Chapter 7
Oxidative Stress and *C. elegans* Models

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Abstract Oxidative stress is thought to be an important contributor to cellular and organismal aging. While there are many reports that support this notion, some recent evidence using transgenic animals indicates that oxidative defense systems, including antioxidant enzymes, may not affect life extension. This leads to speculation that oxidative stress does not play a major role in aging. However, it is difficult to ascertain the role of oxidative stress on aging under complex mechanisms of ROS production and the defense systems in normal cells that maintain a favorable redox balance. The nematode *Caenorhabditis elegans* has gained widespread favor for the study of many biological processes, including aging. Several lines of *C. elegans* research relating to oxidative stress and aging are discussed in this review, including the use of transgenic organisms with altered superoxide dismutase levels as well as studies that focus on mitochondrial mutations.

Keywords *Caenorhabditis elegans* • Aging • Oxidative stress • Reactive oxygen species • Electron transport

7.1 Oxidative Stress and Aging

Aging is controlled by a complex interplay of both genetic and environmental factors. Investigators have been examining these factors from a wide variety of viewpoints. Much attention has focused on the hypothesis that oxidative damage plays an important determinative role in cellular and organismal aging (Harman 1956; Jazwinski 1996; Holiday 1997; Liochev 2013; Clancy and Birdsall 2013) (Fig. 7.1). Reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) can readily attack a wide variety of cellular entities, resulting in damage that compromises cellular integrity and

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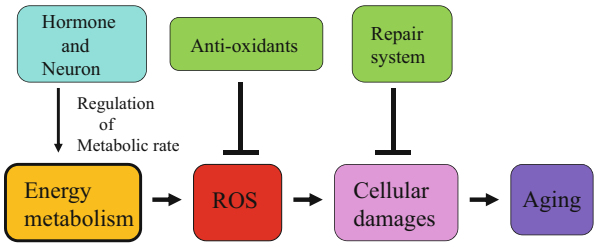

Fig. 7.1 An aging theory that relates to energy metabolism

function (Chance et al. 1979; Vuillaume 1987; Collins et al. 1997). This can cause or at least contribute to a variety of pathologies, including some in humans (Cross et al. 1987; Reddy and Beal 2005; Abou-Sleiman et al. 2006; Valko et al. 2006). To lessen the consequences of this damage, cells have evolved complex defense mechanisms, including enzymatic ones (e.g., superoxide dismutase (SOD) and catalase) as well as various non-enzymatic antioxidants (e.g., vitamins C and E and glutathione) that act to detoxify the offending molecules (Chance et al. 1979; Rajendran et al. 2014). Oxidative damage resulting from an unfavorable balance between oxidative stress and antioxidant defenses may determine the individual aging rate of living things.

Genetic approaches using the small, free-living nematode *Caenorhabditis elegans* have helped elucidate various mechanisms of aging (Houthoofd and Vanfleteren 2007; Kenyon 2010). This review focuses specifically on the role oxidative stress plays in *C. elegans* aging.

7.2 *C. elegans* as a Model System for Aging Research

C. elegans can easily grow in petri plates on a simple diet of *Escherichia coli* and reproduces with a rapid life cycle of approximately 3.5 days at 20 °C. Embryonic development is rapid, taking only 13 h at 20 °C (Sulston 1988; Wood 1988a). The cell lineage has been traced from single-celled zygote to adult, and the entire cell lineage has been determined (Sulston and Horvitz 1977; Sulson et al. 1983; Sulston 1988). After hatching, larval development proceeds through four molts. About 10 % of the cells in the first larval stage undergo further cell divisions during larval development, contributing to the hypodermis, neurons, musculature, and somatic gonadal structures. Adults contain fewer than 1,000 somatic cells.

C. elegans offers several distinct advantages for aging research, which include a short maximum lifespan of approximately 30 days. In addition, *C. elegans* has received much attention as a genetic model, in part because its hermaphroditic mode of reproduction makes for ready isolation of mutants and allows rapid inbreeding. Males, which arise via meiotic non-disjunction can be used to construct stocks and map mutations (Brenner 1974; Wood 1988a, 1988b; Epstein and Shakes 1995; Riddle et al. 1997). Literally thousands of mutants have been isolated that affect virtually all biological processes. These genetic approaches have been useful

