Effects of aging on activities of mitochondrial electron transport chain complexes and oxidative damage in rat heart

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Short title: electron transport chain complexes and aging

Summary

Mitochondrial dysfunction and accumulation of oxidative damage have been implicated to be the major factors of aging. However, data on age-related changes in activities of mitochondrial electron transport chain (ETC) complexes remain controversial and molecular mechanisms responsible for ETC dysfunction are still largely unknown. In this study, we examined the effect of aging on activities of ETC complexes and oxidative damage to proteins and lipids in cardiac mitochondria from adult (6-month-old), old (15-month-old) and senescent (26-month-old) rats. ETC complexes I-IV displayed different extent of inhibition with age. The most significant decline occurred in complex IV activity, whereas complex II activity was unchanged in old rats and was only slightly reduced in senescent rats. Compared to adult, old and senescent rat hearts had significantly higher levels of malondialdehyde, 4-hydroxynonenal (HNE) and dityrosine, while thiol group content was reduced. Despite marked increase in HNE content with age (25 and 76% for 15-mo and 26-mo old rats, respectively) Western blot analysis revealed only few HNE-protein adducts. The present study suggests that non-uniform decline in activities of ETC complexes is due, at least in part, to mitochondrial oxidative damage; however, lipid peroxidation products appear to have a limited impact on enzyme functions.

Key words: Aging, Heart, Mitochondria, Electron transport complexes, Oxidative damage

1. Introduction

The aging heart is characterized by many structural, physiological and biochemical changes. Numerous studies have suggested that age-associated deterioration in heart function can be related to oxidative damage by reactive oxygen species (ROS) produced during mitochondrial oxidative phosphorylation. Complex I and complex III of electron transport chain (ETC) have been recognized as two main sites for the generation of superoxide radical and subsequently other ROS (Barja 1999). As the major cellular source of ROS mitochondria are also the main target of their damaging effects (Endlicher *et al.* 2009). Age-related mutations of mitochondrial DNA, reduced mRNA expression and translation of genes coding for subunits of enzyme complexes were demonstrated in various tissues, including the heart (Mohamed et al. 2006, Preston *et al.* 2008). Besides the gene expression changes the post-translational oxidative modifications of proteins appear to be a key mechanism of age-associated oxidative injury. Protein lesions, manifested as carbonylation, nitration or tyrosine derivatization were shown to accumulate in different tissues during aging (Leeuwenburgh *et al.* 1997, Kanski *et al.* 2005, Choksi and Papaconstantinou 2008). Protein lesions can result also from the formation of adducts with lipid peroxidation (LPO) products, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Choksi and Papaconstantinou 2008, Yarian *et al.* 2006). Many studies have also shown that accumulation of oxidatively modified molecules is coupled to impairment in mitochondrial function. Declines in activities of the ETC complexes (Kumaran *et al.* 2004, Rodríguez *et al.* 2007) and enzymes of citric acid cycle (Yarian *et al.* 2006, Kumaran *et al.* 2005) as well as cytochrome c release from mitochondria (Phaneuf and Leeuwenburgh 2002) of aged heart have been demonstrated and proposed to contribute to dysfunction and death of cardiac myocytes. However, no consistent pattern of activity changes can be discerned. Enzymes were shown to be differentially affected and there are contradictory data on age-related changes of individual ETC complexes I-IV or citric acid cycle enzymes. Moreover, there are also studies showing modest or no changes in mitochondrial enzyme activities or oxidative damage in aged heart (Miró *et al.* 2000, Davies *et al.* 2001).

Therefore, the aim of this study was to examine the effect of aging on activities of ETC complexes in cardiac mitochondria. A subsequent aim was to evaluate the hypothesis that advancing age is associated with gradual accumulation of oxidative damage that contributes to mitochondrial dysfunction. We hypothesized that ROS-induced damage to mitochondria occurs via both LPO-mediated and direct modifications of mitochondrial proteins. To assess potential roles of these two mechanisms we have determined oxidative modifications of lipids and proteins in the rat heart during the aging process. The results obtained suggest that direct protein modification may play a more important role in age-associated decline in mitochondrial function than LPO.

2. Materials and methods

2.1. Animals

Fifteen male Wistar rats (supplied by IEP SAS Dobra Voda, Slovakia) were divided into three groups (5 rats per group) according to age, as adult (6-month-old), old (15-month-old) and senescent (26-month-old). The animals were maintained as described previously (Kaplan *et al.* 2007a). All experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by The US National Institute of Health (NIH publication NO 85-23, revised 1996), and the ethical guidelines of the Jessenius Faculty of Medicine.

