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Cytochrome *c* oxidase loses catalytic activity and structural integrity during the aging process in *Drosophila melanogaster*

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ABSTRACT

The hypothesis, that structural deterioration of cytochrome *c* oxidase (CcO) is a causal factor in the age-related decline in mitochondrial respiratory activity and an increase in H_2O_2 generation, was tested in *Drosophila melanogaster*. CcO activity and the levels of seven different nuclear DNA-encoded CcO subunits were determined at three different stages of adult life, namely, young-, middle-, and old-age. CcO activity declined progressively with age by 33%. Western blot analysis, using antibodies specific to *Drosophila* CcO subunits IV, Va, Vb, VIb, VIc, VIIc, and VIII, indicated that the abundance these polypeptides decreased, ranging from 11% to 40%, during aging. These and previous results suggest that CcO is a specific intra-mitochondrial site of age-related deterioration, which may have a broad impact on mitochondrial physiology.

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1. Introduction

It is widely hypothesized that mitochondrial impairment plays a causal role in the decline of physiological vigor during the aging process [1,2]. The two most frequently reported age-related mitochondrial alterations are (i) that the rate of ADP-stimulated respiration (state 3) declines in old organisms [3,4], (ii) whereas the rates of mitochondrial generation of superoxide anion radical ($O_2^{\cdot-}$) and its stoichiometric product, H_2O_2 increase in the last trimester of life [5–7]. Furthermore, the decrease in mitochondrial respiratory capacity is almost invariably correlated with a concomitant loss in the physiological fitness of the organism, including the loss in locomotive ability. For instance, the wing beat frequency, speed of flying, and the duration of a single flight decline as a function of age in *Drosophila* [8].

Mitochondrial hydrogen peroxide production seems to play a physiological as well as a potentially deleterious role, depending upon its intracellular concentration. At low concentrations, it is believed to be essential for the protein thiol/disulfide-mediated cell signaling mechanisms (reviewed in [9]). For instance, genetic manipulations in *Drosophila*, such as the insertions of ectopic catalase into the mitochondrial matrix, that lower the rate of mitochondrial H_2O_2 release, have been found to result in high mortality rates, thereby demonstrating the physiological usefulness of the mitochondrially-generated H_2O_2 [10]. Nonetheless, mitochondrial H_2O_2 production at rates that exceed the physiological needs may cause elevation of oxidative stress, which is manifested as a pro-oxidizing shift in the thiol redox state and an increase in steady-state levels of macromolecular oxidative damage [9,11]. Mitochondrial hydrogen peroxide production increases ~2-fold during aging in *Drosophila* and is also reported to be inversely related to the life span of different species of flies [5,7].

The nature of the mechanisms underlying these two ubiquitous age-related mitochondrial changes, namely, the decline in respiratory rate and the increased production of $O_2^{\cdot-}/H_2O_2$, is presently unclear; however, the involvement of the components of electron transport chain (ETC) is suspected because they are the primary sites for the production of $O_2^{\cdot-}/H_2O_2$ as well as the trans-membrane extrusion of the protons, required for ADP-stimulated respiration. The mitochondrial respiratory chain and the ADP phosphorylation system consist of two mobile electron carriers, ubiquinone and cytochrome *c*, and five multi-subunit protein complexes embedded in the inner membrane, namely NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:ferricytochrome *c* oxidoreductase (complex III), ferrocytochrome *c*:oxygen oxidoreductase (complex IV), often termed as cytochrome *c* oxidase (CcO), and F_IF_O ATP synthase (complex V). Histochemical studies have shown that the frequency of CcO-deficient cardiac and skeletal muscle myofibers and neurons increases

Abbreviations: CcO, cytochrome *c* oxidase; $O_2^{\cdot-}$, superoxide anion radical; H_2O_2, hydrogen peroxide; ETC, electron transport chain.

