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MOLECULAR ANALYSIS OF THE ROLE OF MITOCHONDRIA IN AGING AND LONGEVITY

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MOLECULAR ANALYSIS OF THE ROLE OF MITOCHONDRIA IN AGING AND LONGEVITY

by

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To my lovely wife, my precious daughter, and my fine parents

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MOLECULAR ANALYSIS OF THE ROLE OF MITOCHONDRIA IN AGING AND LONGEVITY

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The Free Radical Theory of Aging states that there is an increase in reactive oxygen species (ROS) production with aging leading to increase in tissue dysfunction. Mitochondria are the major source of ROS, which damages essential macromolecules by oxidative modification and may lead to mitochondrial dysfunction. Accumulation of oxidative damage caused by ROS has been implicated as a major causal factor in the age-associated decline in tissue function and implicated in many age-associated diseases and sarcopenia. Mitochondrial electron transport chain (ETC) complexes I and III are the principle sites of ROS production, and oxidative modifications to their complex subunits inhibit their in vitro activity. Therefore, we hypothesize that mitochondrial complex subunits are primary targets for oxidative damage which may impair their structure/function leading to mitochondrial dysfunction associated with aging.

In addition, numerous studies have identified long-lived mutant mice (*i.e.*, Ames dwarf mice) that suggest that their longevity correlates with oxidative stress resistance. It has not yet been determined whether the mutant mice have inherently lower levels of ROS and whether there are changes in the electron transport chain function compared to wild-type. Therefore, we hypothesize that the dwarf mice have lower levels of ROS-generated lipid peroxidation markers and there are age-related changes in ETC function from tissues of dwarf and wild-type that may play a role in longevity.

In our studies, we have developed a novel methodology for detecting oxidative modification markers using bovine heart mitochondria. In addition, we found that in various mouse tissues ETC proteins are specifically targeted for oxidation and in most cases increased oxidative modifications with aging correlate with decreased enzymatic activities. Thus, we propose that the specificity of the oxidative modification may play a key role in predicting the consequences of ROS-mediate damage. The results from long-lived Ames dwarf mice show that they have lower levels of ROS markers suggesting delayed aging characteristics and except for kidney very little changes in ETC function are noted. Therefore, our studies provide important insight into physiological effects of oxidative modifications on mitochondrial function as well as role of oxidative stress markers in aging and longevity.

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ACAD1	Acyl-CoA dehydrogenase 1
ACADL	Acyl-CoA dehydrogenase long chain
ACADV1	Acyl-CoA dehydrogenase V1
ACOX1	Acyl-CoA oxidase 1
ALDH2	Aldehyde dehydrogenase 2
ANT	Adenine nucleotide translocator
ATP5A1	Complex V α chain
ATP5B	Complex V β chain
ATP5C1	Complex V γ polypeptide
BN-PAGE	Blue-native polyacrylamide gel electrophoresis
CI	Complex I
CII	Complex II
CIII	Complex III
CIV	Complex IV
CV	Complex V
CS	Citrate synthase
CoQ	Coenzyme Q
COX1	Cytochrome c oxidase subunit 1
COX2	Cytochrome c oxidase subunit 2
COX4	Cytochrome c oxidase subunit 4
Cyt	Cytochrome
DECR1	2,4-Dienoyl CoA reductase 1
DNP	2,4-Dinitrophenylhydrazone
DNPH	2,4-Dinitrophenylhydrazine
DW	Ames dwarf
ETC	Electron transport chain
FH1	Fumarate hydratase 1
GGT1	Gammaglutamyltransferase
HNE	4-Hydroxynonenal
IDH2	Isocitrate dehydrogenase 2
IsoP	F_2-isoprostane
ISP	Rieske iron-sulfur protein
MALDI-TOF	Matrix-assisted laser disorption ionization – time of flight
MDA	Malondialdehyde
MDH2	Malate dehydrogenase 2
MPP	Mitochondrial processing peptidase
NDUFC2	NADH dehydrogenase 14.5 KD subunit
NDUFS1	NADH dehydrogenase Fe-S subunit 1
NDUFS2	NADH dehydrogenase Fe-S subunit 2
NDUFS4	NADH dehydrogenase 18 KD IP subunit

NDUFV1	NADH dehydrogenase flavoprotein subunit 1
ROS	Reactive oxygen species
SDHA	Succinate dehydrogenase subunit 1
SDHB	Succinate dehydrogenase subunit 2
UQCRC1	Core 1 subunit
UQCRC2	Core 2 subunit
UQCRFS1	Rieske iron-sulfur protein
VDAC	Voltage-dependent anion channel
WT	Ames wild-type

CHAPTER I

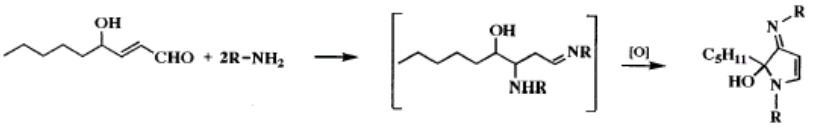
INTRODUCTION

OXIDATIVE STRESS, ROS, AND MOLECULAR MARKERS OF AGING

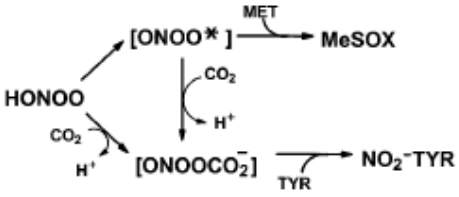
Chronic oxidative stress is a major characteristic of aging, the age-related decline in tissue function and age-associated diseases. The Free Radical Theory of Aging proposes that increasing oxidative stress resulting from increasing mitochondrial dysfunction is a basic mechanism of mammalian aging (Harman, 1956; Harman, 1972). In addition, mitochondria are a major source of ROS production and oxidative stress in the cell and during the aging process they are thought to play a key role in the age-associated decline in tissue function (Finkel and Holbrook, 2000; Huang and Manton, 2004; Lenaz, 1998). Mitochondria are central in cellular metabolism and energy production. In oxidative stress and aging, both metabolism and energy production are decreased (Lenaz, 1998; Wei and Lee, 2002). In addition, oxidative damage to mitochondria increases with age (Cadenas and Davies, 2000; Lee and Wei, 2001; Van Remmen and Richardson, 2001; Wei et al., 1998). Proteins such as aconitase and adenine nucleotide translocator are involved in mitochondrial metabolism and in transport of newly synthesized ATP to the cytoplasm. Both of these proteins have been shown to be oxidized and the degree of modification directly correlates with their impaired enzymatic activities and functional capabilities (Yan et al., 1997; Yan and Sohal, 1998). In addition to these key mitochondrial proteins, oxidative damage to the mitochondrial ETC complexes themselves during aging and oxidative stress may also contribute to mitochondrial dysfunction (Cadenas and Davies, 2000; Ishii et al., 1998; Sandhu and Kaur, 2003; Yan et al., 2000). Although, several *in vitro* experiments have shown that oxidative stress induced in mitochondria leads to a decline in the activities of ETC complexes (Chen et al., 1998; Chen et al., 2001; Lucas and Szweda, 1998; Morikawa et al., 1997; Picklo et al., 1999), the subunits that are oxidatively damaged have not been