2.2. Isolation of cardiac mitochondria

The animals were sacrificed by decapitation after anesthetization by halothane. After cannulation of the aorta the hearts were immediately washed with physiological solution and stored at -80 °C until used. Frozen powdered tissue of the whole heart (about 1g) was thawed in 10 volumes of ice-cold homogenization buffer (30 mM KH_2PO_4, 5 mM EDTA, 0.3 M sucrose, pH 7.0) with 0.3 mM phenylmethylsulfonyl fluoride (PMSF) and homogenized in Potter-Elvehjem homogenizer. Cardiac mitochondrial fraction was isolated from individual tissue homogenates by differential centrifugation as previously described (Babusikova *et al.* 2004).

2.3. Determination of enzyme activities

Complex I (NADH-ubiquinone oxidoreductase)

The oxidation of NADH by complex I was recorded using the ubiquinone analogue decylubiquinone as electron acceptor (Nulton-Persson and Szweda 2001). The basic assay medium (35 mM KH_2PO_4, 5 mM $MgCl_2$, and 2 mM KCN, pH 7.2) was supplemented with 5 μM antimycin A, 60 μM decylubiquinone and 0.1 mM NADH in final volume of 1 ml. The enzyme activity was measured by starting the reaction with 50 μg of mitochondrial protein at 30ºC. The decrease in absorption due to NADH oxidation was measured at 340 nm using molar absorption coefficient $\varepsilon=6.2\ mM^{-1}cm^{-1}$.

Complex II (succinate-ubiquinone oxidoreductase or succinate dehydrogenase, SDH)

Succinate dehydrogenase activity was measured according to Powell and Jackson (2003) as the rate of 2,6-dichlorophenolindophenol (DCIP) reduction at 600 nm ($\varepsilon=21\ mM^{-1}cm^{-1}$) upon addition of 0.2 M KH_2PO_4 (pH 7.6), 0.1 M NaCN, 0.02M phenazine methosulfate, 0.5 M succinate and 1mM DCIP to mitochondria (0.15 mg protein per ml) incubated at 30ºC.

Complex III (ubiquinol- cytochrome c reductase)

Activity of cytochrome c reductase was determined as the rate of antimycin A-dependent reduction of cytochrome c at 550 nm (ε=18.5 $mM^{-1}cm^{-1}$) (Nulton-Persson and Szweda 2001). Mitochondria (0.05 mg protein per ml) were incubated at 30ºC in medium containing 35 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM KCN and 0.05% Triton X-100, pH 7.2 and reaction was started with 60 μM reduced decylubiquinone and 50 μM of cytochrome c.

Complex IV (cytochrome c oxidase, COX)

Activity of cytochrome c oxidase was determined spectrophotometrically by monitoring the rate of cytochrome c oxidation at 550 nm (ε=19.6 $mM^{-1}cm^{-1}$) as previously described (Racay *et al.* 2009). Mitochondria (0.05 mg protein per ml) were incubated at 30ºC in medium containing 50 mM Tris-HCl (pH 8.0), 0.01% (w/v) n-dodecyl β-D-maltoside and reaction started with 50 μM of reduced cytochrome c.

Citrate synthase

Citrate synthase activity was measured spectrophotometrically by monitoring formation of CoA-SH with 2,2-dithiobisnitrobenzoic acid (DTNB). Mitochondria (0.04 mg protein per ml) were incubated at 25ºC in assay buffer containing 0.3 mM acetyl CoA and 0.1 mM 2,2-dithiobisnitrobenzoic acid (DTNB). The reaction was started by the addition of 0.5 mM oxaloacatate and reduction of DTNB was followed at 412 nm (ε=13.6 $mM^{-1}cm^{-1}$).

2.4. Western blot analysis

For detection of HNE-modified proteins the tissue homogenates (aliquots of 20 μg proteins) were separated on 10% SDS-polyacrylamide gels under denaturing conditions and then transferred to nitrocellulose membranes using Mini Trans-Blot cell. Membranes were blocked in 5% milk with Tris-buffered saline Tween-20 (TBS-T) overnight and incubated with monoclonal anti-HNE (Oxis International Inc.) for 90 min. They were then washed with TBS-T, incubated with biotinylated anti-mouse secondary antibody (Vector, 1:10 000) for 90 min, washed with TBS-T and incubated for 30 min with ABC (Vector, 1:1000). After last washing with TBS-T, immunoreactive proteins were visualized using the enhanced chemiluminescence reagent (Amersham) and quantified using NIH image software.

