a prime oxidation target. While all amino acids can be oxidized, sulfur-containing cysteine and methionine are particularly easily oxidized. For example, methionine is physiologically oxidized to form methionine sulfoxide (met-O) by the addition of an oxygen atom to the S atom (Brot and Weissbach 1991; Vogt 1995). The identification of individual proteins that undergo age-associated oxidation and functional impairments is receiving increasing attention. Such a relationship has been documented for the mitochondrial enzymes aconitase (Das et al. 2001) and adenine nucleotide translocase (Yan and Sohal 1998) in flies, murine α -ketoglutarate dehydrogenase (Sadek et al. 2002) and the high mobility group chromosomal protein HMG-D, a DNA-binding protein present during early embryogenesis of *Drosophila* (Dow et al. 1997).

Organisms have evolved a multi-layered protection scheme to manage the deleterious effects of ROS (Vatassery 1998; Mates and Sanchez-Jimenez 1999; Blokhina et al. 2003), whose production may depend on the metabolic rate (Abele et al. 2002; Keller et al. 2004). Once produced, excess ROS can be neutralized by non-enzymatic scavengers, such as certain vitamins, or converted into non-harmful species by the action of 'antioxidant' detoxifying enzymes, such as superoxide dismutases/catalases, peroxidases and glutathione/thioredoxin reductases. Some ROS, however, do escape the scavenging system and inflict oxidative damage to macromolecules. The damaged components may be enzymatic repaired by reductases or preferentially eliminated by proteases.

Longitudinal studies on oxidative damage and aging using mammals are time-consuming and expensive, and the number of subjects available is often limited. Because of its short generation time, high reproduction rate, ease of animal husbandry, the availability of sophisticated genetic tools and well-measurable age-related physiological changes such as motility and fertility, *Drosophila* is a widely used model for research on aging (Helfand and Rogina 2003). Indeed, studies on aging using flies range from temperature experiments at the beginning of the 20th century (Pearl 1928) up to the current creation of animals with multiple transgenes (Orr et al. 2003). In this review we summarize strategies to decrease the level of ROS by enzymatic and non-enzymatic ways, but also to repair ROS oxidized proteins. The experiments described include environmental and genetic manipulations and as different they are by themselves, as various are their outcomes on lifespan and organismal performance. Some of them only act under stressful situations or have gender-dependent impact, others reveal unpleasant side effects under sub-optimal environmental conditions, and yet others suffer from loss of life quality even under favorable laboratory conditions. We have classified these strategies in (1) prevention of ROS generation, (2) reducing of free ROS level, and (3) repair and elimination of ROS-damaged proteins (see Figure 1). We discuss their success and relevance to human applications considering both, the beneficial and deleterious roles of ROS in cellular function and health.

Before discussing oxidative damage and aging, it must be noted that the definition of normal aging has

not been established and it is not entirely clear which experimental parameters one must measure to characterize the process of normal aging. For example, lifespan measurements and the resulting survival distributions are often employed but which parameters of the survival distributions, such as the instantaneous death rate and median survival time, best reflect the underlying aging process is far from certain (e.g., Driver 2001).

Another potential complication is that, in some experimental systems, such as flies and worms, the cause of death of an individual cannot be easily determined and the limiting factors for survival under normal experimental conditions are not clear. Moreover, it is not known whether the same factor(s) are equally important in aging and lifespan determination in laboratory cultures and in wild. For example, some postulate that infection may be a primary cause of death in laboratory *Drosophila* strains (Pletcher et al. 2002).

Prevention of ROS generation: caloric restriction and single-gene mutation

ROS are generated during normal aerobic metabolism, primarily in mitochondria. According to the oxidative damage theory of aging (Harman 1956) derived from the rate of living theory of Pearl (Pearl 1928), reducing the metabolic turnover decreases ROS production, lowers accumulation of damaged macromolecules, and retards the aging process. A variety of experiments have manipulated the meta-

bolic rate of *Drosophila* in two major ways: (1) quality of food and physical activity and (2) single gene mutation of metabolically essential enzymes.

Caloric restriction

The relationship between caloric restriction (CR) and aging/lifespan determination in *Drosophila* and other flies has been investigated extensively (Chapman and Partridge 1996; Sohal and Weindruch 1996; Mair et al. 2003; Rauser et al. 2004). Typically, the animals housed under gender-separated conditions are fed *ad lib*. To induce CR, the food is unchanged in nutrient composition, but has a 40% to 60% reduced concentration of nutrients. Although CR generally increases the lifespan, the extent of extension differs in range between 36% and 82% (Mair et al. 2004; Pletcher et al. 2002). Unfortunately, it is problematic to compare results from different studies, because they often use different levels of caloric restriction and its effect on longevity depends on other factors, like the reproduction activity of the flies (Mair et al. 2004). Moreover, offering a diluted food indeed may not reduce the amount of affiliated nutrients consumed and hence cause caloric restriction. Flies could eat twice the amount of a 50% diluted food source to compensate the reduced concentration of nutrients. Crop-filling assays, which monitor the amount of affiliated food, and body mass measurement are useful and occasionally used controls (Wood et al. 2004), but are not yet widely adopted. Nevertheless, the results from many of these

