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Age-related changes of mitochondrial structure and function in *Caenorhabditis elegans*

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Abstract

A number of observations have been made to examine the role that mitochrondrial energetics and superoxide anion production play in the aging of wild-type *Caenorhabditis elegans*. Ultrastructural analyses reveal the presence of swollen mitochondria, presumably produced by fusion events. Two key mitochondrial functions – the activity of two electron transport chain complexes and oxygen consumption – decreased as animals aged. Carbonylated proteins, one byproduct of oxidative stress, accumulated in mitochondria much more than in the cytoplasm. This is consistent with the notion that mitochondria are the primary source of endogenous reactive oxygen species. However, the level of mitochondrially generated superoxide anion did not change significantly during aging, suggesting that the accumulation of oxidative damage is not due to excessive production of superoxide anion in geriatric animals. In concert, these data support the notion that the mitochondrial function is an important aging determinant in wild-type *C. elegans*.

Keywords: Aging; *C. elegans*; mitochondria; oxidative stress

1. Introduction

It is known that energy metabolism figures prominently in the aging process (Balaban et al., 2005). In aerobic organisms, mitochondria are intimately responsible for ATP production via the electron transport chain [oxidative phosphorylation (OXPHOS) system]. Mitochondria also produce reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) as byproducts of energy metabolism. Oxygen is converted to O_2^- by electron leakage from complex I and, to even a larger degree, complex III (Turrens et al., 1985; Lenaz, 1998; Finkel and Holbrook, 2000; Raha and Robinson, 2000). Such endogenously generated ROS readily attack a wide variety of cellular entities, resulting in damage that compromises cell integrity and function (Vuillaume, 1987; Collins et al., 1997). Aging and age-related degenerative diseases may be due to oxidative damage that results from an unfavorable balance between oxidative stress and antioxidant defenses, although it is difficult to distinguish causal events from the myriad of their secondary consequences (Beckman and Ames, 1998; Lenaz, 1998; Finkel and Holbrook, 2000; Raha and Robinson, 2000). For example, both carbonylated protein and lipofusucin accumulate with advancing age, but there is little straightforward evidence to suggest that they strongly act to limit life span (Gerstbrein et al., 2005; Yan et al., 1997; Yan and Sohal, 1998). However, accumulation is more rapid in short-lived mutants and slower in long-lived mutants, thus making these molecules excellent biomarkers of aging if nothing else (Hosokawa et al., 1994; Adachi et al., 1998; Yasuda et al., 1999; Gerstbrein et al., 2005).

Using oxygen consumption as a proxy of metabolism, several groups have demonstrated that metabolism in *Caenorhabditis elegans* decreases rapidly with aging (Braeckman et al., 2002a, 2002b; Houthoofd et al., 2002; Suda et al., 2005).

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We employed the novel method of Suda and co-workers for the measurement of energy metabolism, because this method is very simple and accurate. Interestingly, the decrease in respiration began soon after maturation, which is long before significant mortality occurred. In this study, we systematically examined age-related changes in mitochondrial structure and function, including energy metabolism and ROS production in aging populations of wild-type *C. elegans*. We document significant changes in mitochondrial structure and functionality with aging, thus supporting the notion that this organelle plays a key role in life span determination.

2. Methods

2.1. General methods

Wild-type *C. elegans* (N2) animals were cultured as previously described (Brenner, 1974). Embryos (eggs) were collected from nematode growth medium (NGM) agar plates using alkaline sodium hypochlorite (Emmons et al., 1979). The released eggs were allowed to hatch by overnight incubation at 20 °C in S basal buffer (100 mM NaCl, 50 mM potassium phosphate (pH 6.0)) (Sulston and Brenner, 1974). The newly hatched larvae (LI-stage larvae) were cultured on NGM agar plates (3-fold Bacto-peptone) seeded with the *Escherichia coli* (*E. coli*) strain NA22. In order to prevent progeny production, 5-fluoro-2′-deoxyuridine (FudR) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to a final concentration of 40 μM after the animals reached adulthood. The life spans of adult hermaphrodites were determined at 20 °C on NGM agar medium plates seeded with the *E. coli* strain OP50 (Honda et al., 1993; Adachi et al., 1998; Ishii et al., 1998). The surviving fractions for 4-, 8-, 12-, and 15-day-old animals were 100, 100, 99.2 and 97.6%, respectively (data not shown). Experiments were performed five times with starting populations of 69–100 animals. Results were consistent among these experiments.

2.2. Electron microscopy

Staged animals were fixed overnight in 2.5% gluteraldehyde/0.05 M phosphate buffer (pH 7.4) at room temperature. The samples were then washed with 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% OsO_4/0.05 M phosphate buffer (pH 7.4) for 1 h at 4 °C. After fixation, the samples were dehydrated with a graded ethanol series and embedded in Quetol 812 (Nissin EM Co. Ltd., Tokyo, Japan). Thin sections were stained with 2% uranyl acetate and lead solution and examined under a JEOL 1200 EX electron microscope operating at 80 kV.

2.3. Measurement of energy metabolism

Energy metabolism was determined indirectly by assaying the oxygen consumption rate. Oxygen concentrations were measured using 300 μm aluminum jacketed fiber optic probe that acted as a spectrometer-coupled chemical sensor for full spectral analysis of dissolved or gaseous oxygen pressure (FOXY-2000, Ocean Optics, Inc., FL) (Suda et al., 2005). A fluorescence method measured the partial pressure of dissolved or gaseous oxygen. An optical fiber carried the excitation light produced by the blue LED to the thin film (ruthenium complex) coating the probe tip. The probe collected fluorescence generated at the tip and carried it via the optical fiber to a high-sensitivity spectrometer. When oxygen in the gas or liquid sample diffused into the thin film coating, it quenched the fluorescence. The degree of quenching correlated to the level of oxygen pressure. The oxygen consumption rate was measured on groups of 40 animals in a small closed chamber of about 3 μl volume (1 mm height and 2 mm in diameter). Animals were immersed in a 0.5 μl liquid solution whose composition was the same as the animals' culture medium without agar, including *E. coli*. All oxygen measurements were carried out within 30 min at 22 ± 1 °C. Energy metabolic rate per animal (W) was calculated from oxygen consumption rate per animal by using an energy equivalent of 20.1 J/ml oxygen.

2.4. Isolation of mitochondria

A flotation method was used to remove debris and dead animals from living animals (Lewis et al., 1995). In brief, NGM plates were washed and the contents were suspended in ice-cold S basal buffer and mixed with an equal volume of ice-cold 60% sucrose. After centrifugation for 15 s at 3000 rpm, the floating animals were transferred to a fresh tube. They were washed three times with S basal buffer and once with isolation buffer (210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA and 5 mM Tris–HCl, pH 7.4). The animals were homogenized in isolation buffer using a glass-glass homogenizer with the inclusion of glass beads (0.10–0.11 mm). The debris was removed by a differential centrifugation at 600 × *g*. The supernatant was then centrifuged at 7200 × *g* and the mitochondria-containing pellet was suspended in TE buffer (50 mM Tris–HCl (pH 7.4), 0.1 mM EDTA). The supernatant was also retained and served as the cytoplasmic fraction.

2.5. Measurement of activities of complex I and complex II of electron transport chain

The activities of NADH-coenzyme Q oxidoreductase (complex I) and succinate-coenzyme Q oxidoreductase (complex II) in mitochondria were measured as previously described (Trounce et al., 1996). Animals were homogenized in isolation buffer (10 mM Hepes (pH 7.4), 0.15 M NaCl). The resulting homogenate was centrifuged at 250 × *g* for 10 min to remove debris. The supernatant was further centrifuged at 31,000 × *g* for 20 min. The pellet was suspended in isolation buffer. Complex I activity was assayed by measuring NADH-sensitive NADH-cytochrome *c* reductase activity at 37 °C in 200 μl 0.1 M Tris–SO_4 buffer pH 7.4, containing 0.32 mg cytochrome *c* and 1 mM sodium cyanate. Complex II activity was assayed by measuring malonate-sensitive succinate-cytochrome *c* reductase activity. The reference cuvette contained 20 μl of 20% sodium malonate solution.