in identifying and mapping genes that regulate aging (Guarente and Kenyon 2000; Finkel and Holbrook 2000; Kenyon 2010). Mutations can be readily analyzed at the molecular level, thus providing specific insights to the various biochemical and physiological elements of lifespan determination. Techniques include the process of germ-line transformation, which is readily accomplished through microinjection, and enables the creation of transgenics, including those with reporter genes. *C. elegans* has also proven susceptible to the phenomenon of RNAi. By exposing animals to double-stranded RNA, an organismal response is triggered that can mimic the null phenotype of the gene corresponding to that particular RNA (Fraser et al. 2000). Because virtually all putative genes have been identified *in silico*, RNAi provides the opportunity to at least crudely determine the phenotypes resulting from inactivation of many *C. elegans* genes. In addition to these advantages, an adult soma consisting of fewer than 1,000 cells, all of which are postmitotic, offers the ability to detect cumulative age-related cellular alterations (Hosokawa et al. 1994; Adachi et al. 1998; Ishii et al. 2002).

7.3 Oxidative Stress and Defense Systems

There are numerous reports dealing with oxidative stress defense systems and their relationship to lifespan. For example, SOD activity is positively correlated in several organs with the maximum lifespan for various animal species, including primates (Tolmasoff et al. 1980). Similar correlations were observed for several other radical scavengers, including plasma urate, carotenoids, and vitamin E (Cutler 1985). There are some good recent articles that review defense systems against oxidative stress and aging of *C. elegans* (Gems and Doonan 2009; Back et al. 2012; Honda et al. 2010). Some evidence indicates that the defense systems, including antioxidant enzymes, may not affect life extension, which leads to speculation that oxidative stress does not play a major role in aging. For example, *sod-1* knockdown/out or transgenic *C. elegans* do not particularly affect lifespan (Back et al. 2012). Specifically, despite oxidative stress increases (Yang et al. 2007; Raamsdonk and Hekimi 2009), MnSOD knockdown/out mutants did not reduce (Yang et al. 2007; Doonan et al. 2008; Honda et al. 2008; Yen et al. 2009) or extended their lifespans (Raamsdonk and Hekimi 2009; Yang and Hekimi 2010; Dingley et al. 2010). Overexpression of MnSOD and Cu/ZnSOD extended lifespan but did not decrease oxidative damage (Cabreiro at al. 2011). Conversely, overexpression of catalase unexpectedly reduced lifespan (Doonan et al. 2008). These results do not support the oxidative stress hypothesis. However, there are some complexities inherent to interpreting these experiments. Specifically, superoxide anion converts superoxide anion to H_2O_2, which can be catalyzed to H_2O and oxygen by catalase or peroxidase. However, H_2O_2 also produces ·OH in response to superoxide and metal ions (Fig. 7.2). The amount and mixture of ROS produced in cells depends on site (cytoplasm)/subcellular organelle (mitochondria or endoplasmic reticulum) and the balance of anti-oxidative enzymes that are present. Thus, overexpressing one

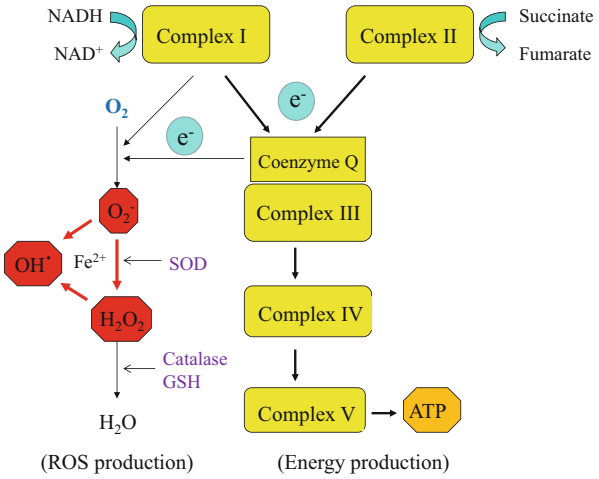

Fig. 7.2 Energy and ROS production from the electron transport chain; *SOD* superoxide dismutase, *GSH*: reduced glutathione

enzyme might have more complex effects than simply depleting its target substrate. Further, the redox balance of reduction and oxidation are finely tuned in healthy cells. Consequently, the redox imbalance in the SOD transgenics may lead to metabolic change including in energy metabolism. In animals which only one defense gene specific to one kind of ROS is knocked down or out or over-expressed, other ROS may be overproduced or imbalance of redox state may result. Just as Orr and Sohal (1994) demonstrated that lifespan was significantly longer in transgenic flies carrying both SOD and catalase genes, experiments using combinations of anti-oxidation enzymes may be necessary to clear up the relation between oxidative stress and aging. In addition, it is critical to determine the cellular amount and distribution of ROS in these transgenic animals.