identified. Furthermore, such protein modifications may lead to increased production of ROS from ETC complexes and cause more oxidative damage producing a deadly cycle resulting into the accumulation of mitochondrial dysfunction. The mitochondrial ROS is thought to be produced by *in vivo* electron leakage from ETC complexes during normal respiration, particularly from Complex I and Complex III (Chen et al., 2003; Lenaz et al., 2002; Liu et al., 2002). Although free radicals from these two complexes have been identified by electron paramagnetic resonance (Han et al., 2001; Lenaz, 1998; Yano et al., 2000), the short half-life of many of these radicals makes their accurate measurement difficult. In contrast, the resultant covalent modifications of macromolecules, such as proteins, caused by reactions with free radicals are more stable and easily detectable, and thus can be used as molecular markers of oxidative stress (Choksi et al., 2007; Wei, 1998). The relative abundance of modified proteins has, therefore, been used by our laboratory to indicate the level of oxidatively damaged macromolecules that accumulate in aged tissues. Protein modifications caused by ROS include the formation of lipid peroxidation adducts (4-hydroxynonenal or HNE and malondialdehyde or MDA) on lysine, histidine and cysteine, nitration of tyrosine and cysteine, and carbonylation of lysine, arginine, proline, and threonine (Beal, 2002; Berlett and Stadtman, 1997; Uchida and Stadtman, 1992a; Uchida and Stadtman, 1992b) (Figure 1.1). Oxidatively damaged proteins have been detected by immunoblotting using antibodies specific for these modifications and subsequently identified by mass spectrometry in our laboratory (Choksi et al., 2004; Choksi et al., 2007; Rabek et al., 2003). In addition, such oxidative modifications to proteins can result in reduction of normal function associated with aging and age-associated diseases (An et al., 1996; Beal, 2002; Berlett and Stadtman, 1997; Choksi et al., 2007; Stadtman, 2001; Yarian et al., 2005). Recently, numerous studies have also shown that increased mitochondrial dysfunction may be the most important mechanism in muscle sarcopenia, one of the first physiological signs of normal aging in humans (Kamel, 2003; Marzetti and Leeuwenburgh, 2006; Roubenoff, 2000; Volpi et al., 2004).

(A) HNE



(B) Nitration



(C) Carbonylation

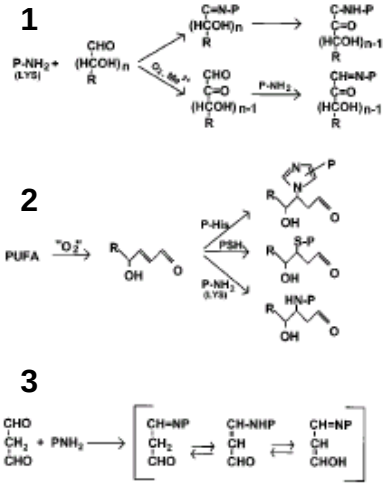


Figure 1.1. Molecular markers of ROS

(A) HNE fluorophore adduct is formed on lysines (Tsai et al., 1998). (B) Tyrosine nitration and methionine oxidation is caused due to reactive nitrogen species (Berlett and Stadtman, 1997). (C) Various reactions of carbonylation adducts on amino acid side chains are shown (Berlett and Stadtman, 1997). 1 – oxidized sugars can be bound to

lysines; 2 – oxidation of fatty acids leading to carbonyl adduct formation on histidines, cysteins and lysines; 3 – MDA adduct on lysine.

Therefore, the oxidative modifications are important molecular markers that provide insight into the cumulative effect of oxidative stress on the molecular mechanisms of aging and development of age-associated diseases.

LONGEVITY AND ISOPROSTANES

Mouse models that carry specific mutations affecting levels of oxidative stress (ROS production) and also exhibit increased life span have been identified. These models strongly support the hypothesis that levels of ROS production may play a key role in longevity determination. In particular, the Snell and Ames dwarf mutants, which have very low levels of growth hormone, thyroid stimulating hormone, and prolactin, and live ~40-60% longer than their normal littermates (Murakami, 2006), have been extensively used in studies of the role of oxidative stress in aging. These long-lived mutants have provided basic information on the role of oxidative metabolism and oxidative stress in longevity determination. Recent studies also suggest that these dwarf mice are more resistant to oxidative stress generated by environmental factors such as UV, hydrogen peroxide and paraquat (Bartke and Brown-Borg, 2004; Brown-Borg et al., 1996; Flurkey et al., 2001; Flurkey et al., 2002; Hsieh and Papaconstantinou, 2006; Murakami et al., 2003; Salmon et al., 2005). These mice also have higher levels of antioxidant enzyme activities compared to the wild-type (WT) mice (Bartke and Brown-Borg, 2004; Brown-Borg et al., 1999; Brown-Borg and Rakoczy, 2000), thus, suggesting a possible mechanism of resistance to external oxidative stress in these long-lived mice. Although there is no direct evidence suggesting that resistance to oxidative stress by the DW mice is due to lower levels of endogenous ROS production our studies have shown that the ROS activated stress response pathway, p38 MAPK, is significantly down-regulated in young and aged Snell dwarf mutants (Hsieh and Papaconstantinou, 2006). We, therefore,

propose that these data strongly suggest lower endogenous levels of ROS in the long-lived mutant.

Studies have indicated that F_2-isoprostanes (IsoPs) are potentially important markers of the status of oxidative stress in eukaryotes (An et al., 1996; Montuschi et al., 2004; Montuschi et al., 2007; Roberts and Morrow, 2000). IsoPs are products of arachidonic acid catalyzed by non-enzymatic free radicals and are present in both tissues and extracellular compartments (Roberts and Morrow, 2000). Thus, IsoPs reflect the level of oxidative stress due to lipid peroxidation *in vivo* (Pratico et al., 1998). Measurement of the levels of these lipid molecules in biological fluids such as serum and urine, and in tissues is well established (Morrow and Roberts, 1999), and their levels are very reliable markers of the status of oxidative stress in normal aging, disease processes and pre- and post-acute injuries (Montuschi et al., 2004; Montuschi et al., 2007; Roberts and Morrow, 2000; Ward et al., 2005). Based on these studies, we hypothesize that serum and tissue IsoP levels may be lower suggesting lower levels of oxidative stress in Ames DW mice (compared to WT) at all ages. In addition, we further hypothesize that the lower levels of ROS-mediated oxidative damage to ETC complexes may lead to improved mitochondrial function which may be a factor in extended life-span in Ames DW mutants.

CHAPTER II

EXPERIMENTAL PROCEDURES

ANIMALS

Young (3-5 months), middle-aged (12-14 months) and old (20-22 months) male C57BL/6 mice were purchased from the National Institute on Aging (Bethesda, MD). Mice were maintained with a 12h light/dark cycle and fed *ad libitum* on a standard chow diet before sacrifice.