2.6. Measurement of carbonylated proteins

Carbonylated proteins were measured using 2,4-dinitrophenyl hydrazine (DNPH) antibodies. Each fraction was treated with DNPH as described by Levine et al. (1990) with some modifications (Nakamura and Goto, 1996; Ishii et al., 2005). Total protein concentrations and protein concentrations of each fraction were determined using a BCA protein assay kit (Pierce, Rockford, IL). For Western analysis, the samples were transferred to nitrocellulose membranes by a Slot blot method using Milliblot-S (Millipore Co., Tokyo, Japan). The carbonylated proteins were detected with affinity purified anti-DNPH antibodies. Immunoreactive proteins were visualized using the enhanced chemiluminescence system (ECL, Amersham Biosciences, Uppsala, Sweden). A Cool Saver densitometer (ATTO Co., Tokyo, Japan) was employed to quantify results.

2.7. Measurement of superoxide anion (O_2^-)

O_2^- production was measured using the chemiluminescent probe MPEC (2-methyl-6-*p*-methoxyphenylethynyl-imidazopyrazinone)(ATTO Co., Tokyo, Japan) (Shimomura et al., 1998; Ishii et al., 2005). MPEC has an advantage of low background rather than MCLA (3,7-dihydro-2-methyl-6-(4-methoxyphenol) imidazol [l,2-a]pyrazin-3-one) that is generally used. Ten microgram of intact mitochondria was added to 1 ml of assay buffer (50 mM HEPES-NaOH, pH 7.4, 2 mM EDTA) containing 0.7 μM MPEC. Solutions were placed into a photon counter with an AB-2200 type Luminescencer-PSN (ATTO Co., Tokyo, Japan) and measured at 37 °C. The rates of O_2^- were expressed as counts per second.

2.8. Measurement of manganese-supeoxide dismutase (Mn-SOD), voltage-dependent anion channel 1 (VDAC) and cytochrome b large subunit of complex II in electron transport (CYT-1)

Total protein was obtained from animals that were homogenized in isolation buffer with a teflon homogenizer. The debris was removed by centrifugation at 600 × *g*. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of sample were run on 12.5%

polyacrylamide gels, transferred to polyviniliden difluoride membranes (ATTO Co., Tokyo, Japan), and immunoblotted. Anti-Mn SOD and anti-VDAC polyclonal antibodies were employed as the primary antibodies. They were purchased from Stressgen Bioreagents (British Columbia, Canada) and Santa Cruz Biotechnology, Inc. (CA), respectively. Peroxidase-conjugated secondary antibodies were then used. CYT-1 was used as a reference since levels remain constant during aging (Senoo-Matsuda et al., 2003).

3. Result

3.1. Ultrastructural examination of mitochondria in aging C. elegans

The structures of mitochondria in the body wall muscles of 4-, 10-, and 15-day-old animals were observed using transmission electron microscopy (Fig. 1). In the 4-day-old animals, most mitochondria were relatively small while only a few had long and thin morphologies. In the 10-day-old animals, the structure of some mitochondria was enlarged and swollen. The mitochondria in the 15-day-old animals, which still constituted 90% of the initial population, were more conspicuously enlarged and swollen. In order to prevent progeny production, FudR was routinely used, however, experiments using animals grown in the absence of FudR yielded a similar spectrum of morphological changes (data not shown).

3.2. Oxygen consumption

Fig. 2A shows the profile of energy metabolism for our novel method that was applied to the *C. elegans* system (Suda et al., 2005). We could significantly detect the signal of oxygen consumption even at the extremely low rate of 0.01%/min. Oxygen consumption was measured in adult populations that were 4-, 8- and 12-days old (Fig. 2B). As indicated in Section 2, metabolic energy was a conversion unit that was directly proportional to the amount of oxygen consumed by groups of 40 adults in a small chamber. Consumption dropped by about 60% between 4- and 8-day-old animals, with another drop of ca. 35% between 8- and 12-day-old animals. Interestingly, there was little mortality during this time, as the survival of 12-day-old animals was over 95%.

3.3. Age-dependent changes in activities of complex I and complex II of electron transport chain

The inner membrane of mitochondria contains five multisubunit complexes that comprise the electron transport system. The enzyme activities of two of these complexes were assayed in staged populations of *C. elegans* (Fig. 3). These two complexes were of particular interest because they represent the two entry points of electrons into the electron transport chain. Electrons then move further downstream from these two complexes to coenzyme Q (ubiquinone), at which point they are passed to complex III. The activity of complex I was reduced by 60% in 12-day-old animals as compared to the 4-day-old cohort ($P < 0.0001$). The percent survivals of these two staged populations were both greater than 95%. Thus, there was a significant decline in complex I activity even though most animals were still living. The activity of complex II decreased by 33%, which was not statistically significant at a 0.05 level (Fig. 3).

3.4. Carbonylated proteins accumulate in mitochondria and cytoplasm as C. elegans ages

The levels of carbonylated proteins have been shown to increase with age in many organisms, including *C. elegans* (Sohal et al., 1993, 1995; Stadtman, 1992). Given that carbonylated proteins are generated by oxidative stress, and that most endogenous free radical generation occurs in mitochondria, carbonylated protein concentrations were determined in mitochondria and cytoplasm isolated from 4-, 8-, 12-, and 15-day-old animals (Fig. 4). The carbonyl content was

Fig. 1. Age-related changes in the structure of body wall muscle as visualized by transmission electron microscopy. (A), (B) 4-day-old animals; (C), (D) 10-day-old animals; (E), (F) 15-day-old animals. The white arrows indicate mitochondria. Scale bars, 1 μm.

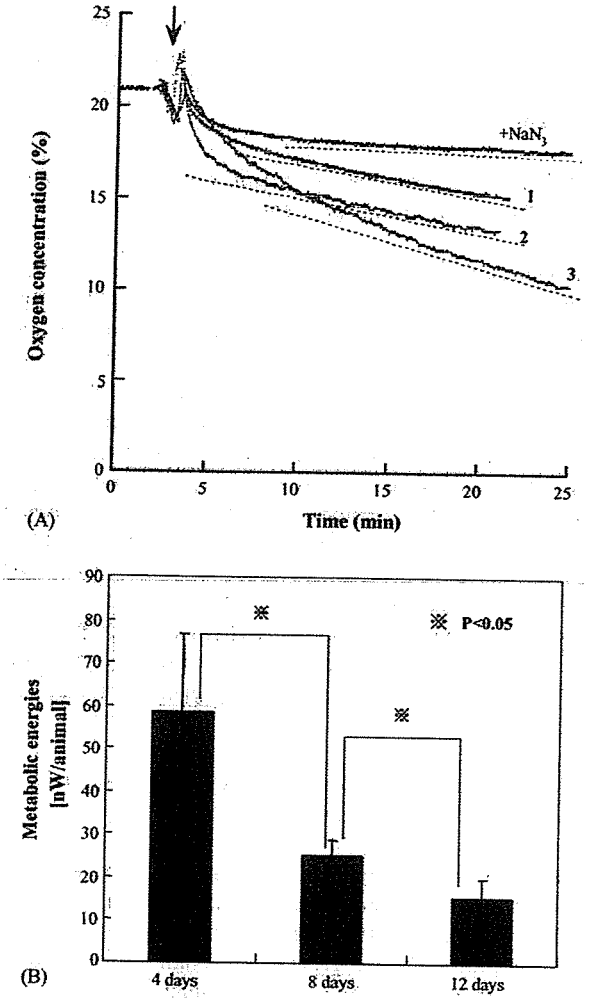

Fig. 2. (A) Typical data showing the oxygen consumption of wild-type *C. elegans*. The number of animals transferred from a Petri dish to a small chamber, which were cultured for four days at 20 °C, was 40. Three independent measurements (1, 2, 3) and a control experiment are shown. The control experiment was performed by adding a 0.5-μl solution of sodium azide (NaN_3, 10 mM) into the animal-immersed medium after the measurement of the case 3. Recording of data was started about 10 min after the addition of sodium azide. The oxygen consumption rate per animal and the metabolic energy per animal for three measurements were calculated from the slope of the dashed line, where case-1 was 134 pl/min, 44.8 nW, case-2 was 153 pl/min, 51.2 nW and case-3 was 236 pl/min, 79.1 nW, respectively. Note that every time course was adjusted at the sealing position, which is marked with an arrow. (B) Oxygen consumption as a function of age in wild-type *C. elegans*. The term metabolic energies directly correlate to oxygen consumption. The vertical bars indicate the standard deviation of three separate experiments.