2.5. Lipid peroxidation

Conjugated diene formation was estimated from the absorbance ratio A_{233nm}/A_{215nm} of mitochondria (20 μg/ml) dispensed in 10 mM phosphate buffer containing 1% Lubrol (Kaplan *et al.* 2003).

Levels of LPO products, malondialdehyde and 4-hydroxynonenal, were measured according to LPO-586 Kit (Oxis International) in heart tissue homogenates.

2.6. Total thiol group content

Total thiol group content in cardiac mitochondria (aliquots of 0.15 mg proteins) was determined spectrophotometrically as described previously (Sivoňová *et al.* 2007). Samples were incubated in medium containing 30 mM imidazole (pH 7.4), 5 mM EDTA, 0.4 mM 2,2-dithiobisnitro-benzoic acid (DTNB). After 10 min incubation at room temperature the sample absorbance at 412 nm was measured together with the absorbance of reagent blank. The thiol group content was calculated using molar absorption coefficient ε=13,600 $M^{-1}cm^{-1}$.

2.7. Fluorescence studies

All fluorescence measurements were performed in solution containing 50 μg of mitochondrial protein per ml, 10 mM HEPES and 100 mM KCl, pH=7.0 at room temperature on Shimadzu RF-540 spectrophotofluorimeter as shown previously (Kaplan *et al.* 2003). Emission spectra of dityrosine, a product of tyrosine oxidation, were recorded in range 380-440 nm (5 nm slit width) at excitation wavelength 325 nm (5 nm). Emission spectra (425-480 nm) of lysine conjugates with LPO-end products were recorded after excitation at 365 nm (5 nm). Binding of fluorescent probe 1-anilino-8-naphtalenesulfonate (ANS) to mitochondrial membranes was measured following 10 min incubation of samples with the probe at 480 nm (5 nm) with excitation at 365 nm (5 nm).

2.8. Statistical analysis

Data are expressed as arithmetic means±SEM. One-way analysis of variance with post-hoc comparisons by Student-Neuman-Keuls test was carried out to test for differences among groups. A value of $p<0.05$ was considered to be statistically significant.

3. Results

3.1 Body weight and heart weight

The effects of aging on body and heart weights are shown in Table 1. Both, body and heart weights of aged rats were significantly greater then those of adult rats. However, heart weight-to-body weight ratio, an indicator of cardiac hypertrophy, was unchanged.

3.2. Age-related changes in activities of electron transport chain complexes

To evaluate the effect of aging on mitochondrial function we measured enzyme activities of complexes I-IV in cardiac mitochondria isolated from adult (6-month old), old (15-month old) and senescent (26-month old) rats (Fig. 1). There were no significant difference in rotenone-sensitive complex I activities between adult and old rats, but the activity of senescent rats decreased to 76±1% of adult ($p<0.01$). Also complex II activity was significantly changed only in senescent rats, however, the decrease was less pronounced (to 86±3% of adult, $p<0.01$) than that of complex I. On the other hand, antimycin A-sensitive complex III activity decreased significantly in both old (to 90±4% of adult, $p<0.05$) and senescent rats (to 83±2% of adult, $p<0.01$). The most pronounced age-related changes were observed in KCN-sensitive complex IV (COX) activity, comparing to adult the activity decreased to 79±3% ($p<0.01$) in old and 63±4% ($p<0.001$) in senescent rat heart. To avoid possible effects of different yield or stability of mitochondria on aging activities of complexes were normalized relative to citrate synthase activity (Davies *et al.* 2001). The age-related changes in normalized activities were qualitatively similar to those of absolute activities but were more pronounced (Fig. 2) since citrate synthase activity progressively increased with age (6-month old 309±10, 15-month old 364±13 and 26-month old 403±13 nmol/min/mg protein). In contrast to absolute values, normalized activities of complexes I and II in old rats were significantly lower than those of adult rats.

3.3 Mitochondrial oxidative damage during aging

To assess if oxidative stress contributes to inhibition of enzyme activities the thiol group content and dityrosine fluorescence were measured as markers of protein oxidative damage. As shown in Fig. 3, total thiol group content decreased by 20±1% ($p<0.001$) at the age of 15 months and by 30±1% ($p<0.001$) at the age of 26 months. Aging significantly affected also protein dityrosine levels; compared with adult rats the level increased by 23±2% at 15 months and by 29±1% at 26 months (Fig. 3). These lesions were not accompanied by structural changes of proteins and/or membrane surfaces, since the binding of ANS probe was not significantly altered by aging (Table 2).