The Ames mouse colony was maintained at UTMB. Ames mice were generated by mating of *Prop1*$^{+/-}$ heterozygous males and females. The progeny were weaned at 1 month of age and tail DNA was collected to genotype *Prop1*$^{+/+}$ (wild-type), *Prop1*$^{+/-}$ (heterozygous), and *Prop1*$^{-/-}$ (dwarf) mice. Real time RT-PCR was performed in Bio-Rad iCycler (Bio-Rad, CA) using TaqMan® probes for allelic discrimination of *Prop1* gene.

PCR probes: Anti-sense, 5'-CGA GCC CAG ATG TCA GGA TAC-3'

Sense, 5'-CAC CGC ACC ACC TTC AAC C-3'

TaqMan® probes: Dwarf probe 5'-TCC CAA AGG CTG **G**CT CCA GC-3'

WT probe 5'-TCC CAA AGG CTG **A**CT CCA GCT-3'

PCR protocol was as follows: 95°C for 1.5 min (1X), 95°C for 0.5 min, 63°C for 0.5 min, and 70°C for 0.5 min (50X). Reaction volume was 25 μL.

Mice were separated according to their genotype and wild-type and dwarf mice were aged for further use. Young (4-5 months), middle-aged (10-12 months) and old (20-26 months) male wild-type and dwarf mice were maintained in UTMB animal research facility with a 12h light/dark cycle and fed *ad libitum* on a standard chow diet before sacrifice.

SERUM AND LIVER SAMPLE COLLECTION

Immediately after sacrificing the animals by cervical dislocation and decapitation, blood was collected from body cavity and allowed to coagulate on ice. Serum was collected as the supernatant by centrifugation of coagulated blood at 2500 X g for 10 min.

The serum samples were then snap-frozen in liquid nitrogen and stored at –80^{o}C for further use. All steps were carried out quickly until the samples were frozen. Following the removal of blood, the liver of the animal was removed and ~100 mg of liver piece was put in cryogenic tubes, snap-frozen in liquid nitrogen and stored at –80^{o}C for further use.

MEASUREMENT OF F_2-ISOPROSTANE LEVELS

F_2-isoprostane (IsoP) levels were determined by extraction of isoprostanes from the serum and liver samples as described previously (Morrow and Roberts, 1999). IsoP values are reported as pg/mL of serum when obtained for serums from 7 young, 8 middle-aged, and 8 old wild-type and 12 young, 13 middle-aged, and 8 old dwarf mice. IsoP values are reported as ng/g of tissue when obtained for liver pieces from 5 young, 7 middle-aged, and 8 old wild-type and 12 young, 13 middle-aged, and 4 old dwarf mice. Statistical significance was calculated using the Student's T-test with $p<0.05$ and $p<0.005$ considered significant and highly significant, respectively.

MITOCHONDRIAL ISOLATION FROM BOVINE HEARTS

Bovine hearts were a kind gift from Sam Kane Meat Packing Co., Corpus Christi, TX. Hearts were cooled prior to processing for mitochondrial isolation. The fat and connective tissue were removed from the hearts prior to homogenization of the cardiac muscle. Mitochondrial isolation was carried out by published methods (Crane et al., 1956; Hatefi, 1978). The crude mitochondria were stored in aliquots at –80^{o}C for further use. For each analysis, fresh aliquots were thawed, centrifuged once and resuspended in the isolation buffer (Hatefi, 1978) The mitochondria were then sonicated briefly to generate submitochondrial particles (SMPs) and their oxygen consumption activities were measured using an oxygen monitoring system (Strathkelvin Instruments Ltd., Glasgow UK) using both NADH and succinate as substrates to verify active respiratory activity.

ISOLATION OF MOUSE MITOCHONDRIA

Mice were sacrificed by decapitation and their tissues were harvested immediately, rinsed in ice-cold PBS, and prepared for subcellular fractionation. Mitochondria were prepared from the pooled tissues of 9 young, 10 middle-aged and 8 old C57BL/6 male mice. In the study with Ames mice, mitochondria were prepared from the pooled tissues of 8 young, 8 middle-aged and 8 old Ames WT and 12 young, 13 middle-aged, and 8 old Ames DW male mice. Mitochondrial isolation was carried out at 4^{o}C as described (Rajapakse et al., 2001) with minor modifications (Choksi et al., 2007). Briefly, tissues were blended using a Brinkman Polytron PT 3000 with large blade for 10-15 seconds in isolation buffer (250 mM Sucrose, 0.5 mM EGTA, 2 mM EDTA, 10 mM HEPES-KOH, pH 7.4, and 1 μg/mL final concentration of Antipain, Chymostatin, Leupeptin and Pepstatin A). The blended tissues were homogenized 20 times with Teflon pestle glass homogenizer and the homogeneous solution was centrifuged at 800 X g for 20 minutes. The supernatant was collected in a separate tube and the pellet was resuspended in half volume of isolation buffer, homogenized again as described above and centrifuged at 800 X g for 20 minutes. The supernatant was combined with the one from the previous step and centrifuged 2 additional times at 800 X g for 20 minutes. Each time the supernatant was transferred to anew tube and pellet was discarded. The final supernatant was centrifuged at 8000 X g for 20 minutes to pellet the mitochondria. The mitochondrial pellets were washed twice with half volumes, centrifuged at 8000 X g and the supernatants were discarded. The final mitochondrial pellets were resuspended in a minimal volume of isolation buffer and stored in aliquots at – 80^{o}C. For each analysis, fresh aliquots were used. Sonicated mitochondria were generated with Branson Model 250 Digital Sonifier (2 cycles of 1.8 seconds each, 6 second total time for each cycle, one minute between each cycle, amplitude 30% pulse time on for 0.3 seconds, pulse time off for 0.7 seconds).

ENZYME ACTIVITIES

Enzyme activities were assayed at room temperature using a Beckman Coulter DU 530 Spectrophotometer (Beckman Coulter, Fullerton CA). Citrate synthase activity was measured as described (Choksi et al., 2007; Jarreta et al., 2000). Briefly, in each 1 mL assay reaction mixture containing reaction buffer (50 mM Potassium Phosphate, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 0.1% Triton X-100 and 1 mg/mL BSA) 7-25 μg of sonicated mitochondria (as described above) were added followed by addition of 0.1 mM Acetyl-CoA and 2 mM DTNB. The reaction was initiated with the addition of 40 μM oxaloacetate and the enzyme activity was recorded at 412 nm (ε = 13.6 mM^{-1} cm^{-1}). Rotenone-sensitive Complex I (CI) activity, Malonate-sensitive Complex II (CII) activity, Antimycin A-sensitive Complex III (CIII) activity, KCN-sensitive Complex IV (CIV) activity, and Oligomycin-sensitive Complex V (CV) activities were assayed as described (Choksi et al., 2007; Kwong and Sohal, 2000; Yarian et al., 2005). Briefly, CI activity was measured at 340 nm (ε = 6.81 mM^{-1} cm^{-1}) in 1 mL reaction mixture containing reaction buffer (50 mM Potassium Phosphate, pH 7.4, 5 mM MgCl2 and 1 mg/mL BSA), 7-25 μg of sonicated mitochondria, 2 mM KCN, 3.7 μM Antimycin A and 100 μM Q_1. The reaction was initiated by the addition of 140 μM NADH and after 3 minutes 20 μM Rotenone was added to inhibit the enzyme activity. The final rate was measured by subtracting Rotenone-insensitive rate from the initial rate. CII activity was measured at 600 nm (ε = 19.1 mM^{-1} cm^{-1}) in 1 mL reaction mixture initially incubated at 30^{o}C for 20 minutes containing reaction buffer (50 mM Potassium Phosphate, pH 7.4, 5 mM $MgCl_2$ and 1 mg/mL BSA), 7-25 μg of sonicated mitochondria, 20 mM succinate and 0.2 mM ATP. The reaction was initiated by the addition of 20 μM Rotenone, 2 mM KCN, 3.7 μM Antimycin A, 50 μM DCPIP and 100 μM Q_1. After 3 minutes, 10 mM Malonate was added to inhibit the enzyme activity. The final rate was measured by subtracting Malonate-insensitive rate from the initial rate. CIII activity was measured at 550 nm (ε = 19 mM^{-1} cm^{-1}) in 1 mL reaction mixture containing reaction buffer (50 mM