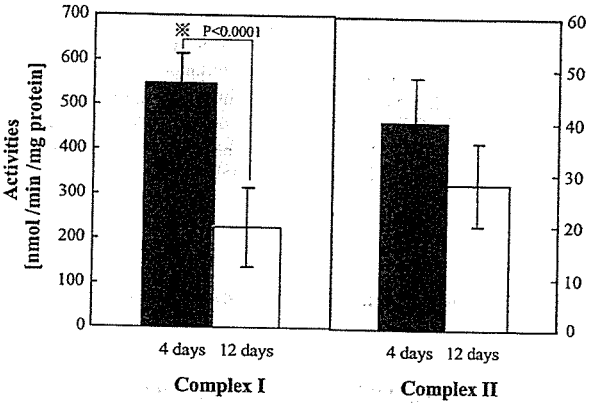

Fig. 3. The activities of complex I and complex II in 4- vs. 12-day-old animals. (A) NADH-cytochrome *c* oxidoreductase as an enzymatic indicator of complex I activity and (B) succinate-coenzyme Q oxidoreductase as an enzymatic indicator of complex II activity. The vertical bars indicate the standard deviation of seven separate experiments.

approximately four-fold higher in mitochondria than cytoplasm in young adults (i.e., the 4-day-old cohort). As expected, the mitochondrial concentrations of carbonylated proteins increased in aging animals. The increase was relatively steady and resulted in an approximate doubling of mitochondrial levels over the 11-day span of the experiment. Conversely, cytoplasmic levels remained relatively constant as there was a statistically insignificant increase in cytoplasmic levels between 4 and 8 days, although there was a slight difference between 4 and 12 days ($p < 0.01$). As a result, the ratio of mitochondrial to cytoplasmic carbonylated proteins increased from ca. 4 to 7.5 between 4 and 11 days of age in wild-type *C. elegans*.

3.5. Amount of O_2^- production in mitochondria

Superoxide anions are generated in mitochondria because of the inappropriate single-electron reduction of diatomic oxygen (Raha and Robinson, 2000). To ascertain superoxide anion levels as a function of age, mitochondria were isolated from 4- and 12-day-old animals. O_2^- levels were quantified using the chemiluminescent probe MPEC. Superoxide anion levels were slightly lower in mitochondria isolated from the 12-day-old animals, although this difference was not statistically significant (Fig. 5).

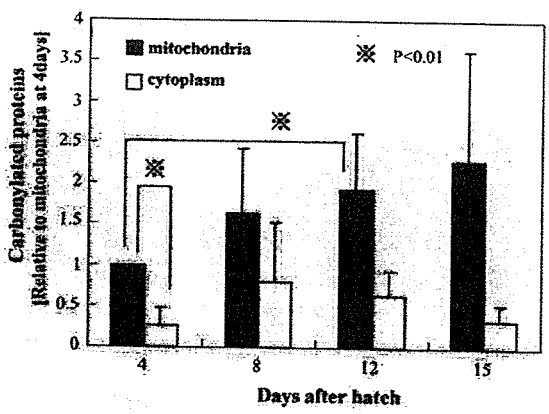

Fig. 4. The relative accumulation of carbonylated protein in the mitochondrial and cytosolic fractions of *C. elegans*. Carbonylated protein content was measured as described in Section 2. All values are relative to that of mitochondrial levels in 4-day-old animals. Comparison of the means by *t*-test reveals significant differences ($p < 0.05$) between the mitochondrial and cytosolic levels at each age. There were statistically differences between 4 days and 12 days in mitochondria ($p < 0.01$), but no difference for 8 days ($p < 0.1$) and for 15 days ($p < 0.2$) as compared with four days, respectively. The vertical bars represent standard deviations from between three to seven separate experiments except two experiments for the cytoplasmic fraction at 15 days.

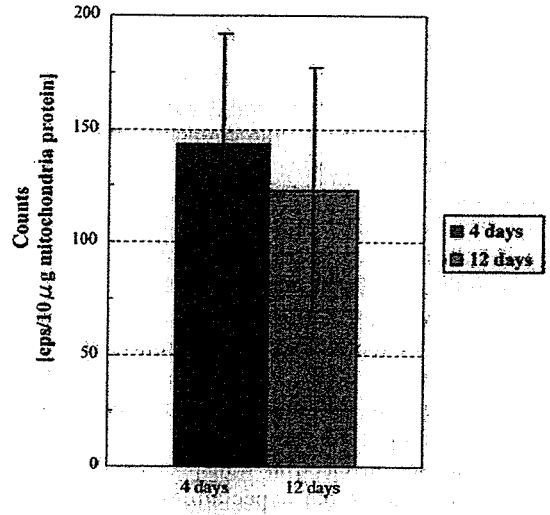

Fig. 5. Superoxide anion production in 4- vs. 12-day-old animals. The error bars indicate the standard deviation of four separate experiments. There was no statistical difference between 4- and 12-day-old animals.

3.6. Mn-SOD, VDAC and CYT-1 levels

It was initially surprising that superoxide anion levels did not increase as animals aged (Fig. 5). To eliminate the possibility that this result might be due to increased levels of the mitochondrial-specific scavenging enzyme Mn-SOD that would counterbalance increased superoxide anion production, we measured Mn-SOD levels using Western blots loaded with whole animals extracts isolated from 4-, 8- and 12-day-old animals (Fig. 6). Although Mn-SOD levels was slightly increased in 8-day-old animal, the levels compared between 4- and 12-day-old animals were unchanged, indicating that the steady state in superoxide anion levels during aging (Fig. 5), reflected reduced production rather than increased removal via enzymatic action in old animals.

CYT-1 is a complex II subunit in mitochondrial inner membrane. VDAC is an integral protein in the mitochondrial outer membrane. Levels of both protein levels held more-or-less constant as animals aged (Fig. 6). In addition, VDAC and CYT-1 protein levels held steady with aging when the input was 150 animals per sample rather than a fixed amount of total protein (data not shown). These results suggest that the decline of mitochondrial function is not caused by a reduction in mitochondrial mass.

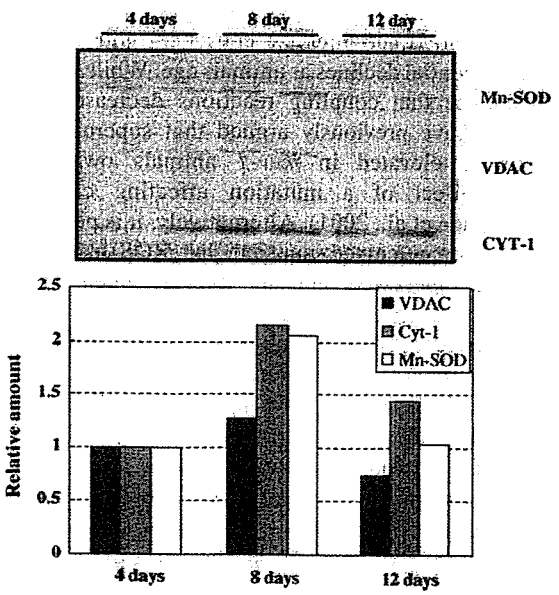

Fig. 6. Age-related changes of mitochondrial protein level. Upper level: an immunochemical assay. Thirty microgram of total protein was applied in each lane. Mn-SOD, VDAC and CYT-1 were detected by described in Section 2. Lower level: quantitative analysis. The detection employed A Cool Saver Densitometer (ATTO Co., Tokyo, Japan).

4. Discussion

Mitochondria play an indispensable role in the generation of ATP in aerobic eukaryotes. Almost paradoxically, mitochondria are also the primary source of cellular oxidative stress, owing to endogenously generated ROS that result from the combination with free electrons with molecular oxygen. Once generated, ROS can readily attack a wide variety of cellular entities, resulting in cellular, tissue and organ damage that ultimately compromises organismal viability (Beckman and Ames, 1998; Demple and Halbrook, 1983; Vuillaume, 1987; Collins et al., 1997; Balaban et al., 2005). Damage is particularly acute in mitochondria, which presumably results in the progressive deterioration of mitochondrial structure and function. The purpose of the current study was to examine mitochondria and certain of their key reactions as a function of aging in wild-type *C. elegans*.