Effect of aging on lipid peroxidation was assessed by measurements of conjugated dienes and LPO end-products. All parameters increased progressively with age (Table 2, Fig. 4). Compared to adult rats, MDA level increased by 13±7% (NS) and 30±4% ($p<0.05$) at the age of 15 and 26 months, respectively (Fig. 4). HNE level increased by 25±7% ($p<0.01$) at 15months and further increased by 76±3% ($p<0.001$) in 26-month old rats (Fig. 4). To see if accumulation of reactive aldehydes results in protein modification we measured fluorescence spectra corresponding to conjugates of LPO products with amino groups of proteins. As shown in Table 2, fluorescent products also significantly increased with aging. To further unravel the contribution of LPO to protein modifications, formation of HNE-protein adducts was investigated by Western blot analysis. Using specific anti-HNE antibodies we detected three bands with HNE-modified proteins (Fig. 5A). Densitometric analysis of Western blots suggests that these proteins exhibit different pattern of age-dependent changes (Fig. 5C). While protein(s) with molecular weight ~57 kDa showed progressive formation of HNE adducts with age, HNE modifications of low molecular weight proteins (19-23 kDa) culminated at the age of 15 months. To control a loading error, β-actin was used as an internal control. There were no differences in content of this housekeeping-gene coded protein among adult, old and senescent rat hearts (Fig. 5B), suggesting that Western blot analysis of HNE-protein adducts was not affected by experimental errors.

4. Discussion

Results of this study demonstrate that cardiovascular aging is associated with accumulation of oxidative damage to lipids and proteins and progressive decline in activities of mitochondrial enzymes. However, the extent of inhibition of individual enzymes was different. While there were relatively small age-related changes in complex II and III activities the decline in complex IV activity was twice as big as those of complexes II and III.

Effect of aging on mitochondrial enzymes in rodent hearts was examined in a number of studies, but with contradictory findings. For example, 42-46% reduction in complex I activity was demonstrated in hearts of 24-month-old rats (Preston *et al.* 2008, Kumaran *et al.* 2004), whereas other studies have reported no age-related decrease in activity (Davies *et al.* 2001, Cocco *et al.* 2005). Our results are in agreement with recent study, which showed small but significant ~9% decrease in activity of 12-14- and ~13% decrease in 20-22-month old mouse heart mitochondria (Choksi and Papaconstantinou 2008). Surprisingly, literature data on complex II show declined (Kumaran *et al.* 2004, Cocco *et al.* 2005) unchanged (Preston *et al.* 2008) and also increased (Choksi and Papaconstantinou 2008, Davies *et al.* 2001, Kwong and Sohal 2000) activity in aged hearts.

Activities of complexes III and IV have been also shown to decline (Kumaran *et al.* 2004, Kwong and Sohal 2000), remain unchanged or increased (Choksi and Papaconstantinou 2008, Davies *et al.* 2001, Cocco *et al.* 2005) during aging. Our data are similar to those reported by Kumaran *et al.* (2004) who also showed age-dependent decrease in all activities, although the pattern and extent of inhibition was different. To avoid species- and tissue-specific changes we compared our results only to literature data on rodent hearts. Therefore, inconsistencies among the studies can be mainly due to differences in the experimental approaches such as mitochondrial populations or electron donors/acceptors used for measurements of complex activities (Kwong and Sohal 2000, Fannin *et al.* 1999) and differences in aging groups. In some studies (Davies *et al.* 2001, Cocco *et al.* 2005) the aged groups (24-28 mo) were compared to young 2-3-month-old rats. This may lead to underestimation of the observed aging effect, since activities of ETC complexes in hearts from young rats might be lower than those observed in adult 6-month-old rats. In our study, cardiac mitochondria were isolated from rats anesthetized with 2% halothane, a known inhibitor of ETC complexes (Hanley *et al.* 2002). However, this mechanism most likely did not affect our findings since this volatile anesthetic was eliminated during heart perfusion and isolation of mitochondria. Regardless of the contradictory data on activity changes of individual ETC complexes, majority of studies demonstrates some age-related impairment in ETC. Such non uniform inhibition of ETC complexes can result in altered electron flow through the chain, impaired ATP production and finally in altered cardiomyocyte function during aging.