Potassium Phosphate, pH 7.4, 5 mM $MgCl_2$ and 1 mg/mL BSA), 1-5 μg of sonicated mitochondria, 20 μM Rotenone, 2 mM KCN, 0.2 mM ATP and 40 μM cytochrome c. The reaction was initiated by the addition of 100 μM decyl benzoquinol with or without 7.4 μM Antimycin A. The final rate was measured by subtracting Antimycin A-insensitive rate from that without addition of the inhibitor. CIV activity was measured at 550 nm (ε = 19 mM^{-1} cm^{-1}) in 1 mL reaction mixture containing reaction buffer (10 mM Tris-HCl, pH 7.4, 20 mM KCl and 1 mg/mL BSA), 0.5-5 μg of sonicated mitochondria, and 1 mM dodecyl-β-D-maltoside. The reaction was initiated by the addition of 11 μM ferrocytochrome c with or without 2 mM KCN. The final rate was measured by subtracting KCN-insensitive rate from that without addition of the inhibitor. CV activity was measured at 340 nm (ε = 6.2 mM^{-1} cm^{-1}) in 1 mL reaction mixture containing reaction buffer (50 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$ and 250 mM sucrose), 7-25 μg of sonicated mitochondria, 25 units of pyruvate kinase, 25 units of lactate dehydrogenase, 20 μM Rotenone, 2 mM KCN, 5 mM phosphoenolpyruvate, and 175 μM NADH. The reaction was initiated by the addition of 2.5 mM ATP and after 3 minutes 15 μM Oligomycin was added to inhibit the enzyme activity. The final rate was measured by subtracting Oligomycin-insensitive rate from initial rate.

CI-III and CII-III coupled assays were performed as described (Choksi et al., 2007; Kwong and Sohal, 2000; Yarian et al., 2005). Briefly, CI-CIII activity was measured at 550 nm (ε = 19 mM^{-1} cm^{-1}) in 1 mL reaction mixture initially incubated at 30^{o}C for 10 minutes containing reaction buffer (50 mM Potassium Phosphate, pH 7.4, 5 mM $MgCl_2$ and 1 mg/mL BSA), 7-25 μg of sonicated mitochondria, 350 μM NADH and 2 mM KCN. The reaction was initiated by the addition of 80 μM cytochrome c and after 2 minutes, both 20 μM Rotenone and 7.4 μM Antimycin A were added to inhibit the coupled activity. The final rate was measured by subtracting inhibitor-insensitive rate from the initial rate. CII-CIII activity was measured at 550 nm (ε = 19 mM^{-1} cm^{-1}) in 1 mL reaction mixture initially incubated at 30^{o}C for 20 minutes containing reaction buffer

(50 mM Potassium Phosphate, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA and 1 mg/mL BSA), 7-25 μg of sonicated mitochondria, 20 mM succinate, 20 μM Rotenone, 2 mM KCN and 0.2 mM ATP. The reaction was initiated by the addition of 40 μM cytochrome c and after 2 minutes 10 mM Malonate was added to inhibit the coupled activity. The final rate was measured by subtracting Malonate-insensitive rate from initial rate.

All activity measurements reported are averages of 4 assays with the pooled samples for each age group. Citrate synthase assay results from young C57BL/6 or Ames WT mice were used to calculate ratios of young to middle-age and young to old as well as WT to DW mitochondrial protein levels and these ratios were multiplied to normalize each enzyme activity for specific age group. Statistical significance was calculated using the Student's T-test with $p<0.05$ and $p<0.001$ considered significant and highly significant, respectively.

MEASUREMENT OF COENZYME Q

Mitochondrial coenzyme Q was quantified using an HPLC method (Boitier et al., 1998; Choksi et al., 2007; Duncan et al., 2005). Briefly, coenzyme Q was extracted from mitochondrial preparations at 4°C with ethanol-n-hexane (2:5, v/v). Samples were dried under a stream of Argon and reconstituted in ethanol in preparation for reversed-phase chromatography. HPLC analysis was carried out on an ESA CoulArray (ESA Biosciences, Chelmsford MA) system equipped with a UV-Visible (Model 528, ESA Biosciences) detector. Isocratic separations were performed by pumping mobile phase consisting of 50 mM $NaClO_4$ dissolved in ethanol-methanol-70% $HClO_4$ (700:300:1, v/v) through a C18 column (150 mm x 4.6 mm, 5 μm Alltech Associates, Deerfield IL) while continually monitoring absorbance at 275 nm. Elution positions of reduced and oxidized coenzyme Q_9 (CoQ_9) and coenzyme Q_{10} (CoQ_{10}) were determined with appropriate standards. Integrated peak area was calculated for peaks corresponding to the pertinent species and molar concentrations were determined for oxidized and reduced CoQ_9 and CoQ_{10} using appropriate calibration curves. To account for variations in extraction

efficiency, decylubiquinone (~20 nmole) spiked in the mitochondrial preparations and served as an internal standard. Statistical significance was calculated using the Student's T-test with $p<0.05$ and $p<0.001$ considered significant and highly significant, respectively.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Blue-native PAGE (BN-PAGE) and SDS-PAGE were carried out according to established methods (Schagger, 1995) with minor modifications (Choksi et al., 2004; Choksi et al., 2007). Briefly, a 5 to 12% acrylamide gradient was used for the first dimension BN-PAGE, imidazole instead of Bis-Tris was used as a buffer, and Criterion® 10-20% 2D-well gels (Bio-Rad, CA) were used for the second dimension SDS-PAGE.

IMMUNOBLOTTING

Immunoblot analysis was performed as described (Choksi et al., 2004; Choksi et al., 2007; Rabek et al., 2003). Briefly, all immunoblots were generated after overnight transfer and were blocked with 5% non-fat blocking grade milk (Bio-Rad, Hercules CA) in TBS-T (Tris base saline, pH 7.4, and 0.05% Tween-20) and incubated with appropriate antibody diluted with blocking solution, for 1 hour or overnight. The blots were washed three times for 5 minutes each with TBS-T and probed with appropriate secondary antibodies conjugated with HRP (Alpha Diagnostic, San Antonio TX). Immunoreactive bands were detected by chemiluminescence using the Immobilon Western HRP substrate (Millipore, MA), and images recorded using Kodak X-Omat AR films. Films were analyzed using Alpha Innotech FluorChem IS-8900 imager (Alpha Innotech Corporation, San Leandro CA) and density values were calculated according to the manufacturer's instructions.