◀ **Figure 1.** Metabolism of reactive oxygen species (ROS) and intervention possibilities for lifespan extension by manipulating ROS induced macromolecular damage. Metabolism of ROS is regulated by its generation during aerobic respiration and its clearance by degradation and neutralization. A multilayered defense system, that contains detoxifying enzymes (e.g. superoxide dismutases, catalases) and antioxidant compounds (e.g. certain vitamins), effectively reduces the level of free radical species by converting or neutralizing most of ROS. Remaining amounts of ROS are required as second messengers in cellular signal transduction and for immune response of neutrophils. Excessive ROS can interact with a variety of other cellular molecules. If this interaction does not cause damaging effects, these molecules act as buffers for ROS and are described as scavengers. Cellular scavenger capacity is controlled by repair and degradation of oxidized scavengers. Uncontrolled attack of ROS on lipids, DNA and proteins can cause damaging effects by changing their structure and subsequent functionality. Specific detection and manipulation systems have evolved to handle oxygen damaged macromolecules. Oxidized proteins either become repaired by enzyme reductases or degraded by the proteasome. Some oxidized protein species escape detection and can form non-soluble protein aggregates that accumulate inside the cell. According to the free radical theory of aging (Harman 1956), reducing ROS oxidative damage can extend individual lifespan. This can be achieved by interventions that reduce the overall level of ROS or by strategies that reduce the amount of ROS damaged macromolecules, such as proteins: ROS production can be in part prevented by caloric restriction or mutations of different genes, that cause down regulation of metabolic rate (i). Mitochondrial and cytoplasmic level of ROS can be reduced by an enhanced activity of detoxifying enzymes or dietary supply with antioxidant compounds (ii). Increasing the activity of components of the repair and elimination machinery, like protein reductases or heat shock proteins, quickly restores or removes oxidized proteins and causes beneficial effects for cell viability and subsequent organismal lifespan (iii).

experiments generally confirm those obtained in other systems; mild to moderate caloric restriction tends to shift the survival distribution to the right along the age axis such that on the average the animals live longer (Sohal and Weindruch 1996; Magwere et al. 2004). These observations are consistent with the hypothesis that reduced caloric intake affects energy production during aerobic respiration in the mitochondria and thereby decreases free radical leak. This idea was tested in rat (Lopez-Torres et al. 2002) and recently in fly (Miwa et al. 2004) yielding opposite results; whereas long-term CR produced a 47% decrease in ROS in rat liver mitochondria (Lopez-Torres et al. 2002), it did not significantly lower mitochondrial ROS production in *Drosophila* (Miwa et al. 2004). Furthermore, experimentally suppressed mitochondrial ROS production by overexpression of mitochondrial adenine nucleotide translocase failed to prolong lifespan of the fly (Miwa et al. 2004).

If CR extends lifespan by decreasing the cumulative oxidative damage, the life extension effect should be gradual in onset and should decrease the long-term death rate. However, the demographic analysis of the effects of CR on the mortality rates indicates that the observed reduction in death rate is immediate and reduces the short-term risk of death: transferring the flies from the CR condition to the normal diet condition increases the mortality rate within 48 h. Similarly, induction of starvation at middle-aged flies immediately decreases the risk of death (Mair et al. 2003; Rauser et al. 2004). These observations are not easily reconciled with the idea CR reduces the cumulative long-term oxidative damage and we may be still far from understanding the molecular basis and consequence/importance of caloric restricted life extension. At least two additional aspects must be considered to explain the life extension effect of CR. First, the reciprocal relationship between caloric restriction and reproductive activity likely exist (Chippindale et al. 1993; Chapman and Partridge 1996). There is an ongoing and intensive debate about the origin of this trade-off. Moreover, it is reported that extension of lifespan by resveratrol, a substance that mimics CR mechanism, is associated with a modest increase in egg production (Wood et al. 2004). Second, caloric restriction appears associated with global changes in gene expression as demonstrated by a genome-wide transcription profile in aged and caloric restricted *Dro-*

sophila melanogaster (Pletcher et al. 2002). In their study Pletcher and colleagues found that the age-associated change of 23% in transcript representation was significant delayed under CR conditions, but the underlying mechanisms remain still speculative.

A recent study shows that the lifespan extension effect of CR may not be truly universal; different dietary food mixtures failed to extend longevity of the housefly, *Musca domestica* (Cooper et al. 2004). In this study, the authors speculate that not all organisms are able to respond to the decrease in source of energy by adjustment/improvement of their metabolic rate, so that even mild caloric restriction can lead to starvation (Cooper et al. 2004).

In addition to CR, decreased physical activity may delay death at least in flies. Prevention of flying resulted in a dramatic lifespan elongation of about 3-fold for mean and maximum lifespan in houseflies (Yan and Sohal 2000). While it is not possible to draw a firm causal connection between solely walking flies and their physical fitness, these investigators demonstrated the crucial importance of oxygen consumption for the magnitude of oxidative damage and, in the end, for the control of the aging process.

Single-gene mutation

A variety of single gene mutations in *Drosophila* that extend longevity of the fly have been reported and are summarized in Aigaki et al. (2002). Many of these longevity genes were originally found in *Caenorhabditis elegans* and are well conserved from worm to human. The products of these longevity genes share a function as constituents of cellular signal transduction and cell metabolism. The kinase Insulin-like receptor (INR) (Tatar et al. 2001) and its substrate, CHICO (Bohni et al. 1999; Clancy et al. 2001), are mediators for the insulin/IGF-like signaling pathway. A genetic disruption of the *InR* or *chico* gene increases female fly lifespan up to 85% for receptor mutants and 48% for substrate mutants (Tatar et al. 2001; Clancy et al. 2001). However, both mutations are associated with reproductive diapause. Lifespan extension without a loss of fertility was generated by mutations of a sodium dicarboxylate cotransporter, *Indy* (Rogina et al. 2000), and a G-protein coupled transmembrane receptor, *methuselah* (*mth*) (Lin et al. 1998). Knock out of the plasma membrane transporter *Indy* by P-element insertional

mutation affected the exchange of Krebs cycle intermediates in the digestive and reproductive system (Rogina et al. 2000). Notably, long-lived *Indy* heterozygote flies did not show a decrease in metabolic or physiological activity (Marden et al. 2003). The mutants full name, *I am not death yet*, reflects the dramatic increase in mean lifespan, which approached 100% at 18 °C and mutant females even showed an extension of the fertility period under normal conditions (Rogina et al. 2000). Thus, the change in uptake, utilization, or storage of metabolites in flies with one mutated allele for the sodium dicarboxylate cotransporter could be sufficient to reduce ROS production, but remains high enough to warrant normal metabolism under non-stressed conditions. The mechanism of lifespan extension for *mth* is unknown but it is speculated that the receptor is involved in the cellular stress response cascade, because *mth* flies are also more resistant to various stresses like heat, starvation and the oxygen radical generator paraquat (Lin et al. 1998). Indeed, stress resistance and longevity seem to be positively correlated. However, whether an improved stress response primarily causes lifespan extension, as suggested by Johnson et al. (1996), or is a 'side-effect' of an enhanced longevity is not known.