In addition to altered energy transduction, imbalance among ETC complexes may cause increased electron leakage from ETC and oxidative stress. Under normal conditions, ETC produces low levels of ROS, which are eliminated by scavenging mechanisms so that no substantial oxidative damage occurs. ROS, elevated due to imbalance in ETC, can attack molecules near the sites of their generation, thus strengthening ETC dysfunction. Accumulation of oxidative damage during aging observed in our study demonstrates that oxidative stress has indeed occurred in mitochondria. Mitochondria isolated from old and senescent rats showed lower thiol group and increased dityrosine contents, indicating age-related modifications of mitochondrial proteins. These results are consistent with previously reported data showing age-related changes in protein dityrosine, nitrotyrosine, carbonyl and thiol group content (Leeuwenburgh *et al.* 1997, Choksi and Papaconstantinou 2008, Babusikova *et al.* 2004, Cocco *et al.* 2005) and differs from those showing no protein oxidative damage in heart mitochondria (Davies *et al.* 2001). Function of ETC complexes depends also on structural integrity of membrane lipid bilayer. Increases in three biomarkers of LPO, conjugated dienes, MDA and HNE, in 15- and 26-month old rats indicate that aging is associated with significant lipid oxidative damage. Although elevated lipid peroxidation in myocardial aging has been demonstrated in several studies (Cocco *et al.* 2005, Judge et al. 2005), the consequences of this process are not clear, since several studies have failed to show correlation between LPO and inhibition of ETC complexes (Miró *et al.* 2000, Zhang *et al.* 1990). We have not tested the role of LPO in detail but unchanged binding of ANS probe to mitochondria suggest that aging is not accompanied with substantial conformational changes in membranes. Although generalized LPO appears not to be involved in functional derangement of mitochondria, processes, such as peroxidation of specific membrane lipid components (Lesnefsky and Hoppel 2008, Petrosillo *et al.* 2009) or modification of proteins by reactive aldehydes are believed to be involved in the inhibitory mechanism. The MDA levels and their age-dependent increase observed in our study (~0.6-0.8 nmol/mg protein) were lower than those observed earlier (~1.2-3.6 nmol/mg protein) (Cocco *et al.* 2005), possibly because instead of nonspecific TBARS method we used method for specific determination of free MDA. Both findings are, however, more than 2-3 orders lower than those inhibiting ETC complexes. Using isolated rat liver mitochondria Long *et al.* (2006) have shown that ETC complexes I and II are significantly inhibited by exogenous MDA from the concentrations of 800 nmol/mg protein and complexes III and IV were not inhibited even at 6.4 μmol/mg protein. Data on HNE content and its age-related changes in cardiac tissue are lacking. Our study shows that in adult hearts the HNE content is similar to MDA content but it increases more substantially with age; in 26-month old rats it was almost twice as big as in 6-month old rats. Several studies have shown that ETC complex activities were reduced when mitochondria were treated with 50-200 μM HNE (see Long *et al.* 2006), but few studies have demonstrated inhibition of enzymes and respiration at much lower concentrations (Kaplan *et al.* 2007b, Chen *et al.* 1998, Humphries *et al.* 1998). Previously we have shown (Kaplan *et al.* 2007b) that complex IV activity is significantly inhibited when mitochondria were shortly exposed to 1 μM HNE. This concentration corresponds to 2 nmol HNE/mg protein and is close to HNE level, which we found in aged hearts. The ability of HNE to affect mitochondrial proteins was confirmed by measurements of HNE-protein adducts. Our results are in agreement with previous studies showing only few HNE-modified proteins in cardiac mitochondria (Choksi and Papaconstantinou 2008, Judge *et al.* 2005, Suh *et al.* 2003). In addition, HNE adducts with low-molecular weight proteins did not increase progressively with age, but peaked in 15-month- and decreased back in 26-month-old rats. Similar profile for some proteins was observed by Choksi and Papaconstantinou (2008). In contrast to their study we have not identify HNE-modified proteins, therefore the effect of this modification on ETC is unknown. Nevertheless, observation that only few mitochondrial proteins showed age-dependent HNE modification suggests that this is not a common mechanism for inhibition of ETC complexes with age.

In conclusion, our study provides further evidence that aging results in non-uniform decline in activities of ETC complexes, which may be due, at least in part, to mitochondrial oxidative stress. Oxidative damage to ETC may occur directly by reaction with ROS or indirectly through LPO products, however, MDA and HNE appear to have a limited impact on enzyme functions. Further studies are needed to identify proteins subjected to oxidative modifications and the impact of these modifications on mitochondrial function.

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Tables

Table 1 Effect of aging on body weight, heart weight and heart to body weight ratio

	Body weight (g)	Heart weight (g)	Heart/body weight (mg/g)
6-months old	352±18.9	1.06±0.08	3.02±0.30
15-months old	435±20.6***	1.25±0.10**	2.88±0.31
26-months old	435±27.9***	1.31±0.10**	3.02±0.43

Values are expressed as means±SEM of 5 experiments. ** P<0.01, *** P<0.001; significantly different as compared to 6-months old rats.