Intact mitochondrial ETC complex bands were visualized with antibodies against CI (NDUFA9 subunit), CII (SDHA subunit), CIII (UQCRFS1 subunit), CIV (COX1), and CV (ATP5A1 subunit) (Invitrogen, Carlsbad CA). The CIV-specific antibody is for mitochondrially encoded subunit COX1. All other complex-specific antibodies are

against nuclear encoded subunits. Several types of oxidative modifications were detected using a mouse monoclonal anti-nitrotyrosine antibody (Millipore, Billerica MA), anti-MDA goat polyclonal antibody (Academy Bio-Medical, Houston TX) and anti-HNE Fluorophore rabbit polyclonal antibody (EMD Biosciences). Carbonylated proteins were derivatized with 2,4-dinotrophenylhydrazine (DNPH) to generate a stable 2,4-dinitrophenylhydrazone (DNP) adduct at the carbonyl group (Levine et al., 1994). Anti-DNP rabbit polyclonal antibody (Invitrogen) was then used to detect DNP-derivatized proteins. All oxidative modification detecting immunoblots were stripped using Restore™ western blot stripping buffer (Pierce Biotechnology, Rockford IL) per manufacturer's recommendations and re-probed with complex-specific antibodies as mentioned above to normalize protein loading. The density values were background subtracted, normalized to protein loading using ratios from anti-complex antibodies, and converted to percentage using density of young protein bands as 100%. Data represented in the figures are from the same samples for each age group where C57BL/6 tissues from 9 animals were pooled in the young group, 10 animals in the middle-aged group and 8 animals in the old age group. We believe that having pooled these many tissues minimizes any experimental error and the differences seen are true biological variations.

MALDI-TOF-TOF

Individual ROS-modified protein bands were excised from second dimension SDS-PAGE run simultaneously with the gels that were immunoblotted and analyzed by the Proteomics Core Facility at UTMB. The proteins were eluted from the gel and digested with trypsin (Promega, Madison WI); the tryptic peptides were then analyzed by MALDI-TOF-TOF (Choksi et al., 2004; Choksi et al., 2007). Mass spectral peak data were submitted to the ProFound (Rockefeller University) online search engine for protein identification using the NCBI database.

CHAPTER III

OXIDATIVELY DAMAGED PROTEINS OF HEART MITOCHONDRIAL ELECTRON TRANSPORT COMPLEXES

SUMMARY

In this study, we employ blue-native polyacrylamide gel electrophoresis (BN-PAGE) to resolve intact the respiratory chain Complexes I–V (Schagger, 1995) of adult bovine heart submitochondrial particles (SMP), and second dimension denaturing SDS-PAGE to resolve individual subunits of these complexes. Immunoblotting analysis has been used to detect proteins that are modified by HNE, nitration, or carbonylation (Levine et al., 1994; Uchida and Stadtman, 1992a). Proteins shown to be oxidatively damaged were subjected to both MALDI-TOF and LC/MS/MS mass spectrometry to establish their identity. Our studies have identified specific protein subunits of the mitochondrial respiratory chain complexes that are susceptible to and targeted for oxidative damage caused by ROS-mediated protein modification.

RESULTS

The oxygen consumption activities of bovine heart SMPs were measured to be 638.8 μmol of O_2/min/mg and 101 μmol of O_2/min/mg with NADH and succinate as substrates, respectively. The respiratory rates were completely sensitive to KCN inhibition. These SMPs were solubilized and subjected to BN-PAGE to resolve Complexes I–V (Crane et al., 1956; Hatefi, 1978; Schagger, 1995). Figure 3.1A shows the first dimension electrophoretic resolution of these SMPs. The complexes were identified by immunoblot using antibodies specific for complex components as seen in Figure 3.1B. Previous reports showed that Complex I is made up by 46 subunits and has an approximate molecular mass of 980 kDa (Carroll et al., 2003); Complex II is made up of 4 protein subunits and has an approximate molecular mass of 130 kDa (Righetti and Caravaggio, 1976); Complex III is made up of 11 protein subunits, and in the

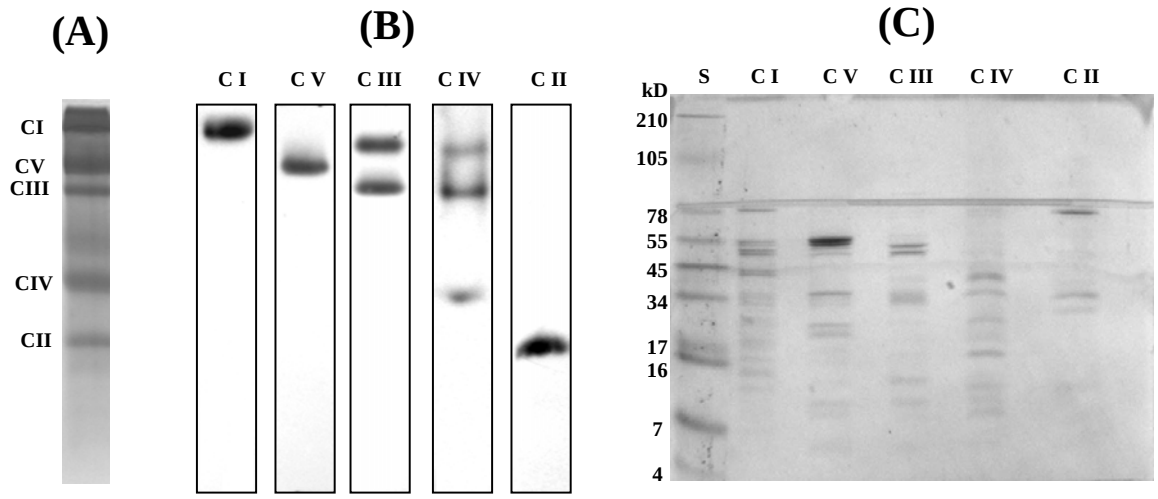


Figure 3.1. Separation of bovine heart SMP respiratory chain complex subunits

(A) Bovine Heart SMPs were solubilized and the respiratory chain complexes were separated on a blue-native first dimension gel as described in Materials and methods. Lane 1 represents the blue-native first dimension electrophoretic separation of mitochondrial complexes from bovine heart SMPs. (B) Complex specific antibodies were used in immunoblots performed after the blue-native gel to confirm the identity and position of each complex on the blue-native gels. (C) The individual Complexes I –V, resolved in (A), were excised and the subunits of each complex were separated on a second dimension SDS-PAGE. The abbreviations are: S = molecular weight standard, C I = Complex I, C II = Complex II, C III = Complex III, C IV= Complex IV and C V= Complex V.

mitochondria, the functional complex occurs as a dimer with an approximate molecular mass of 500 kDa (Schagger et al., 1995); Complex IV is made up of 13 protein subunits and has a molecular mass of 200 kDa (Kadenbach and Merle, 1981); and Complex V is made up of 16 polypeptides and their isotypes, and has a molecular mass of 600 kDa (Gaballo et al., 2002). In the BN-PAGE, these complexes separate in the following order, from highest to lowest molecular mass (Figure 3.1A): Complex I, Complex V, Complex III (dimmer), Complex IV, and Complex II. The immunoblot of C III showed reactivity to not only the dimer but also to a trimer. However, since the dimer form of C III is the predominant species this band was used as C III band for further experiments. Each complex band was excised and run separately in the order of their separation on the

second dimension denaturing gel and the resolved protein bands were visualized by staining with Coomassie blue (Figure 3.1C).