We first employed transmission electron microscopy to reveal numerous deteriorations in tissue and cellular integrity as wild-type nematodes age (Fig. 1). Herndon et al. (2002) have noted similar changes using both transmission electron and Nomarski DIC microscopy. In particular, we both observed the presence of vacuoles that increase in number and size over time (data not shown). Garigan et al. (2002) employed Nomarski DIC microscopy to this same end.

Given our long-standing interest in mitochondrial structure and function, we were particularly interested in the observation that most mitochondria were relatively small while only a few were long and thin in young adults. In contrast, many mitochondria were enlarged and swollen in 15-day-old animals (Fig. 1). Similar ultrastructural abnormalities have been previously observed in the mitochondria of the precociously aging mutant *mev-1* (Senoo-Matsuda et al., 2003). This also occurs as mice age; specifically, a mitochondrial size increase of 60% was reported in 30-month-old mice as compared to 6-month-old mice (Wilson and Franks, 1975). Quantitative alteration of mitochondria by swelling could lead to ultrastructural pathologies that compromise energy metabolism and the functionality of the various constituent complexes of the electron transport chain.

Mitochondrial swelling could be caused by individual mitochondria increasing in size. Alternatively, mitochondria may undergo fusion events to reduce the number of

mitochondria per cell but increase the size of individual mitochondria. To distinguish these two, the levels of two mitochondrial proteins (CYT-1 and VDAC) per animal were measured in Western blots. The levels of both proteins were unchanged between 4- and 12-day-old animals (Fig. 6), suggesting that overall mitochondrial mass did not change as animals aged. This supports notion that the increased mitochondrial size was the consequence of mitochondrial fusion rather than actual mitochondrial enlargement. In turn, this suggests that the reduction of energy metabolism and complex activities of electron transport chain were due to qualitative rather than quantitative changes in mitochondria.

These ultrastructural changes were accompanied by significant decreases in mitochondrial function. Specifically, oxygen consumption, which we employed as a proxy of respiration, decreased significantly throughout the life span of *C. elegans* (Fig. 2). Similar age-correlated decreases have been noted by the Vanfleteren group (Braeckman et al., 2002a, 2002b; Houthoofd et al., 2002). It is worth noting that a number of different methods have been employed for these determinations (Van Voorhies and Ward, 1999; Braeckman et al., 2002a, 2002b; Suda et al., 2005). There is some controversy as to the reliability of the various experimental approaches (Van Voorhies and Ward, 1999; Braeckman et al., 2002a, 2002b). Nonetheless, the general conclusion that mitochondrial function declines as *C. elegans* ages appears to be on firm footing. This notion is fortified by our observation that the activity of both complexes I and II of the electron transport chain decreased as animals aged (Fig. 3). Interestingly, the decrease in oxygen consumption between 4 and 12 days (ca. 70%), which is presumably a reflection of overall mitochondrial efficiency, was greater than the decreases in two individual components, complex I (ca. 60%) and complex II (ca. 42%), over the same time.

Protein carbonyl derivatives are formed *in vivo* under oxidative stress. Most reports in the literature indicate that protein carbonyls accumulate with aging (Starke-Reed and Oliver, 1989; Sohal et al., 1993, 1995; Beckman and Ames, 1998), including in *C. elegans* (Adachi et al., 1998; Yasuda et al., 1999). In *C. elegans*, they accumulate more rapidly in short-lived mutants and more slowly in long-lived mutants (Adachi et al., 1998; Yasuda et al., 1999) thus making them good "aging markers." While these relationships are informative, we thought it important to determine the intracellular distribution of protein carbonyls. As predicted, we found that the level of carbonylated proteins was significantly higher in mitochondria than in the cytosol of 4-day-old animals (Fig. 4). More interestingly, the level of carbonylated proteins was unchanged in the cytosol but significantly increased in mitochondria as animals aged. Consistent with our present findings, Nagai and co-workers reported age-related increase in mitochondrial protein carbonyls with no significant change in the cytoplasmic proteins (Nagai et al., 2000). This could simply be due to continued accumulation of carbonylated proteins in mitochondria since ROS is mostly produce in mitochondria and therefore produces more damage there than in cytoplasm. Alternatively, it is possible that the export or degradation of mitochondrial carbonylated proteins decreased. Decreased rates have been reported in the cytoplasmic fraction as rodents age (Goto et al., 2001). The observation that superoxide anion production does not increase with aging (Fig. 5) argues the latter is true. We are aware of one other report in which protein carbonyls were quantified in mitochondria and the cytosol (Davies et al., 2001). Somewhat surprisingly, not only did they report that carbonylaton of proteins did not increase with aging, but levels were equivalent to or even slightly lower in the mitochondria than in the cytosol. We are at a loss to reconcile our results to those of Davies and co-workers.

While not universally the case, age-associated increases in ROS production is reported in the literature (reviewed in Beckman and Ames, 1998). It has been suggested that oxidative damage to the mitochondrial membranes and proteins is the culprit for this effect, with some sort of resultant imbalance in electron transport as the molecular consequence. Counter to our expectations, superoxide anion production did not increase as wild-type animals grew older (Fig. 5). This may be explained as follows. It is generally held that superoxide anion production is dependent upon two primary factors; namely, the membrane potential (or proton motive force) and the degree of coupling reactions as electrons flow through the electron transport system (Hafner et al., 1990; Nicholls, 2002). Specifically, superoxide anion production is favored by a high membrane potential and electron flow that is not tightly coupled. It is possible that these two agents of superoxide anion production have an inverse relationship as *C. elegans* ages; namely, as animals grew older, the relative decline in membrane potential was counterbalanced by the decreased efficiency in coupling reactions between the various complexes. If true, the decline of membrane potential would lead to a decrease in superoxide anion production, while the decline of coupling reaction would lead to an increase. The age-specific decreases in oxygen consumption as well as complexes I and II activities are consistent with notion that membrane potential declines as animals age. While we have no direct evidence that coupling reactions decrease in aging animals, we have previously argued that superoxide anion production is elevated in *mev-1* animals owing to an uncoupling effect of a mutation affecting complex II (Senoo-Matsuda et al., 2001). Alternatively, it is possible that ROS production remained constant, but ROS leakage from mitochondria increased in an age-dependent fashion. Hence, even though the net amount of mitochondrial ROS produced may be the same in young and old worms, the amount leaking from remaining functional complexes varied quite substantially. Such a localized increase in ROS by old mitochondrial ETC complexes may provide an alternate explanation for why protein carbonylation increases with age.

We find it interesting that the age-correlated changes in mitochondrial structure and function manifest themselves long before animals become decrepit and die. For example, oxygen consumption dropped by approximately 56% between four and eight days, with another 39% decrease between 8 and 12 days. Yet survival remained well over 95% over this time span. Others have also noted that structural deterioration proceeds significant mortality (Herndon et al., 2002; Garigan et al.,

2002). This could mean that these various mitochondrial changes are irrelevant to aging, or at the very least secondary consequences of the aging processes. Alternatively, it is possible that they play a causal role in aging, but there is a lag between the onset of cellular- and tissue-level pathologies and the actual time of death. We favor the latter explanation, although it is certainly possible that some of the effects noted are correlative rather than causal. The future challenge is to separate the two.

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The role of the electron transport gene SDHC on lifespan and cancer

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Abstract

Much attention has been focused on the hypothesis that oxidative damage contributes to cellular and organismal aging. A mev-1 mutation in the cytochrome *b* large subunit (SDHC) of complex II results in superoxide anion (O_2^-) overproduction and therefore leads to apoptosis and precocious aging in the nematode *Caenorhabditis elegans*. To extend these data, a transgenic mouse cell line was constructed with a homologous mutation to mev-1. Many of the mutant nematode phenotypes (e.g., increased superoxide anion production, apoptosis) were recapitulated in the mouse. In addition, a significant fraction of the cells that survived apoptosis were transformed. These data support the notion that oxidative stress from mitochondria play an important role of both apoptosis, which leads to precocious aging, and cancer

Keywords: Aging; Cancer; Mitochondria; Electron transport chain; Oxidative stress; SDHC

1. Introduction

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, 1998).