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in aged mammals [12]. The number of mitochondria with disoriented cristae and CcO deficiency also increases with age in dipteran flies [13,14]. CcO activity has a direct effect on the mitochondrial trans-membrane proton gradient and the redox state of upstream components of the ETC, whose auto-oxidation results in $O_2^{\cdot-}/H_2O_2$ generation [15,16].

In this context, the main objective of the present study was to understand the role of CcO in the age-related changes, such as the decline in mitochondrial respiratory capacity and increased ROS production. In higher eukaryotes, CcO consists of 13 subunits in each of its two homodimers [17]. Subunits I, II, and III, encoded by mitochondrial DNA, form the catalytic core of CcO, whereas the other ten subunits (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII), that are encoded by the nuclear genome, are involved in the assembly/stability of the CcO holoprotein and the modulation of its catalytic activity. A previous investigation in this laboratory indicated that the levels of mitochondrial DNA-encoded CcO subunits II and III are severely depleted during aging in *Drosophila melanogaster* [18]. The present study reports the age-associated changes in the catalytic activity of CcO holoenzyme and the abundance of seven different nuclear DNA-encoded CcO subunits.

2. Materials and methods

2.1. Rearing of flies and isolation of mitochondria

The male *y w* strain of *D. melanogaster* were housed in groups of 125 in 170 ml bottles at 25 °C under constant light and fed on cornmeal-sucrose-yeast-agar medium, refreshed every 2 days until 30 days of age and daily thereafter [4].

Mitochondria were isolated from the thoracic flight muscles at three different ages, representing the young (10 days), the middle age (30 days) and the aged (47 days). CcO activity was measured spectrophotometrically by monitoring the oxidation of ferrocytochrome *c* at 550 and 580 nm as the reference wavelength ($\varepsilon = 27.7\ mM^{-1}\ cm^{-1}$), as described previously [4].

2.2. Production of antibodies and Western immunoblot analysis

Coding regions of the CcO subunits were amplified by PCR, using appropriate cDNAs as templates (the primer sequences used for amplification are listed in Supplementary Material). DNA fragments containing the complete COX subunit coding regions were ligated into the open reading frame of the pProEX HT procaryotic protein expression vector (Invitrogen) and the recombinant constructs were then transformed into *Escherichia coli* DH5-alpha competent cells. Recombinant proteins were purified from inclusion bodies by the following procedure. Briefly, 3L of the LB media were inoculated with bacteria and cultured until the late exponential phase, then induced with 1 mM IPTG for 3 h. *E. coli* cells were collected by centrifugation, re-suspended in 20 ml of 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM EDTA, 2 mM EGTA and disrupted by sonication in an ice/water bath. The insoluble aggregates were sedimented at 20,000*g* for 20 min at 4 °C. Pellets were re-suspended in the disruption buffer, sonicated and adjusted to 0.2% Triton X-100. After 15 min incubation at room temperature inclusion bodies were recovered by centrifugation. This last cycle was repeated two more times. Inclusion bodies were subsequently solubilized in 8 M urea with 50 mM dithiothreitol and recombinant proteins were purified away from minor contaminants by gel filtration on Sephacryl S-200 HR column equilibrated with 8 M urea and 2 mM DTT. Collected fractions were dialyzed against PBS containing 5 mM EDTA and 1 mM EGTA. Aggregated protein was pelleted by centrifugation and re-suspended in a minimal volume of PBS by sonication. Small aliquots were solubilized in 50 mM Tris–HCl (pH 6.8) buffer, containing 1% SDS, 50 mM dithiothreitol and used for assessing protein purity and concentration (RC DC Protein Assay, Bio-Rad, Hercules, CA). Polyclonal antisera were generated through the services of the Proteintech Group, Inc. (Chicago, IL).