Immunoblot analyses of the gels after second dimension separation were carried out to identify oxidatively modified proteins (Figures 3.2 and 3.3). These analyses revealed proteins that were modified by HNE (Figure 3.2A) and nitrotyrosine (Figure 3.2B) adducts. The proteins modified by carbonylation were detected by DNP-derivitization followed by immunoblot analysis (Figure 3.3). These correspondingly modified proteins were cut out from the Coomassie-stained gels (Figures 2C and 3B) and identified by MALDI-TOF and LC/MS/MS mass spectrometry (Table 3.1).

Proteins modified by HNE-adduct formation include: Complex I, NUCM subunit (49.2 kDa), NUEM subunit (39 kDa); Complex III, the mitochondrial processing peptidase subunits a (49.2 kDa) and h (46.5 kDa) also known as Complex III Core 1 and Core 2 subunits, respectively; Complex IV, subunit VIb (10.2 kDa), and Complex V, β chain (51.5 kDa). Proteins modified by carbonylation include: Complex I, 75 kDa subunit, Complex II, 70 kDa subunit, Complex III, Core 1 (49.2 kDa) and Core 2 (46.5 kDa) and Complex V h chain (51.5 kDa). Finally, the only protein found to be nitrated is the β chain (51.5 kDa) of Complex V.

The β chain (51.5 kDa) of Complex V (F1 portion) reacted with all three antibodies suggesting multiple sites that are susceptible to oxidative damage. This h chain was also identified in Complex I (by anti-HNE and anti-DNP) and in Complex IV (by anti-nitrotyrosine; Table 3.1).

Both anti-mouse and anti-rabbit secondary antibodies cross-reacted to cytochrome c1 (27.2 kDa) as seen by the strong signal in secondary antibody blot at an apparent mass of 30 kDa (present in Complex I, V and III in Figure 3.2D). However, no other bands were present in this blot compared to blots in Figure 3.2A and B, suggesting that all other bands in Figure 3.2A and B represent the interactions of oxidatively modified proteins with primary antibodies. Thus, the cytochrome c_1 band interacting with secondary antibody represents the abundance of this protein in the complexes and because the

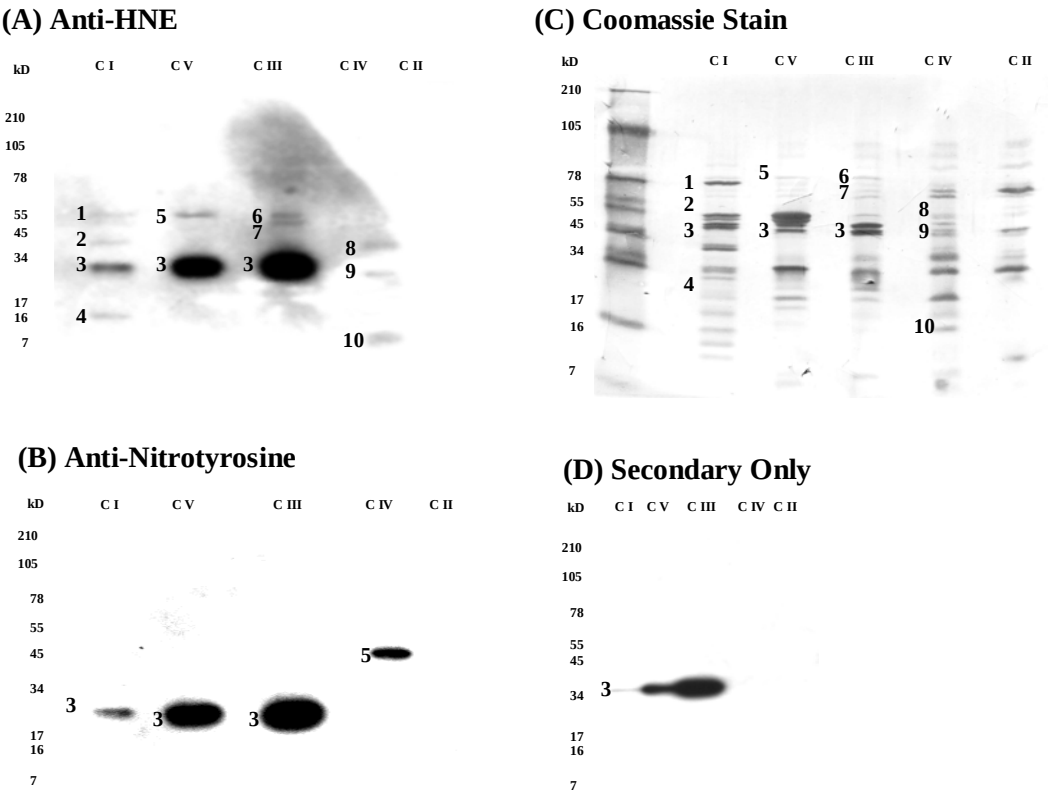


FIGURE 3.2. IDENTIFICATION OF OXIDATIVELY DAMAGED PROTEINS OF BOVINE HEART SMP RESPIRATORY CHAIN COMPLEXES RESOLVED BY SECOND DIMENSION SDS-PAGE
Bovine heart SMP complexes were resolved into their subunits as described in Materials and methods and immunoblots were performed using (A) anti-HNE; (B) anti-nitrotyrosine and (D) secondary only antibodies; (C) Coomassie blue G-250 stain of the second dimension SDS-PAGE used in immunoblots (A) and (B). The molecular weight markers of (A) and (D) correspond to those shown in (C) and (D) and were obtained by overlaying the Coomassie-stained gels over the Western blots. The abbreviations are as follows: C I = Complex I, C II = Complex II, C III = Complex III, C IV= Complex IV and C V= Complex V. Identification of each numbered band is summarized in Table 1.

protein modification is expected to be lower than total protein present, this accounts for the significantly higher intensities of cytochrome c_1 bands compared to all other oxidatively damaged proteins. Although, it is possible that cytochrome c_1 may be modified by oxidation, the recognition by secondary antibodies makes it impossible to determine such modifications. Therefore, further investigation is required to determine