The genetic mutation experiments revealed that the activities of specific genes can be associated with the longevity of a species, and especially *mth* and *Indy* may hold promises as anti-aging interventions. However, it can be argued that genetic down regulation will extend a healthy life only at the cost of other biometric parameters like fecundity, growth, or physical activity. For *InR* and *chico* mutations these trade-offs are obvious under optimized laboratory conditions. How the genetic changes in *Indy* or *mth* affect lifespan in the wild, where environmental conditions are often suboptimal, is uncertain. Indeed, *Indy* flies, in comparison to wild-type Canton S *Drosophila* strain, showed a greater decrease in egg laying under poor food situations (Marden et al. 2003). This could explain why *Indy* mutations have not manifested in nature; the evolutionary pressure may strengthen populations where individuals have a balanced relationship between living rate and age-specific fecundity, potentially representing another case of antagonistic pleiotropy (Rose and Graves 1989). As a consequence, maximization of individual lifespan becomes impeded in favor of handling stressful periods (Marden et al. 2003).

Uncoupling mitochondrial respiration

Lifespan extension based on decreased generation of ROS was recently achieved by overexpression of the human uncoupling protein 2 (hUCP2) in the nervous system of *Drosophila* (Fridell et al. 2005). This carrier protein is located at the inner membrane of mitochondria and mediates protons influx into the matrix. Overexpression of hUCP2 increased the respiration rate under state 4 condition by 54%. Mitochondrial uncoupling was associated with a decrease in mitochondrial membrane potential thereby reducing ubiquinone (QH•) and subsequent ROS generation. Consistent with the free radical theory of aging (Harman 1956), Fridell and colleagues indeed observed a 32% decrease in oxidative damage as measured by the lipid peroxidation-derived aldehyde 4-hydroxy-2-nonenal (HNE), and a 22% increase in lifespan of female flies. Notably, this lifespan extension was achieved by pan-neuronal overexpression of the carrier but not by overexpression in muscle tissue (Fridell et al. 2005). Furthermore, the beneficial effect of the protein was dependent on its activity in early age, because induction of hUCP2 expression in adult flies older than 20 days only caused little extension of lifespan. Long-living animals showed no changes in fecundity and physical activity, and even improved resistance against paraquat induced oxidative stress. However, as noted for single gene mutations, changes in mitochondrial metabolism may be associated with deteriorations in organismal performance; transgenic flies were more sensitive against other stresses such as starvation (Fridell et al. 2005).

Reducing the ROS level: antioxidants and detoxifying enzymes

Some electrons do inevitably 'leak' out of the cellular reaction pathways, such as the electron transport chain in mitochondria, leading to formation of ROS. These reactive molecules could be prevented from damaging cellular macromolecules by the action of small ROS 'scavengers' or by 'detoxifying' enzymes.

Antioxidants

One approach to control the level of ROS is to use dietary antioxidants that scavenge ROS. The dietary

antioxidants, while structurally diverse, have various degrees of 'spin trap' abilities; they have one or more reactive groups on their molecular surface that can be easily modified by ROS without significantly altering the overall structure. The ROS-mediated attacks on the reactive groups in fact neutralize the ROS thereby protecting other sensitive molecules from ROS-mediated damage.

Some of these antioxidants are well known vitamins (vitamins A, C, and E) while others are naturally occurring peptides such as melatonin and glutathione and their related thiol-containing amino acids (thiazolidine carboxylic acid (thioprolin, TP) and *N*-acetylcysteine (NAC)). Among the naturally occurring compounds, ubiquinone-10, widely known as coenzyme Q₁₀, is a commonly used ingredient in commercially available anti-wrinkle skin creams. Yet others are synthetic compounds like the tetrapeptide epitalon (Khavinson et al. 2000). In addition to these substances cells have a catalytic antioxidant system that uses the amino acid methionine and methionine sulfoxide reductases (MSRs; see below) (Stadtman et al. 2002). This system may modulate the intracellular redox state and act as a highly effective ROS sinking machinery by cyclic oxidation of methionine to methionine sulfoxide (met-O) and its subsequent reduction back to methionine.

According to the free radical theory of aging (Harman 1956), a decelerated accumulation of oxidative damage will result in an extended lifespan. Thus, numerous experiments have been carried out to test whether these scavenging antioxidants slow aging in a variety of model systems, including *Drosophila*. These studies appeal to our own human preference to 'pop a few pills' and by our aversion to make certain lifestyle changes such as limiting dietary caloric intake. To study whether melatonin has any effect to slow aging, Coto-Montes and Hardeland (1999) induced oxidative stress in male flies by feeding the catalase-inhibitor 3-amino-1,2,4-triazole and oxidative protein modification was monitored by measurement of protein carbonylation. Whereas 20 h treatment of the flies with the inhibitor alone induced an increase in protein carbonyl and resulted in death of nearly all flies, the simultaneous application of 2 mM melatonin prevented carbonylation and protected the flies from death (Coto-Montes and Hardeland 1999). Another study quantified the effect of melatonin, 100 µg/ml in the food daily, on *Drosophila* lifespan under normal conditions: an increase

of 13.5% in median lifespan and 33.2% (from 61.2 days for controls up to 81.5 days for melatonin fed flies) in maximum lifespan (Bonilla et al. 2002). Furthermore, those flies fed with melatonin were more resistant against paraquat induced oxidative stress and heat stress. Similar lifespan extension results in *Drosophila* were reported using vitamin E, 2,4-dinitrophenol, nordihydroguaiaretic acid and thiazolidine carboxylic acid (TCA) by Miquel and colleagues (1982). They observed an average increase in median lifespan from about 13% for the first three compounds and even 30.5% for TCA (Miquel et al. 1982).