The electron transport system is the major endogenous source of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) (Nohl and Hegner, 1978). Such endogenously generated molecules can readily attack a wide variety of cellular entities, resulting in damage that compromises cell integrity and function (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; Martin and Grotewiel, 2006; Abou-Sleiman et al., 2006; Valko et al., 2006). It has been estimated that generation of O_2^- and its dismutated product hydrogen peroxide may constitute as much as 1–2% of total electron flow (Chance et al., 1979), although others have placed this value at 0.1% (Fridovich, 2004). 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). We have also demonstrated that O_2^- is produced from complex II in a genetic background that compromises complex II functionality (Senoo-Matsuda et al., 2001). This results suggests that age-related complex II deterioration may also produce O_2^- and consequently accelerate aging.

2. A SDHC mutant of *Caenorhabditis elegans* (*C. elegans*)

The nematode *C. elegans* can be readily cultured in petri plates on a simple diet of *Escherichia coli* and reproduces

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with a rapid life cycle of approximately 3.5 days at 20 °C. *C. elegans* offers several distinct advantages for aging research, not the least of which is a short maximum life span of approximately 30 days. Literally thousands of mutants have been isolated that affect virtually all biological processes including aging. In addition to these advantages, the somatic tissues of adult animal consist of long-lived postmitotic cells, thus offering the opportunity to detect cumulative age-related cellular alterations (Hosokawa et al., 1994; Adachi et al., 1998; Ishii et al., 2002). Genetic approaches have proven useful in identifying the genes and pathways that regulate aging (Guarente and Kenyon, 2000). In addition to these biological advantages, there exists a general spirit of cooperativity and free exchange of information within the "worm community". For example the *Caenorhabditis* Genetics Center at the University of Minnesota distributes a large number of strains free of charge. Finally, there are a number of Internet sites (most notably http://elegans.swmed.edu and http://www.wormbase.org/) that serve as excellent resources.

The electron transport chain of *C. elegans* is composed of about 70 nuclear and 12 mitochondrial gene products. It closely parallels its mammalian counterpart in its metabolism and structure, and *C. elegans* mitochondrial DNA (mtDNA) is similar in size and gene content to that of humans (Murfitt et al., 1976; Okimoto et al., 1992). The *mev-1* (*kn-1*) mutation has been identified as residing in the putative gene *cyt-1* (a human SDHC gene homologue), which is homologous to succinate dehydrogenase (SDH) cytochrome *b* large subunit in complex II (Ishii et al., 1998). The *mev-1* mutant was isolated based upon its hypersensitivity to the ROS-generating chemical methyl viologen (Ishii et al., 1990). In addition to its methyl viologen hypersensitivity, *mev-1* mutants are oxygen hypersensitive with respect to both development and aging. The *kn1* 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 CoQ and contains the Kreb's 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*). *In vivo*, SDH is anchored to the inner membrane with cytochrome *b* and is the catalytic component of complex II. Using separate assays, it is possible to quantify specifically both SDH activity and complex II activity. This was done with wild type and *mev-1* after extracts of each were subjected to differential centrifugation to separate mitochondria and mitochondrial membranes from cytosol (Ishii et al., 1998). The SDH activity in the *mev-1* mitochondrial fraction was experimentally identical to that of wild type. As expected of a mitochondrial enzyme, no SDH activity was observed in the cytosol. Thus, the *mev-1* mutation affected neither SDH anchoring to the membrane nor SDH activity *per se*. However, it dramatically compromised the ability of complex II to participate in electron transport. The cytochrome *b* large subunit is also essential for electron transport to CoQ in complex III. Based upon its position, the mutation site in *mev-1* may affect the domain binding to CoQ.

The mean and maximum life spans of both the wild type and *mev-1* mutant were influenced by oxygen (Honda et al., 1993). Wild-type life spans were not influenced by oxygen concentrations between 2% and 40%. On the other hand, the mean and maximum life spans of the *mev-1* mutant under atmospheric conditions (21% oxygen) were shorter than wild type (Ishii et al., 1990). 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 mitochondrial and sub-mitochondrial particles are 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, perhaps even at complex II. Several experiments suggest the latter. Another biochemical pathology in *mev-1* animals is that of reduced glutathione concentration (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*. Furthermore, the *mev-1; ced-3* double mutant lived longer than *mev-1*, which suggests that the supernumerary apoptoses 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 renders 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 Kreb's 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 Kreb's 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 suggests 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).

3. Mutated SDHC transgenic fibroblast NIH3T3 cell lines (*mev-1* cell)

In order to determine the effects of mitochondrially derived ROS in mammals, we established a transgenic mouse fibroblast NIH3T3 cell line with the equivalent mutation in the SDHC gene as *C. elegans mev-1* (Ishii et al., 2005). After transfection, we selected cell lines that expressed equal amounts of mRNA from the transgene and endogenous, wild-type allele. Knockout cell lines were not obtained, most likely because cells with more than 80% abnormal mitochondrial DNA are inviable (Nakada et al., 2001). In addition, RNAi with *cyt-1* produced an embryonic lethal in *C. elegans* (Ichimiya et al., 2002). The enzymatic activity of complex II in the mouse *mev-1* cells was reduced to 40% whereas the activity of complex I was unaffected. ATP levels were not affected, suggesting that this mutation did not directly compromise cell survival through reduced respiration *per se*.

In the *mev-1* cells, O_2^- production was slightly but not statistically significantly higher in isolated mitochondria. The addition of succinate (a substrate for complex II that stimulates complex II activity) to these mitochondrial preparations resulted in a large increase in O_2^- production in the mutant cell line. Under these conditions, O_2^- levels were significantly higher in intact mitochondria isolated from *mev-1* cells at both one month and three months after establishment. The *mev-1* cells accumulated cytoplasmic carbonyl proteins, a marker of oxidative stress, at a faster rate than wild type (Ishii et al., 2005). In addition, the amount of 8-OHdG, a DNA marker of oxidative stress was two-fold higher in *mev-1* cells (Ishii et al., 2005).

During three months (the time necessary for colony formation the medium plates), wild-type NIH3T3 cells maintained normal fibroblast morphology and grew in a monolayer. Conversely, the *mev-1* cells showed the loss of contact inhibition and had many apoptotic molecule-like granules during the first month after establishment. During the period of colony formation, some clefts characteristics of programmed cell death were found in the center of some colonies. As expected, the activity of the apoptosis marker caspase 3 was 1.3 to 1.8 times higher in *mev-1* cells. In three-month *mev-1* cells, the morphology was changed from the typical solid and elongated fibroblasts to smooth and rounded cells. In addition, the *mev-1* cells formed multiple layers. The doubling time of one-month *mev-1* cells after establishment was 1.5–2 times slower than that of wild-type cells. However, in three-month *mev-1* cells the doubling time had completely recovered to that of wild type. When one-month *mev-1* cells were injected under the epithelium of nude mice, they rapidly disappeared as compared to those of wild type. This suggests that these cells were dying of apoptosis and were phagocytized shortly after injection. Conversely, injecting the same number of three-month cells resulted in the production of tumors.

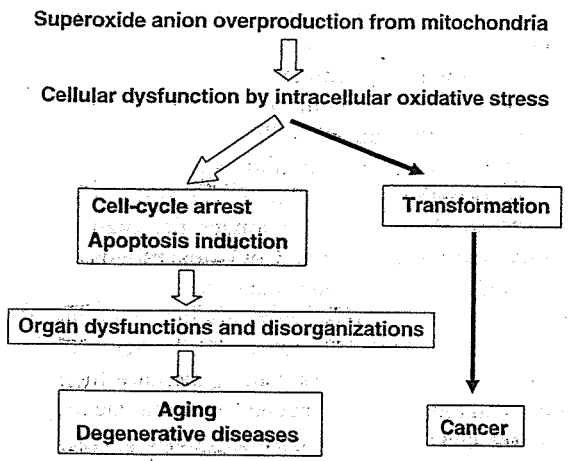

Fig. 1. Cascading effects of superoxide anion production in mitochondria. Superoxide anion production from mitochondria can cause cellular dysfunction by presenting oxidative stress to intracellular components. This can lead to apoptosis or cell-cycle arrest in most cells. The cellular dysfunctions then result in the organ dysfunction. In addition, some cells can be transformed via mutations induced by oxidative stress. Collectively, this ultimately can lead to reduced life span, degenerative disease and cancer.