Table 2 Effect of aging on lipid peroxidation and binding of ANS probe

	Conjugated dienes	Fluorescence intensity of	
	A_{233nm}/A_{215nm}	Lys-LPO	ANS
6-months old	0.290±0.005	30.3±1.2	40.7±1.8
15-months old	0.346±0.005**	39.1±0.9***	37.9±1.3
26-months old	0.448±0.003***	54.3±2.5***	43.9±2.5

Values are expressed as means±SEM of 5 experiments. ** P<0.01,

*** P<0.001; significantly different as compared to 6-monthds old rats.

Figure legends

Fig. 1 Effect of aging on activities of ETC complexes in rat cardiac mitochondria. Values are given as mean±SEM of 5 experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; significantly different as compared to 6-months old rats

Fig. 2 Effect of aging on activities of ETC complexes related to citrate synthase activity. Values are given as mean±SEM of 5 experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; significantly different as compared to 6-months old rats

Fig. 3 Effect of aging on thiol group content and dityrosines in cardiac mitochondria. Values are given as mean±SEM of 5 experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; significantly different as compared to 6-months old rats

Fig. 4 Aging-associated changes in MDA and HNE contents in rat heart homogenates. Values are given as mean±SEM of 4 experiments. * $P<0.05$, *** $P<0.001$; significantly different as compared to 6-months old rats

Fig. 5 Aging-associated changes in HNE-modified proteins in rat heart homogenates. Representative Western blot of HNE-protein adducts (A), actin (B) and HNE-modified protein contents expressed as a percentage of the average value in 6-monthds old rats (C). For detection of HNE-modified proteins 20 μg proteins were applied on SDS-polyacrylamide gels Values are given as mean±SEM of 3 experiments. * $P<0.05$, *** $P<0.001$; significantly different as compared to 6-months old rats.

Fig. 1

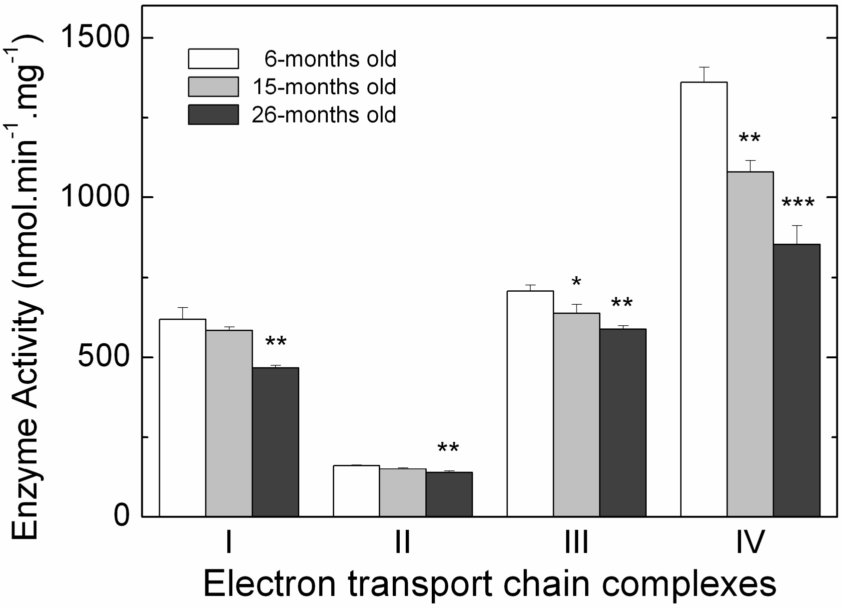

Enzyme Activity (nmol.min^{-1}.mg^{-1})
1500
1000
500
0
6-months old
15-months old
26-months old
**
***
*
**
**
**
I
II
III
IV
Electron transport chain complexes