While the results of these lifespan trials are encouraging, there is growing evidence that the direct ROS scavenging activity *per se* may not be the principle mechanism responsible for the lifespan extension observed when animals are fed with these antioxidant compounds. For example, coenzyme Q₁₀ may directly decrease the *generation* of ROS by improving the efficiency of the mitochondrial respiratory chain (Bliznakov 1999). Another substance with aging retarding activity is epitalon, a tetrapeptide that increases the mean lifespan of male fruit flies by 11–16%. Its geroprotector effect occurs even at an infinitesimal concentration of about $0.001 \times 10^{-6}\%$ of culture medium weight, which is, in comparison to active melatonin doses, up to 80,000,000 times lower (Khavinson et al. 2000). It is supposed that the compound somehow 'optimizes vital functions,' but the detailed mechanism remains to be discovered (Khavinson et al. 2000).

As found in other animals, supplementation of the food with antioxidants capable of scavenging ROS in the fly is associated with changes in gene expression and often longer lifespan. For example, dietary *N*-acetylcysteine (NAC) causes lifespan prolongation on average 16.6% (1 mg/ml NAC) or 26.6% (10 mg/ml NAC) and is associated with increases in the mRNA levels of specific genes (Brack et al. 1997). In another study, a strong increase in the enzymatic activity of the ROS detoxifying enzymes superoxide dismutase (SOD) and catalase is reported in flies fed with 0.5 M magnesium chloride (Matkovics et al. 1997). These observations, however, do not directly address the underlying mechanism. Are the changes in gene expression necessary to produce lifespan extension? Does the enhanced ROS scavenging by these dietary supplements directly extend lifespan by preventing oxidative damage?

Treatments with some compounds, which provide ROS scavenging activity, are associated with lifespan extension, but other scavenging compounds have no effect on the lifespan. What accounts for the differential effects? Antioxidants differ in their efficacy to scavenge ROS and also in their subcellular targeting. Some, because of their hydrophobic nature, may preferentially localize near/in membrane compartments while others may remain in the cytoplasm or the extracellular milieu. Thus, it may not be too surprising that some compounds fail to extend lifespan. For example, the powerful ROS neutralizers coenzyme Q₁₀ and alpha-lipoic acid (ALA) fail to extend lifespan of mice (Lee et al. 2004). Such contradictory results with even negative effects on longevity are also described for a variety of antioxidant compounds tested in the fruit fly, as summarized in Le Bourg (2001). Among the popular antioxidants, Driver and Georgeou (2003) discuss the variability associated with the effects of vitamin E on *Drosophila* lifespan. Depending on the concentration used, vitamin E can extend or shorten the lifespan, suggesting that a small optimal concentration range may exist for lifespan extension. The complicated dose-dependent action of the antioxidant compounds may be a result of the existence of numerous redox-dependent cellular signaling cascades and seriously dampen the simplistic 'the more, the better' philosophy in the dietary supplementation approach for minimizing oxidative stress and promoting longevity in humans.

Recently, investigations on rodents indicated a beneficial role of melatonin, α -lipoic acid, NAC and other antioxidants in prevention of age-related neurodegenerative diseases. One study used intracerebroventricular application of streptozotocin, a free radical generator, as a model of sporadic Alzheimer type dementia (Sharma and Gupta 2001). Streptozotocin injected rats are characterized by the presence of oxidative stress and development of neuronal disorders. In contrast, chronically feeding of injected rats with doses of 10 mg/kg and 20 mg/kg melatonin resulted in a dose-dependent decrease in lipid-peroxidation and a reduced deterioration of cognitive performance (Sharma and Gupta 2001). Similar results, namely prevention of enhanced oxidative damage coupled with cognitive improvement, were obtained with SAMP8 mice after application of α -lipoic acid and NAC by Farr and colleagues (2003). In their model, brain oxidative stress was induced by

elevated levels of amyloid- β , which causes deficits in learning and memory in aged mice of the disease-carrying strain. Even for old, but healthy animals the native loss of memory was partially reversed by feeding with α -lipoic acid (Liu et al. 2002). As for their effect on lifespan extension, the underlying mechanism for cognitive improvement may be lowering oxidative damage. These results indicate that enhancing 'cellular fitness' may positively affect longevity but also other physiological parameters like stress resistance, endogenous disease combating, and cognitive capability.

Detoxifying enzymes

The superoxide anion O_2^- , one of the prominent radical species that could give rise to other reactive compounds, is converted to H_2O_2 by the action of the enzyme superoxide dismutase (SOD). H_2O_2 is in turn converted to H_2O and O_2 by the enzyme catalase (CAT). Alternatively, H_2O_2 may be converted to H_2O by the action of peroxidases in many cells. Antioxidative activity was also shown for the reducing enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR), which restore the intracellular antioxidants glutathione and thioredoxin. The enzymes work in a cooperative manner, but not all of them are simultaneously necessary for an effective antioxidative shield. For example, the lack of glutathione peroxidase/reductase in *Drosophila* is substituted by the activity of the thioredoxin system (Sohal et al. 1990; Kanzok et al. 2001).