Mitochondrial proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), using 4% stacking and 10% separating gels [18]. For each experiment, one gel, containing the separated proteins, was stained with Coomassie blue and the other was processed for immunoblotting, for which the proteins were electrotransferred to a PVDF membrane (Immobilon P^{SQ}, Millipore). The membranes were incubated overnight at 4 °C with the primary antibody at dilutions of 1:3000 for CcO subunit VIII; 1:5000 for anti-subunits Va, VIb, VIc, VIIc; and 1:10,000 for COX subunits IV, Vb. After washing, the secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (Pierce, Rockford, IL) was added at a dilution of 1:50,000 and incubated for 1 h at 37 °C. Immunoreactive bands were detected by enhanced chemiluminescence (ECL reagent; GE). The membranes were then stripped and re-probed with mouse anti-complex Vα and anti-porin antibodies in order to obtain internal loading controls. Blots were scanned and the optical densities of the bands were calculated using Quantity One program (Bio-Rad). Protein content was quantified by densitometric analysis of equal loading amounts of samples from different age groups and expressed as percent of the reactive protein present in the young flies (10-day-old).

3. Results and discussion

3.1. Life span

Survivorship plots of five different populations of flies are presented in Fig. 1A. The average life span was 47 ± 2 (SEM) days. There were relatively few deaths until around 43 days of age, i.e. when the flies had reached ~90% of their average life span, suggesting that the cause(s) of death was related to age rather than some random pathological factor. Thus, the three different ages, 10, 30, and ~47 days, at which the comparisons were made, represented 21%, 56%, and 100%, respectively, of the average life span.

3.2. Cytochrome c oxidase activity at different ages

CcO activity in sonicated mitochondria decreased gradually by 33% ($P < 0.05$) between 10 and 47 days of age (Fig. 1B). There was no significant difference in the slope of the decline during the first and the second half of the average life span. Our previous studies on *Drosophila* indicated that the rate of state 3 respiration, supported by NAD- as well as FAD-linked substrates declined by 31–36% during aging. No significant age-related alterations were detected in the activities of complex I, complexes I/III, or complexes II/III [4]. Thus, the present demonstration that the CcO or complex IV activity declines with age by a magnitude similar to that by which state 3 respiration decreases with age, suggests that CcO is the main site of mitochondrial respiratory dysfunction during aging in *Drosophila* [4,19]. Age-related decreases in CcO activity have also been reported in other species of dipteran flies [20] as well as rodents [21,22]. Histochemical studies have shown extensive, but randomly distributed, losses of CcO activity in striated muscles and brain of monkeys during aging [12]. Thus, the age-associated decline in CcO activity appears to be a ubiquitous feature of aging, transcending phylogenetic boundaries.

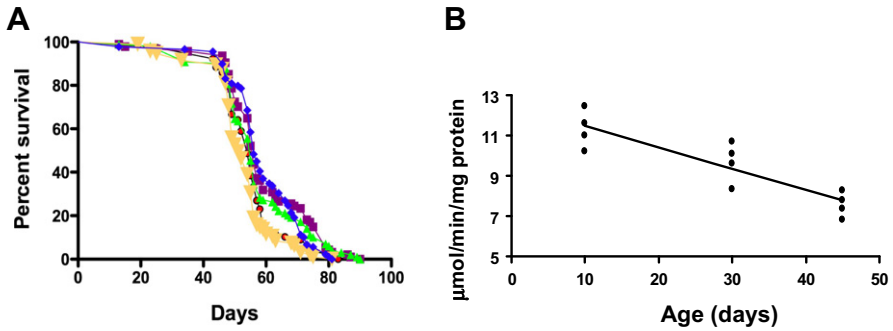

Fig. 1. (A) Survivorship curves of five separate groups of male *Drosophila melanogaster*, each from a different set of parents. After emergence from the pupa, flies were housed in 170 ml, 5.6 × 10.0 cm square bottom polyethylene bottles, with 125 flies per container, at 25 °C. (B) Cytochrome *c* oxidase activity at different ages. Values are based on data from three independent experiments. ANOVA Bonferroni (post-hoc) analysis indicated a significant decline (33%) in activity during 10–47 days of age ($n = 4$; $P < 0.05$).