Fig. 2

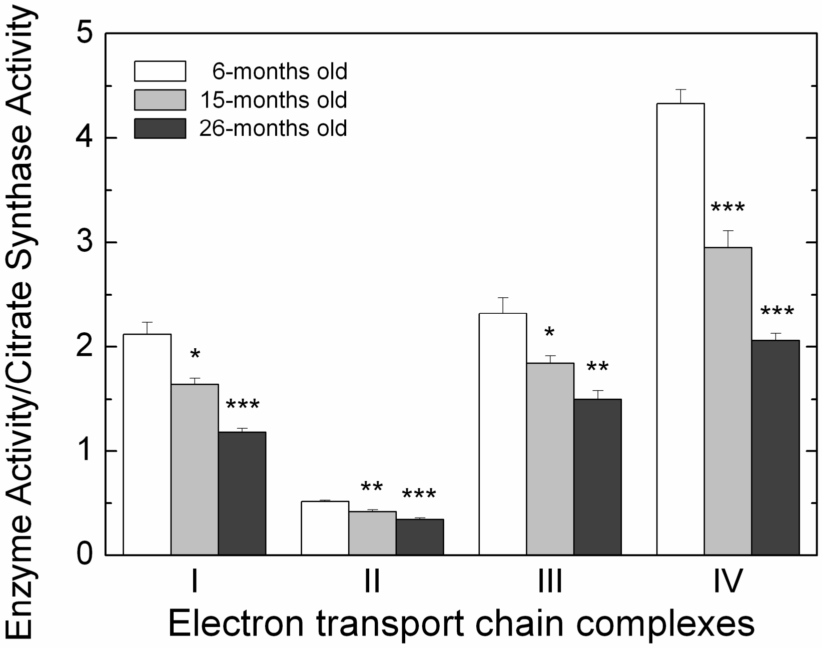

Enzyme Activity/Citrate Synthase Activity
5
4
3
2
1
0
6-months old
15-months old
26-months old
*
***
**
***
*
**
***
***
I
II
III
IV
Electron transport chain complexes

Fig. 3

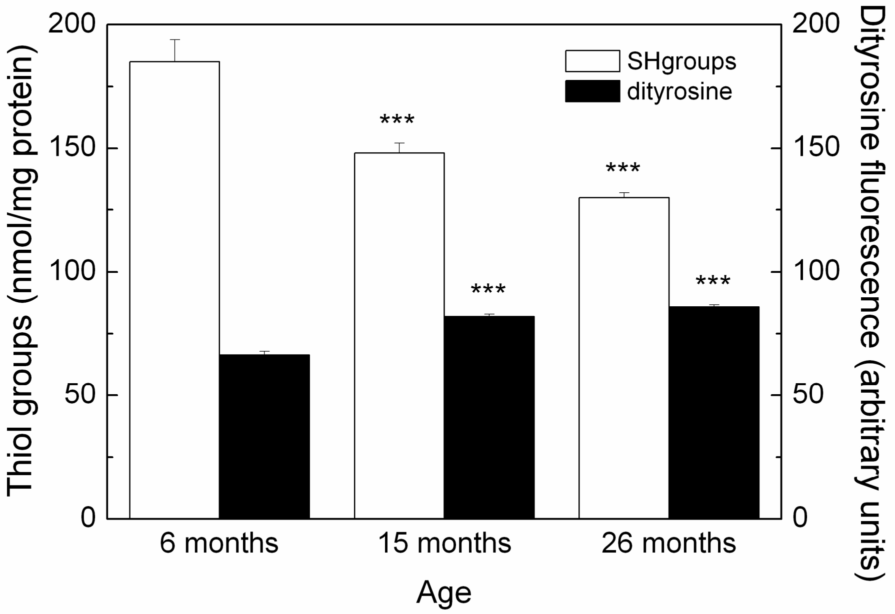

200
150
100
50
0
Thiol groups (nmol/mg protein)
SHgroups
dityrosine
***
***
***
***
6 months
15 months
26 months
Age
Dityrosine fluorescence (arbitrary units)