If the oxidative damage caused by excess ROS contributes to aging and lifespan determination as suggested by the oxidative damage theory of aging, enhancing the enzymatic detoxification system may slow aging and extend lifespan. Furthermore, the long-living animals may possess greater oxidative stress resistance and display less signs of oxidative damage. The initial studies showed that moderate increases in activity of catalase alone (Orr and Sohal 1992) or Cu/Zn superoxide dismutase alone (Orr and Sohal 1993) were associated with no effect or only a slight extension in mean lifespan of male *Drosophila*. However, simultaneous insertion of an extra copy of the *SOD* gene and *CAT* gene by P-element mediated transformation did extend mean lifespan by 15% and maximum lifespan up to 34% of experimental flies (Orr and Sohal 1994). Based on this finding, molecular and physiological parameters of

the doubly transgenic animals were intensively investigated by measuring of carbonyl content, 8-hydroxy-2'-deoxyguanosine (OH8dG) concentration, mitochondrial hydrogen peroxide generation, *in vivo* oxygen consumption, and negative geotaxis. Sohal and colleagues found strong evidence that the extended longevity was associated with a significant reduction of accumulated oxidative protein and DNA damage, a diminished age-related increase in mitochondrial H₂O₂ generation, an increase in later-age oxygen consumption, and a higher physical activity (Sohal et al. 1995).

Comparison of the results of different lifespan trials involving transgenic flies suggested that any effect of enzyme overexpression may depend greatly on the experimental conditions and that lifespan extension may be negligible under more physiological conditions (Le Bourg 2001). Orr and Sohal (2003) summarized data from nine published studies featuring overexpression of antioxidative enzymes in transgenic fruit flies and demonstrated an inverse relationship between lifespan extension and control lifespan in *Drosophila*. More recent studies with flies of long-lived genetic backgrounds failed to extend longevity; in one large-scale study, the mean lifespan of a robust control strain at 25°C remained at ~60 to 70 days after overexpression of either (1) Cu/Zn-SOD and CAT, (2) Cu/Zn-SOD, CAT and Mn-SOD, or (3) Cu/Zn-SOD, CAT and TrxR (Orr et al. 2003). In some long-lived strains, the overexpression may even shorten the lifespan slightly (Mockett et al. 2003; Orr et al. 2003). However, there is one situation in which overexpression of antioxidative enzymes successfully prolongs lifespan of even long-living animals: Under oxidative stress, induced by hyperoxia (100% oxygen), H₂O₂ (73.5 mM), or paraquat (20 mM), overexpression of glutathione reductase or mitochondrial catalase increased the survival time of *Drosophila* by approx. 50% (Mockett et al. 1999b, 2003).

One likely explanation for the failure of overexpressed detoxifying enzymes to extend lifespan is that their endogenous activities, at least in long-lived or non-stressed strains, are normally adequate to control the ROS levels (Mockett et al. 1999a,b). But in stress situations, when enzymatic activities become insufficient, increased activities of these antioxidant enzymes can have beneficial effects. Another possibility is that the optimal concentration of ROS may show spatial variations; its excess and also its

deficiency are equally detrimental. Some ROS are most likely required for organism survival, as exemplified by the use of ROS in immune cells. In addition, other cell types may employ ROS as signaling molecules and an increasing number of examples for both flies (Morey et al. 2001) and mammals (Frank et al. 2003; Williams and Kwon 2004) have been reported. For example, nitric oxide is a radical signaling molecule implicated in a variety of physiological phenomena (Kuzin et al. 2000; Gibbs 2003). Given these physiological roles of ROS, it may not be unexpected that the experimental treatments designed to perturb the normal ROS level by massive supplies of ROS scavengers or by global overexpression of detoxifying enzymes, do not uniformly cause beneficial, protective effects for cellular metabolism.

Even with increased activities of detoxifying enzymes, basal oxyradical attack seems to be inevitable. Actually detoxifying enzymes undergo oxidative damage and lose their function, as reported for the enzyme superoxide dismutase by Strack et al. (1996). These considerations suggest that modulating repair and degradation of ROS-damaged proteins could be an alternative strategy for longevity (Friguet 2002). Up to now there exists no systematic analysis of results obtained from *Drosophila* that take into account a 'repair and elimination strategy'. Therefore, the third part of this article will focus on this issue.

Repair and elimination of ROS-damaged proteins: reductases and the protein degradation machinery

Some ROS inevitably escape the scavenging system, either during normal aerobic cell respiration or during oxidative stress episodes, and inflict oxidative damage to macromolecules. While this review focuses on ROS-induced protein damage, nucleic acids (esp. DNA and RNA), fatty acids (esp. membrane components) and carbohydrates are also targets for oxyradical modifications. Oxidative damage of each of these macromolecules contributes to the aging process potentially with different functional specializations and consequences. For example, DNA is exposed to frequent oxidative hits, estimated to be as high as 9×10^4 per cell/day (Fraga et al. 1990). Oxidative DNA damage, often measured by OH8dG levels, appears to increase with age in rats in a tissue-dependent manner (Fraga et al. 1990). The contribu-

tion of such mutations towards development of senescence-related phenotypes such as weight loss, reduced subcutaneous fat, hair loss, osteoporosis, anemia, reduced fertility, and heart enlargement was demonstrated in mice with aggrandized mitochondrial point mutations and deletions, created by expression of a defective mitochondrial DNA polymerase (Trifunovic et al. 2004). Fortunately, rapid accumulation of oxidative genomic damage is prevented by an efficient repair machinery for nuclear and mitochondrial DNA (Gros et al. 2002; Mandavilli et al. 2002). For example, OH8dG damages are continually removed by DNA-glycosylase and AP nuclease (base excision repair, BER), which perform an estimated repair effort of $\approx 4,300$ residues per cell/day (Fraga et al. 1990).