3.3. Age-associated changes in protein levels of nuclear DNA-encoded CcO subunits

Mitochondrial proteins, resolved by SDS–PAGE, were stained with either Coomassie blue or probed with antibodies against specific CcO subunits. Pilot studies indicated that the commercially available antibodies against nuclear-encoded mammalian CcO subunits had little immunoreactivity with their counterparts in *Drosophila*. Consequently, *Drosophila*-specific antibodies were prepared, as described in Section 2. The Coomassie-stained gels showed ~20 distinct protein bands of variable densities. None of the bands exhibited a notable age-related alteration in staining intensity (Fig. 2A). Similarly, there were no discernable age-related changes in the immunodensities of porin or complex Vα per unit mitochondrial protein, confirming the equal loading of mitochondrial proteins from flies of different ages (Fig. 2B).

Age-related comparisons of the immunoreactivity of seven different CcO subunits, IV, Va, Vb, VIb, VIc, VIIc, and VIII at three different ages, indicated that the amounts of the immunoreactive proteins of all these subunits were lower in 47-day-old flies compared to 10-day-old flies; however, the magnitude of the decreases varied for different subunits, ranging from 11% to 40% (Figs. 2C and 3). The rank order of the age-associated declines among the seven subunits was: VIII > VIIc > VIb = Vb > VIc > Va > IV. Broadly, there were three different levels of age-related losses in the relative abundance of different CcO subunits: the smallest, ranging from 11% to 14%, occurred in subunits IV and Va; moderate declines of 24–30% were encountered for subunits VIc, Vb, VIb, and VIIc; and the severest attenuation, 40%, was in subunit VIII. While levels of the subunits decreased during both the first as well as the second half of the life span, the magnitude of the losses was significantly greater during the 30–47 day than 10–30 day interval. Statistical analysis of the data, using one-way ANOVA, indicated that the effects of age were significant ($P < 0.05$) for all the subunits. Post-hoc comparison showed that the significance of the age-related loss was: subunit Vb, $P < 0.01$; VIc, $P < 0.005$; and for all the other subunits, $P < 0.05$.

Altogether, results of this study indicate that CcO progressively loses activity as well as structural integrity during the aging process in *Drosophila*, which raises two broad issues, namely (i) what are the possible causes of the decline in activity and (ii) what may be the functional consequences of these changes. Differential losses in the relative abundance of CcO subunits during aging may be one of the factors for the loss of catalytic activity. CcO holoprotein is a homodimer and each monomer is composed of three mitochondrial DNA- and 10 nuclear DNA-encoded polypeptides in unimolar stoichiometric ratio [17]. While the mitochondrial DNA-encoded CcO subunits form the catalytic core of the holoenzyme, the nuclear DNA-encoded subunits control the assembly, activity and stability of the holoprotein (Fig. 4). We have previously reported that in *Drosophila* the mitochondrial DNA-encoded CcO