7.4 Mitochondrial Oxidative Stress

The major endogenous source of reactive oxygen species ROS derives from the electron transport system in mitochondria (Nohl and Hegner 1978; Chance at al. 1979) (Fig. 7.2). It has been estimated that generation of O_2^- and its dismutated product H_2O_2 may constitute as much as 1–2 % of total electron flow, although others have placed this value at 0.1–0.2 % (Tahara et al. 2009). It is known that oxygen is initially converted to O_2^- by electrons leaked from complexes I and mainly complex III (Turrens 1997; Lenaz 1998; Finkel and Holbrook 2000; Raha and Robinson 2000).

Energy metabolism in aerobic organisms is almost exclusively the result of glycolysis, the Krebs cycle and electron transport. With respect to electron transport, five membrane-bound complexes within mitochondria form the respiratory chain that sequentially transfers electrons through a series of donor/acceptors, with

oxygen (O_2) as the final acceptor (Wallace 1999; Leonard and Schapira 2000). The eukaryotic mitochondrial electron transport system is composed of more than 80 subunits and requires more than 100 additional genes for its assembly (Attardi and Schatz 1988). The *C. elegans* electron transport is composed of about 70 nuclear and 12 mitochondrial genes products. The metabolism and structure of the *C. elegans* electron transport closely parallel its mammalian counterpart, and its mitochondrial DNA (mtDNA) is similar in size and gene content to the human mtDNA (Murfitt et al. 1976; Okimoto et al. 1992).

The nuclear gene *gas-1* (*fc21*) encodes a homologue of the Ip49 kDa iron protein, a subunit of complex I of the electron transport system (Kayser et al. 1999). The 49-kDa iron protein is abundantly expressed in multiple tissues, including neurons and body wall muscle in *C. elegans* (Kayser et al. 2001). The subunit seems to be implicated in binding CoQ and, therefore, it is believed to be essential for the core function of complex I (Xu et al. 1992; Anderson and Trgovcich-Zacok 1995). Mutations in *gas-1* were isolated based upon their ability to confer hypersensitivity to volatile anesthetics such as halothane or diethyl ether (Morgan and Sedensky 1994; Kayser et al. 1999). The mitochondria isolated from *gas-1* mutant reduced complex I enzymatic activities and increased complex II-dependent metabolism (Kayser et al. 2001). We have determined that *gas-1* mutants are hypersensitive to hyperoxia in a temperature-dependent fashion (Hartman et al. 2001). Specifically, the *gas-1* mutant was three times more sensitive than wild type at 15 °C and over six times more sensitive at 25 °C. These temperatures are typically employed as the permissive and restrictive temperatures for *C. elegans*. In these experiments, survival was defined as the ability of first-stage larvae to complete development. The hypersensitivity of *gas-1* was not restricted to larval development, as the ability to complete embryogenesis was also strongly influenced by oxygen concentration. The *gas-1* mutation also caused a dramatic decrease in lifespan upon exposure to hyperoxia. This was most dramatically observed when animals were reared under atmospheric oxygen and shifted to 60 % oxygen upon sexual maturity.

The question that is most directly related to the scope of this review is as follows: what is the mechanism by which a complex I defect creates hypersensitivity to ROS? This is at least partially answered by the observation that superoxide anion levels in sub-mitochondrial particles are more than two times greater than in wild-type (Ishii and Ishii, unpublished data). Thus, the hypersensitivity and precocious aging caused by the *gas-1* mutation is likely due to excess ROS production.

We have also demonstrated that O_2^- is produced from complex II in a genetic background that compromises complex II functionality (Ishii et al. 2006, 2013). Namely, the *mev-1* mutant is oxygen and ROS-generating chemical methyl viologen (paraquat) hypersensitive with respect to both development and aging (Ishii et al. 1990). The *mev-1*(*kn-1*) mutation, which results in an amino acid substitution at the 71st position from glycine to glutamate (G71E), has been identified as residing in the putative gene *cyt-1* (a human SDHC gene homologue), which is homologous to the succinate dehydrogenase (SDH) cytochrome b large subunit in complex II (Ishii et al. 1998). The mutation results in a greater than 80 %

reduction in complex II activity in the mitochondrial membrane fraction. Complex II catalyzes electron transport from succinate to ubiquinone and contains the citric cycle enzyme succinate dehydrogenase (SDH), which is composed of the flavin protein (Fp), the iron-sulfur protein (Ip) and two other subunits (a small subunit of cytochrome b and a large subunit of cytochrome b encoded by *cyt-1*) (Cecchini 2003; Cecchini et al. 2003; Maklashina and Cecchini 2010). The cytochrome b large subunit is essential for electron transport to ubiquinone in complex III. Based upon its position, the mutation site in *mev-1* may affect the domain binding to ubiquinone.