Repair mechanisms also exist for oxidized phospholipids in cell membranes. Oxyradical attack occurs predominantly at their polyunsaturated fatty acyl chains (Bielski et al. 1983), whereby it alters fluidity and plasticity of the membrane and therefore has impairing effects on cell viability and can induce apoptosis (Sandstrom et al. 1995; Pratico 2002). Phospholipids, bearing a hydro(pero)xy group, are repaired by cleavage of the oxygenated fatty acid residue (Matsuzawa et al. 1997). A strong candidate for an enzymatic sanitizer of oxidized membranes is the enzyme platelet-activating factor-acetylhydrolase II (PAF-AH (II)) (Hattori et al. 1993). In response to the intracellular redox state PAF-AH (II) translocates to membranes where it preferentially hydrolyzes oxidized phospholipids (Matsuzawa et al. 1997).

Thus, repair of oxygen-damaged macromolecules has become well established during evolution and one should expect adequate mechanisms for restoration of oxidized proteins. It should be pointed out that the use of 'repair' for nucleic BER or hydrolysis of oxidized phospholipids does not mean reversal of the *oxidation* process by *reduction* of the oxidized position. DNA and lipid membranes are molecular aggregates and repair of oxidized base pairs (DNA) or fatty acyl chains (membrane lipids) is characterized by excision and replacement of larger molecular groups. In comparison to nucleic and fatty acids, some oxidized atoms in ROS-damaged proteins can literally be repaired and restored by the activity of reductases.

Oxidatively damaged proteins face two consequences. In general, they are degraded more rapidly than their native counter parts (reviewed by Grune

et al. 2003) and they may be preferentially removed from the functional pool. This accelerated removal may, however, at least transiently compromise functionality if the safety margin is small. Alternatively, oxidized proteins may be repaired by the activity of reductases to restore the initial molecular structure. Based on the enzymatic kinetics of reductases, this repair results in a much faster regeneration of damaged proteins than achieved by degradation and replacement processes. Reductases are a diverse group of enzymes with high substrate specificity and an extensive role in cellular metabolism. To our knowledge, their importance in lifespan determination was demonstrated for two members up to now.

Repair of ROS-damaged proteins: reductases

Sniffer

A common result in protein oxidation is the formation of carbonyl groups in the side chains of certain amino acid residues (Stadtman and Oliver 1991). *In vivo*, the carbonyl content increases with age in flies (Yan and Sohal 2000) and mammals (Jana et al. 2002; Soreghan et al. 2003). Evidence for a substantial role of carbonyl reductases in a putative 'protein repair machinery' comes from a recent study using the *Drosophila* reductase *sniffer*, which is a member of the short-chain dehydrogenase/reductase (SDR) enzyme family (Botella et al. 2004). Carbonyl reductases reduce a large number of carbonyl substrates, like ketones, aldehydes and quinones in a NADPH-dependent manner (Terada et al. 2000). The enzyme Sniffer is, so far, the solely identified functional carbonyl reductase in *Drosophila*, and *sniffer* null mutants revealed a distinct 50% reduction in general carbonyl reductase activity (Botella et al. 2004). Although these animals had, in comparison to wild types, no significant change in the overall level of carbonylated proteins, the mean lifespan of *sniffer* mutant flies decreased from ≈ 60 to ≈ 20 days. Morphologically, the shortened lifespan was associated with an accelerated formation of vacuoles in brain neuropil. Vacuolization was also observed in wild-type flies exposed to hyperoxia-induced oxidative stress, but could be prevented by overexpression of the *sniffer* gene. Moreover, increased Sniffer activity under hyperoxia resulted in a considerable extension of mean and maximum lifespan (43% and 44%, respectively). While overexpression of *sniffer* significantly reduced the amount of total carbonyl

groups in flies compared to controls (by 50% and 20% in 12 and 25 days old animals, respectively), it did not extend the lifespan under normoxia conditions. This observation led to the suggestion that *sniffer* protects neurons from enhanced oxidative stress. Because oxidative damage can contribute to the pathogenesis of neurodegenerative disorders (e.g. Alzheimer's and Parkinson's diseases; see below), Botella and colleagues (2004) suggest an involvement of the reductase in the cellular defense mechanism against age-related neurodegeneration. Indeed, they showed that overexpression of the reductase improves physical activity, measured by negative geotaxis experiments, by 63% in 20 days old flies and 79% in 30 days old flies, thus counteracting the age-related decline in locomotor performance.

Methionine sulfoxide reductases (MSRs)

The sulfur-containing amino acid methionine is particularly sensitive to oxyradical attack; it becomes easily oxidized by the addition of an oxygen atom to the reactive side-chain sulfur atom (Brot and Weissbach 1991; Vogt 1995). Oxidation of methionine produces two optical enantiomers of methionine sulfoxide (met-R-O and met-S-O), resulting in a stiffer and more polar side chain. The oxidation of protein-bound, surface exposed methionine residues can lead to functional alterations or activity changes of the protein (Dow et al. 1997). In contrast to oxidative modifications of other macromolecules, oxidation of methionine (and cysteine) is physiologically reversible. Met-S-O and met-R-O are enzymatically reduced back ('repaired') to methionine in a stereo-specific manner by the enzymes methionine sulfoxide reductases A (MSRA) and B (MSRB), respectively, using thioredoxin *in vivo* (Sharov et al. 1999; Arner and Holmgren 2000). Although less is known about the substrate specificity of these reductases it is postulated that methionine sulfoxide of both, free methionine and protein-bound methionine residues at a protein surface, are repaired by the enzymes *in vivo*.