The transformation rate on soft-agar medium for wild-type NIH3T3 cells was less than 1×10^{-6}. On the other hand, the rates were 5×10^{-4} for the one-month *mev-1* cells and 5×10^{-3} for the three-month cells. Thus, the *mev-1* cells had 100- to 1000-fold higher transformation rates than wild-type cells, indicating that *mev-1* cells are hypermutable. These results underscore the notion that mitochondrially generated oxidative stress can contribute to nuclear DNA damage, mutagenesis and, ultimately, tumorigenesis. In conclusion, ROS from mitochondria can promote not only apoptosis leading to aging but also tumorigenesis (Fig. 1).

4. 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 complex II. Moreover, these ROS can then attack all of the electron transport system, damaging complexes that produce 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 structures. 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 life span. 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 caused adult animals with reduced ATP levels and prolonged life spans (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 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 life span 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 life spans (Dillin et al., 2002). These gene functions may not affect electron flow directly but instead lower metabolic rate without electron leakage. 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 life span.

5. Discussion and conclusion

The electron transport chain or oxidative phosphorylation (OXPHOS) system is located within the mitochondrial inner membrane and is intimately responsible for three important processes: (i) production of ATP, (ii) generation of ROS, and (iii) regulation of programmed cell death, or apoptosis. Chronic elevation in ROS levels presumably results in damage to the various components of the electron transport system, which in turn results in the production of ROS at an even higher rate. The net result of this cascade is cellular and organismal aging.

Oberley and colleagues argued the importance of O_2^- in cancer, differentiation as well as aging (Oberley and Buettner, 1979; Oberley et al., 1980; Oberley and Oberley, 1988). Our data imply that a mutation in the *mev-1* (corresponding to *cyt-1* or SDHC) gene of both *C. elegans* and mouse cells leads to apoptosis and high mutation frequency in the nuclear genome, most likely because mitochondrial abnormalities lead to excess ROS production in mitochondria. Mitochondrailly derived ROS can mutate other genes, including tumor suppressor genes and oncogenes, and can lead to cellular transformation. Indeed, a significant fraction of the *mev-1* mouse cells that survived apoptosis were transformed. These data support the notion that oxidative stress from mitochondria play an important role of both apoptosis, which leads to precocious aging, and cancer.

Defective mitochondrial respiratory enzymes also cause myopathy and neurological diseases in humans (DiMauro et al., 1998; Howell, 1999; Wallace, 1999). Compared with defects in the other four complexes, abnormalities in complex II are rare and clinical presentation varies among individuals. In some cases, as in Leigh's syndrome, a mutation occurs in SDHA or SDHB. In addition, a mutation in SDHC or SDHD was found in patients of some familial chromaffin cell tumors (i.e., paragangliomas) (Baysal et al., 2000). It is still unclear whether oxidative stress contributes to the symptoms of these mitochondrial diseases, but in general, inhibition of electron flow causes electron leak from the complexes and consequently increases ROS production.

In conclusion, oxidative stress from mitochondria may lead to pathologies such as precocious aging, neuronal degeneration, and tumorigenesis in humans. The use of *mev-1* mutants in both the nematode *C. elegans* and a mouse cell line has enabled systematic study of the effects of this oxidative stress and its molecular, cellular, and organismal effects.

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フリーラジカルとアンチエイジング医学

Free Radicals and Anti-Aging Medicine

石井直明*

はじめに

「アンチエイジング（抗老化）」はヒトの心身ともに健康な長寿の実現を目指すものであるが，老化をひき起こすメカニズムを明らかにしないかぎり，科学的根拠に乏しい勘や経験によって抗老化の方法を模索せざるをえない．しかし老化は長くて複雑な過程を経るために，そのメカニズムはいまだに未解明で，そのためこれまで多くの老化仮説が考えられてきた（表1）．これらの仮説はどれも魅力的なものであるが，すべての生物や臓器・器官の老化に当てはめることはむずかしかった．そのなかでMartinらは，1996年のNature Genetics誌のなかで，老化のメカニズムを「共通メカニズム」と「個別メカニズム」に分けて考えることを提唱した[1]．「共通メカニズム」はこれまで考えられてきた，すべての生物や臓器・器官に当てはまる老化のメカニズムであり，「個別メカニズム」はある生物や臓器・器官のみに当てはまるメカニズムである．「共通メカニズム」のなかで確信がありそうなのはフリーラジカル（活性酸素）のみであるとMartinらは述べている．個々の寿命は活性酸素の発生量とそれに対する防御機構の能力とのバランスにより決定されている可能性がある．興味あることに，この活性酸素を老化仮説の中心に考えると，他の多くの仮説がそこに関連づけられてしまう（図1）[2]．

細胞内で発生する活性酸素のおよそ90%がエネルギー代謝の過程で電子伝達系から発生し，その量は総酸素消費量の0.1～2%になると考えられている[3,4]．電子伝達系は80以上のサブユニットからなり，それを構築するための遺伝子が100以上も必要となる．複合体Iは40以上ものサブユニットからなり，唯一，立体構造が

表1　さまざまな老化の仮説

個体レベル	神経内分泌説
	ストレス説
	免疫説
細胞機能レベル	体細胞分裂寿命限界説
遺伝子機能レベル	プログラム説
	体細胞突然変異説
	遺伝子翻訳エラー説
分子レベル	老廃物蓄積説、
	高分子架橋説
	フリーラジカル説
	DNA傷害説
分子から個体へ	エラーカタストロフ説

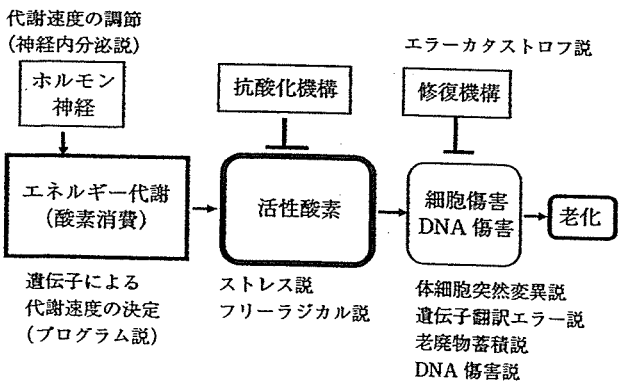

図1　酸素が関わる老化説
〔石井直明：分子レベルで見る老化．講談社ブルーバックスより転載〕

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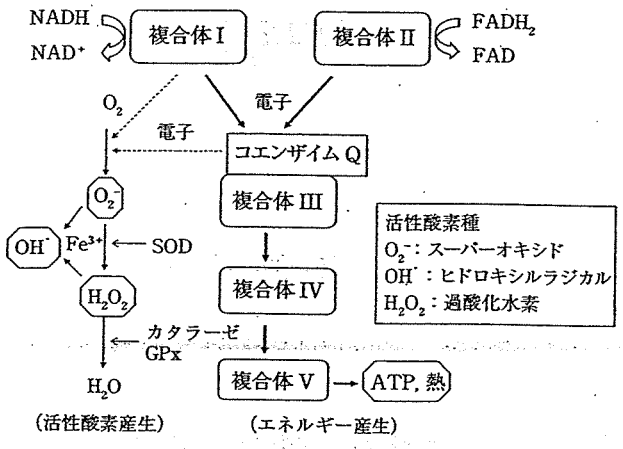

図2　ミトコンドリア電子伝達系と活性酸素

解明されていない大きな複合体である．複合体Iは少なくとも1つのFMN (flavin mono-nucleotide) と8つの硫酸鉄をもち，これらの個所から活性酸素が発生すると考えられている．複合体Iあるいは II から電子が複合体 III に渡される．複合体 III のなかではユビセミキノンが自動酸化されるときに酸素に電子が渡されることで活性酸素が発生すると考えられている (図2)．電子が複合体 III から IV と渡り，ATP (アデノシン三リン酸) は最終段階の複合体 V で合成される．酸素を一番消費するのは，電子伝達系の終末酵素であるシトクローム c オキシダーゼであるので，ここから多量の活性酸素が発生しても不思議ではないが，その証拠は得られていない．