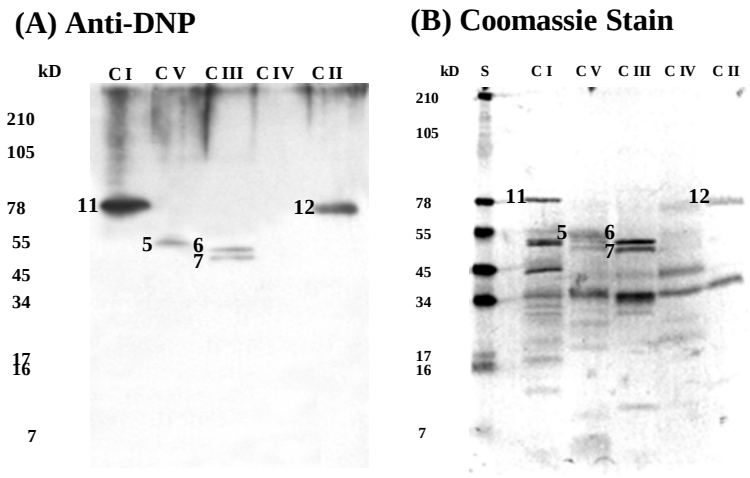


Figure 3.3. Identification of carbonylated proteins of bovine heart SMP respiratory chain complexes resolved by second dimension SDS-PAGE

Bovine heart SMP complexes were derivatized using DNPH and resolved into their subunits as described in Materials and methods followed by immunoblot analysis. (A) Anti-DNP antibody, and (B) Coomassie blue G-250 stain of the second dimension SDS-PAGE used in the immunoblot. Identification of each numbered band is summarized in Table 1.

whether cytochrome c_1 is oxidatively damaged.

In addition to the respiratory chain complex subunits, two proteins, heart-specific T1 isotype of adenine nucleotide translocator (ANT, 32.9 kDa) and voltage-dependent anion channel 1 (VDAC, 30.7 kDa) were also identified among Complex IV proteins modified by HNE formation (Table 3.1). Both ANT and VDAC are part of a complex that forms the mitochondrial permeability transition pore that has been shown to play a key role in cellular apoptosis.

All proteins we detected as oxidatively modified are known to associate with or are in close proximity to the inner mitochondrial membrane (Figure 3.4). These data are consistent with the proposal that ROS generated by dysfunctional/oxidatively stressed respiratory chain complexes may react with the mitochondrial membranes, thereby causing increased lipid peroxidation, and these lipid peroxidation products modify membrane-associated or membrane-proximal subunits of various complexes.

Table 3.1. HNE, nitrotyrosine-modified and carbonylated subunits of bovine heart SMP respiratory chain complexes

Band Number	MALDI-TOF/ ProFound ID	Z score	% Coverage	Mitochondrial Localization
HNE-Modified (Figure 3.2A)				
1	NUCM (49.2 kDa)	2.42	41	Complex I
2	NUEM (39 kDa)	2.37	40	Complex I
3*	Cyt c_1 (27.2 kDa)	2.30	32	Complex III
4*	B17 (15.5 kDa)	1.83	32	Complex I
5	β chain (51.5 kDa)	2.37	52	Complex V
6	Core 1 (49.2 kDa)	2.41	43	Complex III
7	Core 2 (46.5 kDa)	2.38	29	Complex III
8	ANT (T1) (32.9 kDa)	2.25	29	Mitochondria
9	VDAC 1 (30.7 kDa)	2.35	39	Mitochondria
10	VIb (10.2 kDa)	2.31	33	Complex IV
Nitrotyrosine-Modified (Figure 3.2B)				
3*	Cyt c_1 (27.2 kDa)	2.30	32	Complex III
5	β chain (51.5 kDa)	2.37	52	Complex V
Secondary Only				
3*	Cyt c_1 (27.2 kDa)	2.30	32	Complex III
Carbonylated (Figure 3.3A)				
5	β chain (51.5 kDa)	2.37	52	Complex V
6	Core 1 (49.2 kDa)	2.41	43	Complex III
7	Core 2 (46.5 kDa)	2.38	29	Complex III
11*	NUAM (75 kDa)	2.40	30	Complex I
12*	SDHA (70 kDa)	2.31	34	Complex II

* - Identified by both MALDI-TOF and LC/MS/MS.

In addition, these data also suggest differential targeting of specific subunits of the respiratory chain complexes resulting in differences of nitrotyrosine modification, HNE adduct formation and carbonylation, as summarized in Table 3.1.

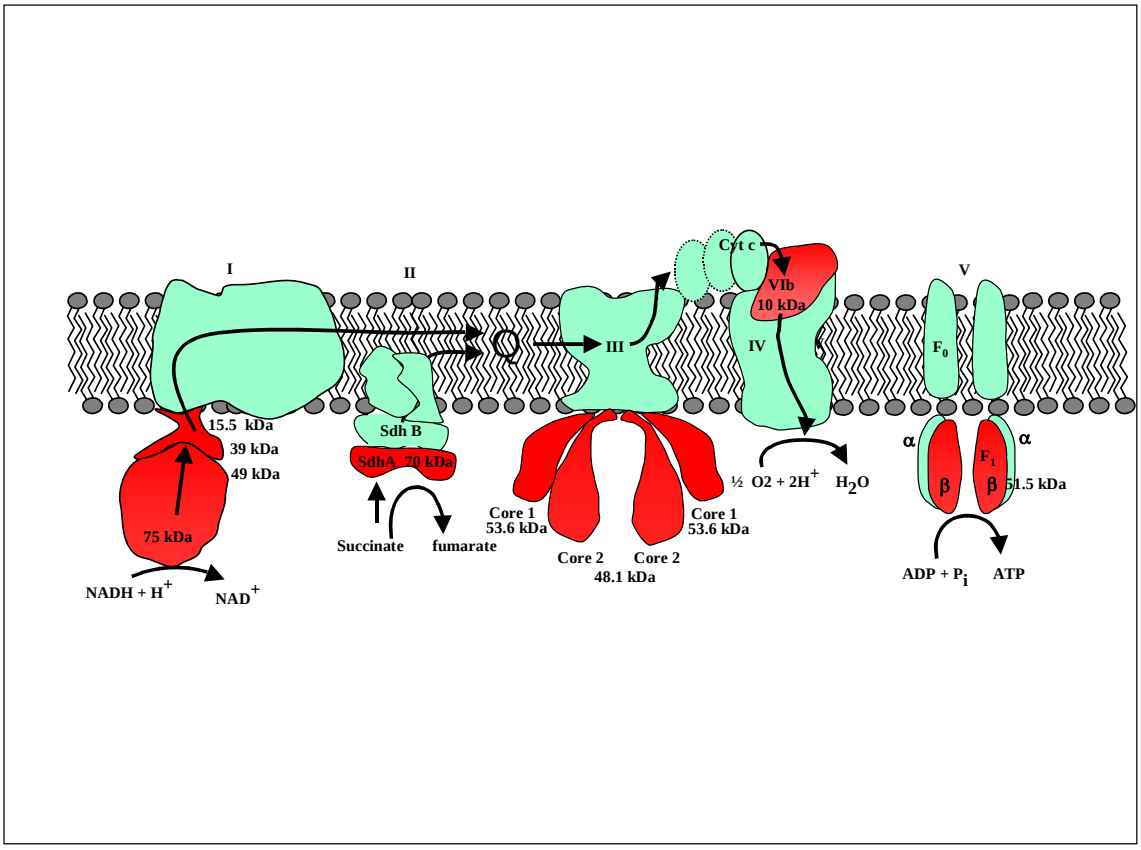


Figure 3.4. A diagrammatic presentation of the localization of oxidatively damaged proteins of Complexes I –V of the mitochondrial respiratory chain
The diagram shows the proteins of each complex identified in our studies to be oxidatively damaged by HNE, nitration and/or carbonylation, and that these proteins are localized in the mitochondrial matrix or intermembrane space. The red portions of complexes depict oxidatively damaged subunits and the green portions represent subunits that are not damaged. The pattern of proteins that are modified follows that of the interactions that are carried out by these extramembrane components of the complexes.