Fig. 4

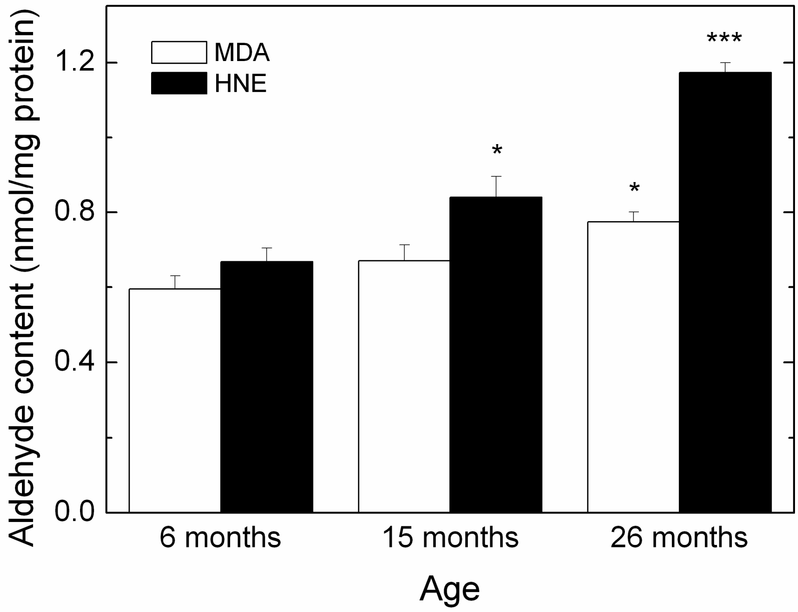
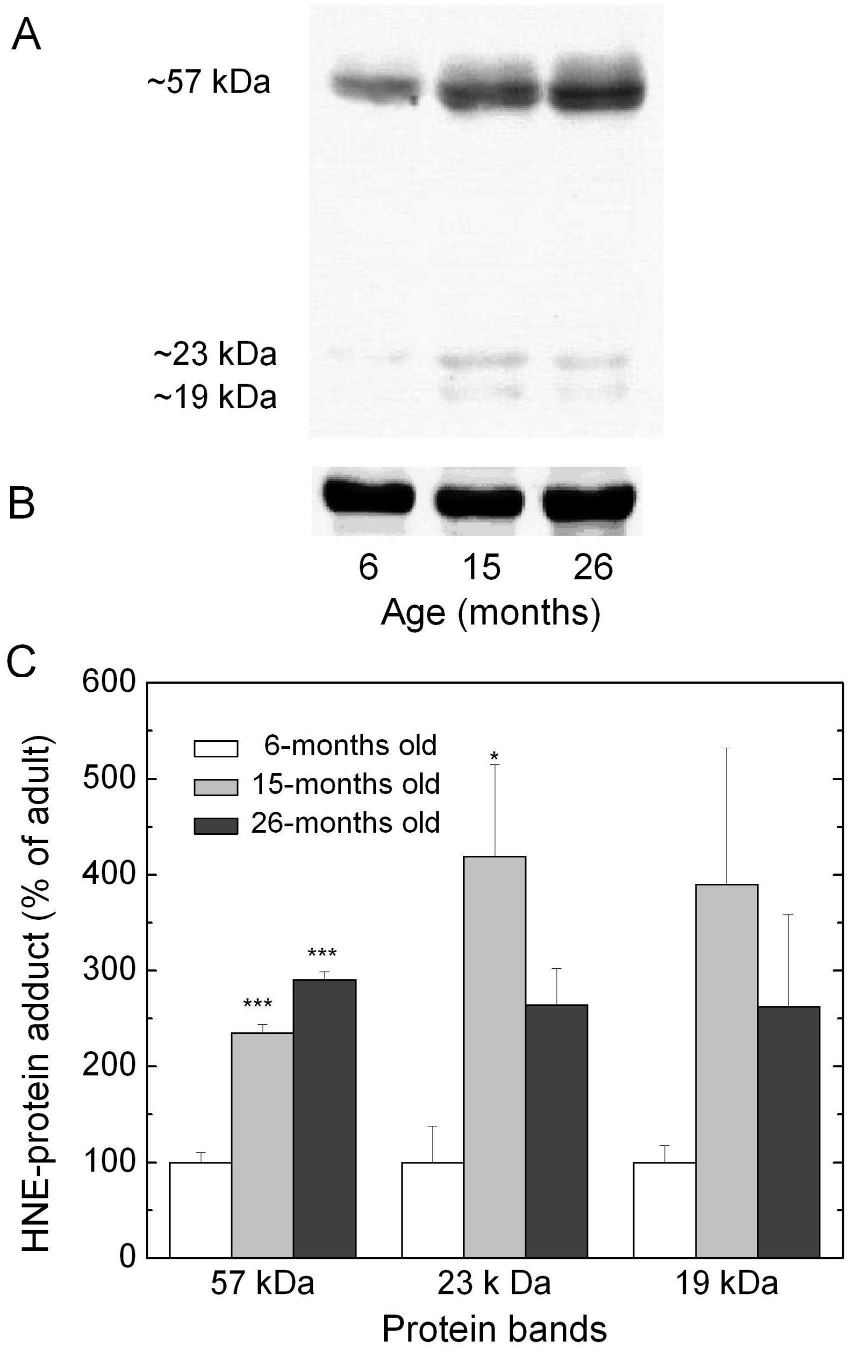

1.2
0.8
0.4
0.0
Aldehyde content (nmol/mg protein)
MDA
HNE
*
*
***
6 months
15 months
26 months
Age

Fig. 5


A
~57 kDa
~23 kDa
~19 kDa
B
6
15
26
Age (months)
C
HNE-protein adduct (% of adult)
0
100
200
300
400
500
600
6-months old
15-months old
26-months old
***
***
*
57 kDa
23 k Da
19 kDa
Protein bands