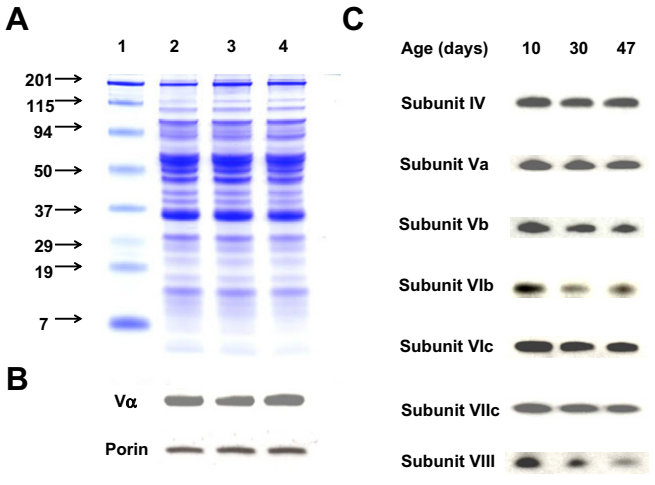

Fig. 2. Comparison of mitochondrial proteins at different ages. (A) Mitochondrial proteins (10 µg) were resolved on an SDS/polyacrylamide gel and stained with Coomassie blue. Lane 1 contains standard markers of known MW; lanes 2, 3, and 4 contain mitochondria from 10, 30, and 47 days of age. (B) Proteins from flies of different ages were transferred from the gel to a PVDF membrane and probed by anti-porin and anti-Vα-complex antibody demonstrating the equal loading of the proteins. (C) Representative immunoblots of different nuclear DNA-encoded cytochrome *c* oxidase subunits in mitochondria of flies of three different ages. $n = 5–7$ independently conducted experiments.

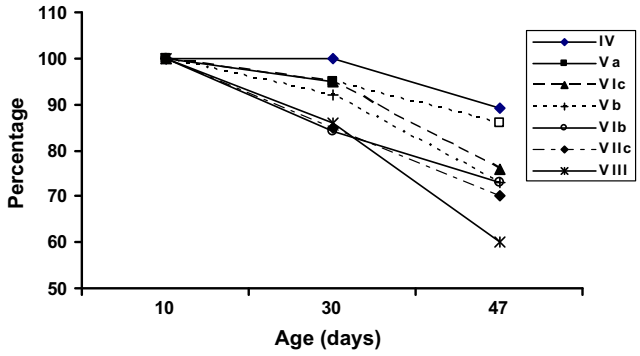

Fig. 3. Effect of age on the abundance of cytochrome *c* oxidase subunits in *D. melanogaster*. Results are means of 5–7 independent mitochondrial preparations. For clarity error bars are not indicated. The coefficient of variation ranged between 4% and 10%.

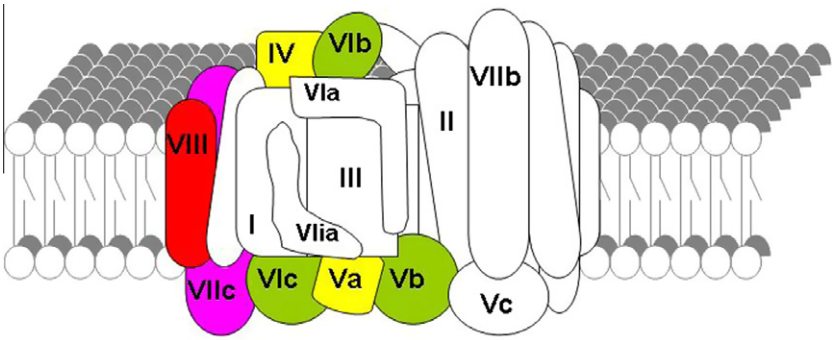

Fig. 4. A schematic sketch of the topography of constituent subunits of cytochrome *c* oxidase. (Adapted from [44]).

subunits I, II, and III undergo losses of, respectively, 15%, 43%, and 75%, in their abundance during aging [18]. Results of experimental manipulations have indicated that decreases in the transcript levels of specific nuclear-encoded CcO subunits affect CcO functions, such as the binding of cytochrome *c*, affinity for oxygen, transfer of electrons between redox centers, and coupling of electron transfer to proton translocation [16,17,23–26].