The mean and maximum lifespans of both the wild type and *mev-1* mutant were influenced by oxygen (Honda et al. 1993). Wild-type lifespans were not affected by oxygen concentrations between 2 % and 40 %. On the other hand, the mean and maximum lifespans of the *mev-1* mutant under atmospheric conditions (21 % oxygen) were shorter than wild type (Honda et al. 1993). Fluorescent materials (lipofuscin) and protein carbonyl derivatives are formed in vivo as a result of metal-catalyzed oxidation and accumulate during aging in disparate model systems (Strehler et al. 1959; Spoerri et al. 1974; Stadman and Oliver 1991; Stadman 1992). The presence of fluorescent materials and protein carbonyl modifications can be a specific indicator of oxidized lipid and protein. The *mev-1* mutants accumulated fluorescent materials and protein-carbonyl derivatives at significantly higher rates than did their wild-type cohorts (Hosokawa et al. 1994; Adachi et al. 1998). Thus, the aging process in *mev-1* animals approximates that of wild type except for its precocious nature. The biochemical pathologies of *mev-1* include elevated ROS. Specificity, O_2^- levels in both intact mitochondria and sub-mitochondrial particles were approximately two times greater in *mev-1* mutants as compared to wild type (Senoo-Matsuda et al. 2001). Given that most O_2^- generation is thought to occur around complex III, this means that the *mev-1* mutation either exacerbates O_2^- production at this location or, in some indirect way, increases O_2^- production at another point in electron transport, even at complex II. Another of the biochemical pathologies is that of reduced glutathione concentration in *mev-1* animals (Senoo-Matsuda et al. 2001). The *mev-1* mutation also caused supernumerary embryonic apoptosis especially under hyperoxia (Senoo-Matsuda et al. 2003). The abnormal apoptosis was suppressed by mutations in either *ced-3* or *ced-4*, indicating that the inappropriate signal in *mev-1* embryos stimulated induction of the normal *ced-9*/*ced-3*/*ced-4* apoptotic pathway in *C. elegans* (Senoo-Matsuda et al. 2003). Furthermore, the *mev-1*;*ced-3* double mutant lived longer than *mev-1*, which suggests that the supernumerary apoptosis contributed to the phenotype of life shortening in *mev-1* (Senoo-Matsuda et al. 2003). In addition, the oxidative stress by hyperoxia in *mev-1* animals rendered them hypermutable to nuclear mutations (Hartman et al. 2001). Finally, a number of biochemical pathologies likely derive from the role played by succinate dehydrogenase in the citric cycle. First, the ratio of lactate to pyruvate is significantly higher in *mev-1* mutants, suggesting that a metabolic imbalance known as lactate acidosis occurs in these animals. Second, a number of citric cycle intermediates are present at abnormal concentrations in *mev-1* mutants.

Conversely, ATP levels are normal in *mev-1* mutants. This was initially surprising but may suggest that *mev-1* animals rely more heavily on glycolysis for energy acquisition, thus explaining the elevated lactate levels. However, it is also possible that ATP consumption is decreased in *mev-1* because of some sort of global decrease in the metabolic rate that acts to counterbalance the compromised ATP generation in *mev-1* (Senoo-Matsuda et al. 2001). These results suggest that age-related complex II deterioration might also produce O_2^- and consequently accelerate aging.

In a similar fashion, Lemire and colleagues constructed transgenic *C. elegans* strains with a series of mutations in the succinate dehydrogenase iron-sulfur subunit (SDHB-1) (a human SDHB homologue) (Huang and Lemire 2009). They also resulted in reduced lifespans. These strains are also more sensitive to oxygen and paraquat. They overproduced superoxide anion with decreased succinate–cytochrome c reductase activity compared to the control strain. Thus, they recapitulate the phenotypes of the *mev-1* mutant. On the other hand, the *gas-1* and *mev-1* mutants do display some divergent phenotypes. For example, mutations of *isp-1* (*qm150*) and *lrs-2* genes, which encode iron sulfur protein of complex III and mitochondrial leucyl-tRNA synthetase, respectively, increased lifespan (Feng et al. 2001; Lee et al. 2003).