Comprehensive information is not yet available in *Drosophila* but the results obtained from other species together suggest that the amount of oxidized methionine residues and its regulation by methionine sulfoxide reductases affect the aging process. One hindrance to a more thorough understanding of this subject is that neither a specific tracer nor an antibody against methionine sulfoxide exists. However,

based on the observed age-associated increase of protein carbonylation (Stadtman 1992), a similar accumulation of met-O could be supposed. This assumption is plausible because an age-associated decline in the activity of the methionine sulfoxide reductase system was observed in fibroblasts (Picot et al. 2004) and organ homogenates from rats (Petropoulos et al. 2001; Stadtman et al. 2002); for human WI-38 fibroblasts, Picot and colleagues reported that endogenous MSRA and MSRB (CBS-1) enzymes become down regulated during replicative senescence (Picot et al. 2004). The remaining methionine sulfoxide reductase activity of 80% in middle-aged cells and 60% in old cells, compared to early cell passages, is a result of a decrease in mRNA levels for both, MSRA and MSRB, enzymes (Picot et al. 2004). A similar decline in MSR activity of 50% and 60%, respectively, was measured for liver and kidney lysates of rats at their median survival age (26 months) (Petropoulos et al. 2001). Surprisingly, the decrease in the MSR activity in late-passage fibroblastic cells was not related to an increase of the overall protein-bound met-O level, which remained unchanged at about 10% during replicative senescence (Picot et al. 2004). However, this could be a phenomenon in cultured cells, which may not completely reflect all age-associated changes that occur within aging of an organism.

Increasing evidence indicates that MSRA and, under special circumstances, MSRB, act as important bidirectional modulators of lifespan; suppression of their activity (which is not lethal) shortens, and enhancement of their activity extends lifespan. The deleterious and lifespan shortening effects of MSRA deletions were demonstrated in several model systems, including yeast (Koc et al. 2004) and mouse (Moskovitz et al. 2001), as summarized in Hansel et al. (2005). Similar results with *msrB* genes have so far only reported for yeast (Koc et al. 2004). Because in mammals three genes comprise the MSRB system, genetic disruption of one *msrB* gene may not necessarily produce noticeable phenotypes. In yeast, overexpression of MSRA extends slightly but significantly the lifespan under oxidative stress (Moskovitz et al. 1998), whereas overexpression of MSRB extends lifespan only under caloric restriction by remarkable 62% (Koc et al. 2004). In *Drosophila* where the cells are largely post mitotic, pan-neuronal overexpression of MSRA extends median lifespan of transgenic flies by 70% (Ruan et al. 2002). This

extension was observed in a relatively long-lived background, with mean lifespans of 58 days for females and 45 days for males. Moreover, the lifespan extension in the flies differs in two important aspects from studies in yeast and others; (1) transgenic flies did not arrest in a hypometabolic state, but even showed increased physical activity and fertility, (2) longevity was observed without experimentally induced oxidative stress and the flies were also more resistant to paraquat-induced oxidative stress. Modulation of lifespan by manipulating the level of this reductase differs from the previous strategies, because the MSR activity may not directly affect the ROS level, but causes the restoration of already oxidized methionine residues. Whether the repaired methionine residues are essential for the function of the respective proteins ('repair hypothesis') or serving as a component of the catalytic antioxidant system that eliminates redundant ROS ('sink hypothesis'; see above) is unclear. However, MSRA indeed is able to partially protect cells from oxidative stress induced cell death (Jung et al. 2003; Kantorow et al. 2004; Yermolaieva et al. 2004) and evidence for a relationship between age-associated accumulation of oxidized proteins and decrease in activity of MSRs was presented by Picot et al. (2004). It would be interesting to see if an increased activity of methionine sulfoxide reductases can diminish the age-dependent increase in oxygen-damaged aconitase (Das et al. 2001) or nucleotide exchange factor (Yan and Sohal 1998) and restore their decreased activity.

However, the aforementioned findings cannot completely explain the lifespan extension mechanism of MSRA. Presumed that ROS-induced methionine oxidation generates equal amounts of both met-O enantiomers, the met-S-O specific reductase MSRA should reduce only approx. 50% of the overall methionine sulfoxide pool. One would expect that likewise reduction of met-R-O, which is the substrate of MSRB enzymes, results in an equal lifespan extension. However, our own results show that overexpression of the MSRB enzyme type CBS1 in the fruit fly does not show a beneficial effect on longevity for flies kept under similar (non-stressed and no food restricted) conditions. Results from both species, yeast and fly, indicate that MSRA and MSRB enzymes are probably involved in different cellular defense systems. Their (protective) effect may depend on the intracellular localization and the

energy intake. In fact, MSRA and MSRB enzymes represent a complex family of enzymes with specific organ, tissue and subcellular localization patterns (Hansel et al. 2005; Kim and Gladyshev 2004). Mitochondria and cytoplasm contain MSRA isoforms (Hansel et al. 2002) and mitochondria, cytoplasm, ER, and nucleus contain MSRBs (Kim and Gladyshev 2004). It should be kept in mind overexpressed MSRs may not be localized to their physiological target loci.

Elimination of ROS-damaged proteins: protein degradation machinery

To avoid an accumulation of oxidized proteins, they are preferentially degraded. For example, Lon protease degrades oxidized murine mitochondrial aconitase at a much higher rate than the non-oxidized control protein (Bota et al. 2002). Any change in elimination of oxidized proteins can contribute to their age-related accumulation. The biological relevance of such alterations was first observed in old mice, where Agarwal and Sohal (1994) described a decline in proteolysis of oxidized proteins.

Hsp22

Before oxidized proteins are selectively eliminated, they have to be recognized and chaperoned. Thus, the increase in protein damage during the aging process should be associated with an enhanced expression/activity of such factors. Growing interest in this context centers on the *Drosophila* heat shock protein Hsp22, whose expression level increases by about 150 fold in flies over 50 days of age (King and Tower 1999). Hsp22 knock-out flies have a 40% decrease in lifespan (Morrow et al. 2004a) and conversely targeted overexpression of Hsp22 within motor neurons increases mean lifespan by more than 30% (Morrow et al. 2004b). Longevity of transgenic flies was further associated with a significant delay in the onset of age-associated decline in physical activity and 35% increased resistance to paraquat-induced oxidative damage (Morrow et al. 2004b).