活性酸素はエネルギー代謝の副産物としてミトコンドリアから発生する以外にも，HAD(P)H オキシダーゼやキサンチンオキシダーゼなどの生体酵素反応から，また放射線やタバコなどの外的要因からも発生する．活性酸素は細胞傷害や細胞死 (アポトーシス) を起こし，それによって老化や老年性疾患が生じ，さらに傷害が遺伝子に及べば癌化が生じることが分子遺伝学的な基礎研究から明らかになってきた．

I　活性酸素と老化

1960年から70年代にかけて行われた若い動物 (細胞) と老いた動物 (細胞)，あるいは寿命の異なる動物間の比較研究のなかから，生物学的な老化のメカニズム解明に重要なヒントを与えてくれたものがある．一つは，体重の大きい動物 (体重の重い動物) ほど長生きする傾向があることが明らかになった．さらに，体重の大きい動物ほどエネルギー代謝が低い傾向がある[5]．つまり，エネルギー代謝が低い動物ほど寿命が長いことになる．このようなデータは，寿命決定の遺伝子とエネルギー代謝の関係を明らかにした最近の分子遺伝学的な研究結果をすでに暗示していたことになる．

線虫の一種，*Caenorhabditis elegans* (*C. elegans*) の長寿突然変異体の *age-1* や *daf-2* の遺伝子解析から明らかになったインスリン様シグナル伝達系はこのエネルギー代謝を制御している．現在では，インスリン様シグナル伝達系を介した寿命制御のメカニズムは *C. elegans* のみならず，ショウジョウバエ，マウス，ヒトと，多くの生物に共通であると考えられるようになってきた[6]．

用語解説

線虫，*C. elegans*

線虫は種の数，生息数ともに他の生物を圧倒するほど多く，ヒトに寄生する回虫や，カミキリムシを媒体として松を枯らすマツノザイ・センチュウなど，その多くは寄生性であり特定の宿主の中で生息している．*C. elegans* は非寄生性の線虫であり，細菌を餌にして地中で自活している．体長1 mmあまりの虫であるが，表皮，神経，筋肉，消化器官，生殖器官という動物に必要最小限の体制をもつ．

Sydney Brenner が遺伝子のレベルで行動のメカニズムを解明するために開発したモデル動物であり，さまざまな突然変異体を分離され，分子遺伝学的手法が確立されている．多細胞動物で初めてゲノムの全塩基配列 (約1億塩基対) が決定されている．*C. elegans* の成虫は，たった959個の体細胞から成り立っており，Johns Sulston は，受精から成虫に至るまでのすべての細胞の分裂様式や運命を記述した細胞系統樹を完成させた．これは他の動物と比べて細胞数が少ない *C. elegans* でのみ可能な偉業であり，世界中で賞賛された．線虫の発生分化の段階ではプログラムされた細胞死が現れるが，Bob Horvitz はそのメカニズムを遺伝子のレベルで解明し，ヒトを含むさまざまなアポトーシスのメカニズムの解明に先駆者的な役割を果たした．この3人はこれらの業績により2002年のノーベル医学生理学賞を受賞している．*C. elegans* は約1カ月の短い最長寿命をもつことから，老化の研究にも盛んに使われるようになった．

さらにもう一つの長寿突然変異体，*clk-1* の原因遺伝子産物は電子伝達系に必要な酵素であり，エネルギー代謝に関与している[7]．興味あることに，これらの遺伝子の突然変異体はエネルギー代謝に関係することに加えて，酸素に耐性を示す．エネルギー代謝は，解糖系から，TCA（トリカルボン酸）サイクル，電子伝達系を介して，体温維持のための熱や，生体内の化学エネルギーであるATPを作りだす生化学反応である．多くの生物はこの反応のなかで酸素を必要とする．体内に取り込まれた酸素の多くはエネルギー代謝の最終反応で無害な水となるが，一部が無差別に細胞構成生物に傷害を与える活性酸素に変化する．生体内で生じる活性酸素の約90%は，このエネルギー代謝の副産物としてミトコンドリアに存在する電子伝達系から発生する．エネルギー代謝が高くなれば，活性酸素の発生量が増加する．これが，エネルギー代謝が高い動物ほど寿命が短くなることや，男性ホルモンによりエネルギー代謝が高い雄のほうが雌よりも寿命が短くなる一因になっていると考えられる．

筆者らは複合体IIのサブユニットの一つである，シトクローム *b* 大サブユニット（CYT-1）に欠損が生じた *C. elegans* の突然変異体（*mev-1*）を分離した[8~10]．この変異は複合体IIから活性酸素の過剰産生をひき起こし[11]，その結果，大気中でも野生株に比べて短命で，酸素濃度に依存して寿命の短縮がみられ，さらにミトコンドリアの形態異常，老化のマーカーとして知られるリポフスチン（老人斑）や酸化蛋白質の早期蓄積などの早老症の兆候を示すようになる[12,13]．さらに，*mev-1* の胚では正常な発生・分化とは無関係な野生株にはみられない細胞死も多数生じる[14]．*C. elegans* ではある細胞が胚発生期にアポトーシス（線虫では「プログラムされた細胞死」と定義）を起こし，それが個体の発生・分化に重要な役割を担っていることが知られている．アポトーシスには実行因子である *ced-3*（カスペース3）が必要となり，これが欠損するとアポトーシスは起こらなくなる．*mev-1ced-3* の二重突然変異体では，細胞死がまったくみられなくなることから，*mev-1* に生じる過剰な細胞死も線虫がもつプログラムされた細胞死の経路が必要になる．*mev-1ced-3* の平均寿命は *mev-1* 単独の寿命より長く，野生株よりも短い[14]．これは細胞死が寿命短縮に少なからず寄与していることがわかる．これらの結果から，加齢とともに電子伝達系に傷害が蓄積すれば，活性酸素が複合体IやIII以外からも発生するようになり，老化が加速されることを示唆している．

線虫の *cyt-1* 遺伝子に相当するSDHC遺伝子に *mev-1* と同様の変異をもつマウスのNIH3T3培養細胞でも，ミトコンドリアから活性酸素が過剰に産生されるようになる[15]．その結果，ミトコンドリアの形態変化や膜電位の低下，酸化蛋白質の蓄積，核のDNA中の oxo^8dG（8-oxo-2′-deoxyguanosine）蓄積量と突然変異頻度の増加が起こる[15]．培養を続けるうちに，細胞はアポトーシスを生じるようになり，増殖速度が低下する．しかし培養をさらに続けると，アポトーシスを逃れた細胞が形質転換を生じ，増殖能力が復帰する[15]．これらの細胞をヌードマウス皮下に移植すると，アポトーシスを盛んに起こしている時期の細胞は短時間で貪食されるが，形質転換を生じた細胞を移植すると腫瘍を形成するようになる[15]．この現象は，個々の細胞の機能低下やアポトーシスにより生じる「老化」と，核DNAの遺伝子変異により生じる「癌化」が，活性酸素の傷害を起因とした同じ過程のなかで生じることを示した初めての例である（図3）．家族性傍神経節腫（パラガングリオーマ）の患者に複合体IIのSDHCやSDHDサブユニットの遺伝子変異が見いだされたことから[16]，最近では，これらの遺伝子

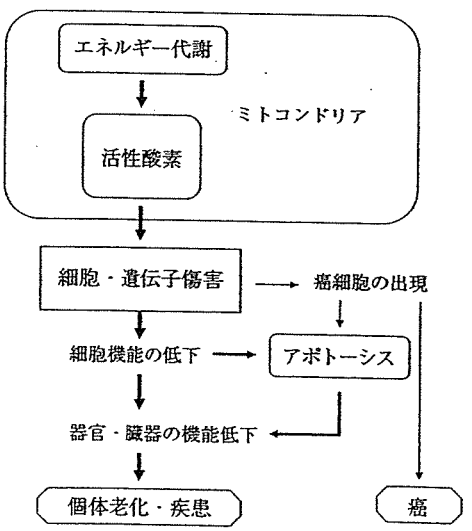

図3　ミトコンドリアが関わる老化や疾患のメカニズム

は腫瘍抑制遺伝子と定義されている[17].