7.5 The Mitochondrial Paradox

While indispensable as a source of ATP generation, mitochondria are also the major endogenous source of ROS. Most of this occurs at complex III, although we have provided evidence that ROS can be generated at complexes I and II. In either case, these ROS can then attack all components of the electron transport system, damaging complexes that then leads to the production of even more ROS. The net result of this cascade is cellular and organismal aging. The metabolic abnormality affects electron flow and leads to a decreased mitochondrial membrane potential ($\Delta\Psi_m$). This may ultimately disrupt the mitochondrial structure and function. It is thought that this metabolic abnormality and ROS generation causes degenerative disease and aging. On the other hand, the reduction of energy metabolism may actually reduce ROS generation from mitochondria and consequently extend lifespan. In addition to the *isp-1* (*qm150*) and lrs-2 mutants described above, for example, RNAi treatment of *atp-3* (a subunit of complex V), *nuo-2* (a subunit of complex I), *cyc-1* (a subunit of complex III) and *cco-1* (a subunit of complex IV) genes resulted in adult animals with reduced ATP levels and prolonged lifespans (Dillin et al. 2002). In addition, a *clk-1* mutant [defective in demethoxy ubiquinone (DMQ)], whose gene encodes hydoxylase, exhibit a longer life than wild type (Lakowski and Hekimi 1996). CoQ biosynthesis is dramatically altered in *clk-1* animals such that mitochondria lack detectable levels of CoQ_9, and instead contain DMQ_9 (Miyadera et al. 2001). Furthermore, Larsen and Clarke (2002) showed that CoQ-less diets, which are the result of growing nematodes on a bacterial strain

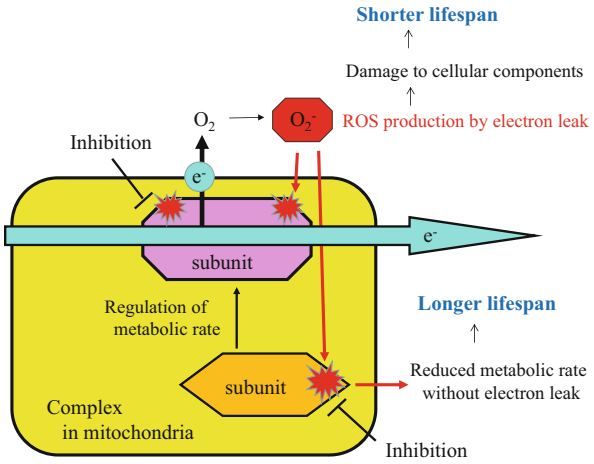

Fig. 7.3 Effects on lifespan by inhibition to subunits of the electron transport complexes (Ishii and Hartman 2003)

lacking CoQ, increase the lifespan of wild type. They also postulated that CoQ-deficient diet may affect aerobic respiration such that less superoxide anion is generated. In the case of the RNAi experiments (Dillin et al. 2002), this is somewhat akin to the effects of caloric restriction. The two contrary results (that is, the reduced lifespan with compromised complex II activity versus the increased lifespan with compromised complex I, III, IV and V activities) may depend on different functionalities of each subunit in the complexes. As described above, the *cyt-1* (= *mev-1*) mutation reduced lifespan and plays a direct role in electron flow from complex II to CoQ. Indeed, this subunit has a binding site to CoQ. Conversely, RNAi of *atp-3*, *nuo-2*, *cyc-1* and *cco-1* gene yielded animals with longer lifespans (Dillin et al. 2002). These gene functions may not affect electron flow directly but instead lower metabolic rate without electron leakage (Fig. 7.3). In addition, the presence of other isoforms may be partially compensatory. Indeed, there are such candidates in the genome (e.g., ceSHDA in complex II). In either case, avoiding electron leakage from electron transport and the resultant ROS production seems to be essential for a normal lifespan.

7.6 Conclusion

Since Harman (1956) postulated in his free-radical theory of aging, much attention has focused on the hypothesis that oxidative damage plays an important determinative role in cellular and organismal aging. In the 50-plus years since then, there are many results in support of this hypothesis, while certain observations could be interpreted as contradicting it. Normal cells, which produce several types of ROS as byproduct of energy metabolism and remove them by the complex defense systems, maintain a balance between reduction and oxidation states. Artificially changing (by drugs or transgenic over- and under-gene expression) can lead to an imbalanced

redox state and then to metabolic changes including energy metabolism via mitochondria, which could ultimately impact cellular and organismal wellbeing. In addition, even if the activity or amount of an antioxidation enzyme or antioxidant are changed, cells have a complex systems to compensate for it. On the balance, the evidence points to the fact that ROS can and do impose considerable damage throughout an organism's lifespan. Thus, the evidence supports. The evidence includes studies using the nematode *C. elegans* in which mutants and transgenics have been subjected to a variety of analyses

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