It is quite plausible that the functional impact of the selective losses of CcO subunits during aging would depend upon the specific role of the relevant subunit as well as the severity of its decrease in abundance (Fig. 4). The maximal loss (40%) of subunit abundance in *Drosophila* occurred for subunit VIII, whose function is presently unknown. Subunit VIIc, which decreased 30% during aging, is thought to interact with conserved residues in subunit I and is required for optimal functioning of the holoenzyme. Subunit VIb plays a structural role in stabilizing the native dimer, and modulates km for cytochrome *c* [16,24,27]. Subunit Vb facilitates assembly of the holoenzyme, enhances the binding affinity of subunit II for cytochrome *c*, and interacts with the regulatory subunit of protein kinase A, which modulates COX activity in a cAMP-dependent process [28]. Over-expression of CcO subunit Vb has been demonstrated to increase the tolerance of HeLa cells to hyperoxia [29]. Subunit Va, located adjacent to subunit IV, has been found in mammals to bind thyroid hormone 3,5-diiodothyronine, for which the likely functional analog in insects is juvenile hormone [16]. Such binding blocks ATP-mediated inhibition of holoenzyme activity. Subunit IV binds to subunits I and II on the matrix side and modulates electron transfer by affecting the heme a_3-Cu_B environment of subunit I. It has two ATP-binding sites, whose phosphorylation under high ATP/ADP conditions can lead to the allosteric inhibition of CcO activity due to an increase in K_m for cytochrome *c*. Knockdown studies have indicated that reduction in the amounts of subunits IV, Va, and VIa decreases the CcO holoprotein number as well the CcO-containing respiratory supercomplexes [30–32]. Although no cause and effect relationship can be established on the basis of present results, the observed age-related changes in the abundance of CcO subunits seem extensive and may have a variety of effects on the activity and structural stability of the holoenzyme.

Regarding whether the observed decrease in CcO activity is likely to have an impact on mitochondrial respiratory function, the classical view, based on studies on isolated mitochondria, was that the normal CcO activity greatly exceeds the level needed to support the maximal respiratory capacity of cells and that CcO activity exerted a "control strength" of only 0.15–0.20 over the respiratory rate [33,34]. However, studies on intact cultured cells have shown that CcO activity is the rate-limiting step in mitochondrial respiration and that maximal CcO activity exceeds the respiratory activity of the cells by merely ~20% [35,36]. Since the transfer of electrons from cytochrome *c* to molecular oxygen is coupled with the electrogenic transfer of protons across the inner mitochondrial membrane, a decrease in CcO activity would likely have a negative effect on the trans-membrane proton gradient, utilized by complex V for ATP synthesis. Furthermore, as CcO is both the terminal component of the electron transport chain (ETC) and its reduction of oxygen is essentially an irreversible reaction, a decrease in its activity may also potentially create a bottleneck effect on the turnover number of the entire electron transport chain [17]. At the organism level, loss of CcO activity and its putative effect on ATP synthesis is likely to have a broad effect on physiological fitness; for instance, the capacity of the flies for sustained flight gradually declines during aging to the point where they are unable to fly [8,37,38].

One of the most ubiquitous alterations during aging is the increase in the rate of mitochondrial production of H_2O_2, with concurrent accumulation of macromolecular oxidative damage [7]. Superoxide anion radical, the progenitor of H_2O_2, is formed by the auto-oxidation of semiubiquinone, associated with the mitochondrial respiratory complexes I and III [15]. A partial blockage of the electron transfers at the CcO terminus would tend to increase the reducing potential of the upstream components of the ETC, including ubiquinone. Experimental studies have shown that a KCN or azide induced decrease in CcO activity by a magnitude similar to that occurring during aging indeed causes a comparable increase in the rate of mitochondrial H_2O_2 generation [4,20].

The nature of the mechanisms that lead to the selective age-related losses in CcO subunits has not as yet been established. A decrease in the levels of specific mRNA transcripts during aging has been reported in the rat heart [39]. *Drosophila* mitochondrial proteins also exhibit an age-related increase in oxidative damage, manifested by adducts with the lipid peroxidation products, 4-hyroxynonenal and malondialdehyde [40]. Cardiolipin in the inner mitochondrial membrane has four fatty acyl chains that are mostly composed of the polyunsaturated fatty acid, linoleic acid (18:2), which is susceptible to the attacks by ROS. CcO activity has been reported to be affected by the cardiolipin content of the inner mitochondrial membrane [41]. Furthermore, peroxidation of cardiolipin can disturb the association between CcO subunits and cardiolipin molecules [42,43].

In conclusion, the results of this and our previous study [18] demonstrate that decline in CcO activity during aging is associated with selective losses in the levels of both nuclear and mitochondrial DNA-encoded CcO subunits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.009.

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