TCAサイクルや電子伝達系を含む細胞小器官であるミトコンドリアは，エネルギー産生と，その過程の副産物である活性酸素を発生させ，さらにアポトーシスも制御しており，老化の鍵を握っているといっても過言ではない．とはいえ，エネルギー代謝・活性酸素と老化との関係には，一つの矛盾がある．活性酸素はエネルギー代謝の副産物としてミトコンドリアから産生されるため，エネルギー代謝が活発になれば，当然，活性酸素の発生量も増えることになる．活性酸素はあらゆる細胞内の分子を攻撃するので，これを産生するミトコンドリア自身にも傷害を与える．その結果，ミトコンドリアの機能低下が生じてエネルギー代謝が低下することになる．エネルギー代謝が低下すれば活性酸素の産生量が低下するはずである．これが正しければ，加齢とともに活性酸素の発生量は低下することになり，ミトコンドリアへの傷害は減少するはずである．現に*C. elegans*の実験で，ミトコンドリア電子伝達系の複合体のサブユニットをコードする遺伝子の発現を抑制すると，寿命が延長することが報告されている[18,19]．これらの遺伝子発現の抑制はエネルギー代謝の低下を招くことから，寿命延長効果は産生する活性酸素の量が低下するためと考えられている．しかし，前述したように，筆者らの研究では電子伝達系の傷害は活性酸素の過剰産生をひき起こし，*C. elegans*を短命にする．これは電子伝達系の複合体の傷害が電子の正常な流れを妨げ，電子伝達経路の先に進めなくなった電子がその場から逸脱し，近傍の酸素と反応して活性酸素を生じるためと考えられる．筆者らは最近，活性酸素の発生量は加齢で変化しないことを突き止めている[20]．これは，老化によるミトコンドリア傷害は活性酸素の産生量に影響を与えることはないためと考えられるが，ミトコンドリアの傷害により電子伝達系のなかで活性酸素量が増える個所と減少する個所が存在するために，総発生量としては変化が表れてこないことが原因であるとも考えられる．

II　活性酸素と老年性疾患

活性酸素は老化のみならず，前述した癌をはじめ，加齢とともに増加するさまざまな疾患にも関与している

表2　活性酸素が関与する傷害や疾患

炎　症
リウマチ関節炎，高尿酸血症，熱傷
癌
癌の発生，癌の増殖，癌の転移
神　経
Alzheimer病，Parkinson病，家族性筋萎縮性側索硬化症
組織・臓器
動脈硬化，潰瘍，糖尿病，白内障，加齢黄斑変性症，虚血，膵炎

（鈴木孳之：老化の原点をさぐる．裳華房，谷口直之，淀井淳司：酸化ストレス・レドックス．共立出版より改変）

（表2）．最近は過食，運動不足によって内臓脂肪が蓄積し，メタボリックシンドロームとよばれる，高血圧症，高脂血症（コレステロールやトリグリセライドの高値），糖尿病（インスリン抵抗性）など複数の生活習慣病を合併する人が増えている．これらの病気はお互いが密接な関係をもって発生しており，多く合併するほど動脈硬化を促進して脳梗塞や心筋梗塞などを起こしやすくなる．

動脈硬化には活性酸素が深く関与している．コレステロールの輸送に関わる低比重リポ蛋白質（LDL）は血中から血管の内皮細胞下に侵入すると，内皮細胞や平滑筋細胞で作られた活性酸素によって酸化LDLに変化する．一方，内皮細胞下に侵入したマクロファージは酸化LDLに結合する受容体をもつために，これを介して酸化LDLを取り込む．すると酸化LDLからコレステロールが切り離され，マクロファージ内に蓄積するようになる．この過程でマクロファージは泡沫細胞を形成する．この泡沫細胞が血管内膜に蓄積し，そこにリンパ球や血小板が集まってくる．血小板からは血小板由来増殖因子（PDGF）が放出され，これが平滑筋細胞の増殖を招くことで，血管の柔軟性が失われたり，血管の内腔が狭められたりしていく．このような経過により生じる，高齢者の大動脈にみられるアテローム性動脈硬化症に活性酸素がトリガーの役目を果たしている．

最近，筆者らは電子伝達系複合体サブユニットであるSDHCの変異遺伝子を導入した遺伝子組換えマウス（トランスジェニック・マウス）の作製に成功した（未発表）．この変異マウスは，同様の変異をもつ線虫や培養細胞と同様にミトコンドリアから活性酸素が過剰に産生

され，さらに視力の低下といった老人性の疾患が野生型に比べて早期に認められるようになった．これにより，このマウスが生体内酸化ストレスを原因とする老化や老人性疾患のモデル動物として，老化の基礎研究のみならず臨床研究にも応用され，老化および老人性疾患の発症機構の解明から治療や予防まで幅広い分野で貢献することが期待される．

III　活性酸素とアンチエイジング

実験動物で長寿を実現させる最も簡単な方法が，「腹八分目」，つまりカロリー制限である．これは単細胞から霊長類のサルまで，実験に使用した生物すべてに当てはまる．このメカニズムはいまだに不明であるが，カロリー制限という一種の飢餓状態を細胞が感知し，そのシグナルが抗酸化や免疫などの生体防御能力を高めることが一つの理由にあげられている[21]．活性酸素は細胞毒性が強いため，生物はそれに対する防御機構を進化させてきた．これらのなかには活性酸素を除去するスーパーオキシドディスムターゼ（SOD）カタラーゼ，グルタチオンペルオキシダーゼ（GPx）などの酵素や，ビタミンCやEなどの低分子物質などが知られており，寿命が長い動物ほど，これらの酵素や低分子物質を多くもつことが知られている．*C. elegans* のインスリン様シグナル伝達系の下流では *daf-16*（ヒトではHOXO）とよばれる転写因子が発生，老化，抗酸化にかかわる遺伝子を制御していると考えられ[6]，マンガン-SODの発現量が長寿突然変異体である *daf-2* で上昇していることが知られている[22]．

最近は，適度な運動が活性酸素に対する防御機構の能力を上げると考えられるようになった．これは運動によるエネルギー代謝亢進で発生する活性酸素の適応応答（ホルミシス効果）である可能性がある[23]．ホルミシスとは，これは軽度な傷害が防御機構をより強くし，大きな傷害に備えるような適応応答とよばれる現象である．ホルミシスは最初に放射線傷害から発見されたが，電離放射線による傷害の原因の90%が，放射線が水と反応して生じた活性酸素によるものであることや，あらかじめ酸素ストレスをかけた *C. elegans* が放射線に耐性を示すことから，ホルミシスの本来の役割は活性酸素に対する生体防御機構と考えられる．つまり，活性酸素は細胞構成成分に傷害を与える一方で，細胞伝達のシグナルとして発生や癌化の過程に関与すると同時に，活性酸素のシグナルが活性酸素に対する防御機構の活性化にも働いている．老化の促進や老年性疾患を防ぐには，活性酸素を必要のない場所で過剰に発生させず，一方で栄養や運動で抗酸化能力を高めることが必要になる．

おわりに

老化の研究者は，誰にでも生じる「生理的老化」以外に，誰にでも生じるわけではないが，加齢とともに増える疾患を「病的老化」として考えている．生理的老化による死亡は老衰であるが，これは総死亡数の3%にすぎない．多くの人たちは老化でなく，病的老化で亡くなるために100歳まで行き着かない．日本人の総死亡数の60%が脳と心臓の血管傷害と癌の疾患であり，これらの病的老化による疾患を克服すれば，「平均寿命」は今よりもさらに延びることになる．最近の研究から，活性酸素は生理的老化のみならず，病的老化にも密接に関与していることが明らかになってきた．生体内の活性酸素を制することは，寿命や老化を制することといっても過言ではないかもしれない．分子遺伝学にもとづいた老化のメカニズムの研究は，確かなヒトの健康的な長寿実現の方法を見いだしてくれるに違いない．

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