Chaperones, like Hsp22 and others, are known to play an essential role in protein metabolism. Morrow and colleagues (2004a,b) hypothesize that Hsp22 chaperones and supports the removal of mitochondrial proteins, which are damaged by leaking ROS, and therefore protects mitochondrial integrity. Although interaction or target partners of Hsp22 are

currently unidentified, the complex relationship between protein oxidation, Hsp activity, and cellular function becomes clear under conditions with impaired chaperone performance. Vertebrate alpha-crystallins, a group of structural proteins in the eye lens with homology to *Drosophila* heat shock protein Hsp22, (and Hsp's 23, 26, 27) (Ingolia and Craig 1982), contribute by their chaperoning activity to the solubility of other crystallins in the lens (reviewed by Derham and Harding 1999). With aging, or after direct application of oxidative stress by peroxide or UV light treatment, alpha-crystallins themselves undergo oxidation of their cysteine and methionine residues (Smith et al. 1997; Fujii et al. 2004). The resulting decrease in chaperone activity has serious consequences on the solubility of the other crystallins and leads to the formation of cataract (Truscott and Augusteyn 1977; Garner and Spector 1980).

Parkin

Degradation of damaged proteins is also important for a class of age-related neurodegenerative disease, called synucleopathies, whose pathogenesis involves oxidative stress. Mice and the fruit fly have become useful models to investigate their causes as well as to find potential therapeutic strategies. For Parkinson's disease (PD), two *Drosophila* models are now available. First, overexpression of human α -synuclein induces PD-like phenotypes in the fly including the formation of Lewy bodies, loss of dopaminergic neurons and locomotor dysfunction (Feany and Bender 2000). Second, disruption of the *Drosophila parkin* gene, whose product Parkin is an ubiquitin-protein ligase with homology to human PARK2, causes a shortened lifespan, infertility, degeneration of indirect flight muscle, decrease in motor capability and reduced resistance against paraquat-induced oxidative stress (Pesah et al. 2004).

Overexpression of *parkin* fails to extend lifespan of fruit flies (Haywood and Staveley 2004) and the animals overexpressing α -synuclein suffer from a dramatic decline in physical activity. However, simultaneous overexpression of α -synuclein and *parkin* protects flies from premature loss of climbing ability and suppresses the PD-like phenotypes on physical deterioration (Haywood and Staveley 2004). The toxic activity of α -synuclein is based on its accumulation and formation of insoluble, toxic inclusions, whereby overexpression of the protein overwhelms the endogenous protection system. The

limiting step in removal of α -synuclein may be its targeting system for degradation, which can become accelerated by overexpression of the ubiquitin ligase (Haywood and Staveley 2004). Thus, enhanced elimination of cytotoxic proteins promoted by *parkin* could be a beneficial strategy in the treatment of PD and other synucleopathies.

Conclusion

Changes in cellular redox state and the accumulation of oxidatively modified macromolecules contribute to lifespan determination of aerobic organisms (Figure 1). Prevention and reduction of ROS as well as repair and degradation of ROS-induced damage can modulate fly lifespan. However, any strategy to increase lifespan involving the cellular redox state must recognize the fact that a certain level of ROS is required for normal cell function. Dietary restriction, single gene mutations, supply with antioxidant compounds, and antioxidative enzymes are often beneficial in lifespan extension, but their effects frequently require the application of extraneous oxidative stress or are associated with unacceptable side effects for fecundity or organismal performance.

Molecular repair and elimination mechanisms for oxidatively modified proteins have not been fully investigated but detailed information about the high efficiency of DNA repair capabilities give promising hope for the existence of such tools. Molecular substrates of protein reductases like *sniffer* or MSRs are yet to be identified and the influence of their redox status on aging has to be proven. Different effects for the two classes of methionine sulfoxide reductases (MSRA and MSRB) on lifespan of yeast (Koc et al. 2004) and *Drosophila* (Ruan et al. 2002; Wassef et al. unpublished) indicate that not only oxidation of met to met-O, but also the specific enantiomer generated, met-R-O or met-S-O, may determine the damaging effect on organisms. However, in contrast to other strategies, repair and degradation of oxyradical damaged proteins may represent a chance to prolong a normal, 'healthy' lifespan. While oxidative protein damage contributes to the aging process, selective reversal of this damage would not only delay, but even may roll back the process of aging.

The model system fruit fly allows us to study and compare this broad variety of anti-aging and lifespan

extension strategies in a single species. Nevertheless, *Drosophila* cultures used for stress resistance and lifespan studies show a wide variation in normal lifespan. Comparison of their biochemical and physiological parameters should reveal insight into nature of the aging process (Mockett et al. 2001). However, fitness of genetically identical cohorts in different laboratories is also influenced among others by the size of the culture vessel (enough space for flying) (Yan and Sohal 2000), gender separation (Chapman and Partridge 1996), growing density in larval stages (Sorensen and Loeschcke 2001), servicing of the culture, and composition of food and temperature. Even changes in gravity may manipulate the rate of aging (Le Bourg and Minois 1997). This represents the complexity of epigenetic age-determining mechanisms and complicates the comparability and quantification of lifespan extension results from different research groups. Moreover, the existence of such complex relationships could explain the difficulties associated with extrapolating laboratory results obtained from yeast and worm, into human practice. For that reason, use of the fruit fly as a more complex 'compromise' model organism will continue to reveal more detailed insights into aging and anti-aging strategies.

Acknowledgments

The work in the authors' laboratories has been supported by NIH and Parkinson's Disease Foundation. We thank Dr W. Johnson, Dr J. Pettus and Dr A. Hansel for valuable discussion.

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