DISCUSSION

Mitochondria are a major source of ROS in mammalian tissues. However, the difficulties inherent in direct measurement of many short-lived ROS radicals have hampered the identification of ROS formation mechanisms and the targets of oxidative damage. In this study, we used an indirect measurement of ROS damage, *i.e.* protein modification, to serve as an indicator of damage from mitochondrial ROS. Here, we

present for the first time, evidence that specific protein subunits of mitochondrial respiratory chain Complexes I–V are susceptible to oxidative damage in normal unchallenged bovine heart mitochondria (Figure 3.4). Modifications due to oxidative damage have been shown to result in either the loss-of-protein function (Stadtman, 2001) or protein malfunction such as toxic-gain-of-function (Rakhit et al., 2002). For example, it has been shown that the in vitro modification by HNE of subunits I and IV of Complex IV inhibits the enzymatic activity of this complex (Chen et al., 1998). In our study, we show that subunit VIb of Complex IV is HNE-modified. This modification may affect the enzymatic activity of Complex IV thereby resulting in mitochondrial dysfunction. Modification of the β chain of F1F0-ATP synthase with all three adducts suggests that this protein is particularly susceptible to oxidative damage. Since the β chain of F1 portion functions to synthesize ATP, it is possible that oxidative damage to this subunit may lead to decreased ATP synthesis. In amyotrophic lateral sclerosis, a gain-of-toxic-function occurs due to point mutations of SOD1 that result in increased enzyme activity and propensity to aggregate (Rakhit et al., 2002). The in vitro oxidation of SOD1 causes an increase in enzyme activity and its propensity to aggregate, thus mimicking the in vivo characteristics of the gain-of-toxic-function.

Many diseases associated with mitochondrial dysfunction result from deficiencies or defects in respiratory chain complex subunits. Mitochondrial myopathy has been attributed to the deficiency in the iron–sulfur cluster containing subunits of Complex I (39 and 49 kDa subunits) (Hall et al., 1993). The disproportionately large deficiency of the 75 kDa iron–sulfur subunit of Complex I plays a major role in mitochondrial encephalomyopathy as well as congenital Complex I deficiency in severe lactic acidosis (Ichiki et al., 1989; Moreadith et al., 1987). Down-regulation of the B17 subunit of Complex I in response to oxidative stress, and deficiency of this subunit has been implicated in the defect in Complex I activity in Parkinson's disease (Yoo et al., 2003). These studies thus suggest that modification due to oxidative damage of specific components of electron transport complexes may play a key role in the gradual decline in

tissue function. Furthermore, it has been reported that inherent defects of mitochondrial succinate dehydrogenase (SDH) enzyme in humans are associated with various clinical presentations ranging from early-onset of devastating encephalopathy to tumor susceptibility in adulthood or optic atrophy in the elderly (Rustin et al., 2002). Thus, modification of the 70-kDa subunit of Complex II, the succinate oxidizing subunit, suggests that oxidative damage to this subunit via carbonylation may inhibit the protein's ability to oxidize succinate, thereby contributing to its dysfunction.

We have observed that proteins that are not involved in electron transport have also been modified by oxidative damage. The mitochondrial processing peptidase subunits (Core 1 and Core 2) of Complex III, which are both carbonylated and HNE-modified, play a critical, non-respiratory role in mitochondrial function. These proteins function as mitochondrial processing peptidases involved in proper protein folding during the maturation and transport of the mitochondrial matrix and inner membrane proteins (Deng et al., 2001). Indeed, recent studies have shown that misfolded proteins within the mitochondria activate a mitochondria-specific unfolded protein stress response, indicating the importance of these protein folding–transport processes (Zhao et al., 2002). In addition, ROS generation from Complex III is believed to occur at the heme b_H of cytochrome b subunit or at the quinone reducing Q_i site (Zhang et al., 1998). Notably, the Core 1 and Core 2 subunits of the mitochondrial processing peptidases are located close to the site of ROS production and their carbonylation and HNE adduct formation suggest that proximity to the site of ROS generation may, therefore, lead to their oxidative damage. Thus, we propose that subunits proximal to ROS generation sites of respiratory chain complexes may be more susceptible to oxidative damage and that their modification may lead to mitochondrial dysfunction as seen in oxidative stress.

Two additional proteins, ANT (Vieira et al., 2001; Yan and Sohal, 1998) and VDAC (Bahamonde and Valverde, 2003), which are not components of the respiratory chain complexes were also oxidatively modified. Recent studies have shown that in vitro modification of ANT by nitric oxide, peroxynitrite and HNE enhances permeability

transition pore opening in proteoliposomes suggesting that this modification may be a regulatory marker for mitochondrial permeability transaction pore opening and cellular apoptosis (Vieira et al., 2001). Although the authors reported that none of the endogenous ANT was modified by such adducts, our results clearly show that there is, in fact, endogenous modification of ANT and VDAC in bovine heart mitochondria. Thus, endogenous modification of these proteins in heart mitochondria may also contribute to regulation of the permeability transition pore opening and control of apoptosis. The VDAC is localized in the outer mitochondrial membrane that provides a major pathway for the transport of metabolites, e.g. ATP, cholesterol and involved in mitochondrial events that lead up to apoptosis (Dirmeier et al., 2002). It is interesting that VDAC proteins are localized only in the peripheral and not to the perinuclear mitochondria (Bahamonde and Valverde, 2003).

Our data show that the proteins of Complexes I–III, and V, which are oxidatively damaged, are localized in the mitochondrial matrix, while subunit VIb of Complex IV is localized in the intermembrane space. This follows the pathway of electron flow and is, therefore, suggestive of selectivity for extramembrane components of the complexes involved in electron transport (Table 3.1, Figure 3.4). Carbonylation of aconitase, VDAC and Core proteins of Complex III have been shown to occur in anoxia-induced oxidative stress in yeast (Bahamonde and Valverde, 2003). Interestingly, we show that the same proteins are oxidatively damaged in bovine heart SMPs. These data suggest that the same proteins, across species, of the electron transport complexes are more susceptible to oxidative damage. We propose that their proximity to the electron transport processes, i.e. their intramitochondrial localization whether yeast or bovine, may contribute to this selectivity. The results presented here support the hypothesis that mitochondrial respiratory chain complexes generate ROS, which can oxidatively damage key proteins thereby contributing to mitochondrial dysfunction. The oxidative damage from ROS includes proteins of the mitochondrial respiratory chain complex subunits as well as proteins involved in other functions. These modifications occur under normal basal

conditions, suggesting a consistent low level of mitochondrial damage in the absence of an oxidative stress challenge.

CHAPTER IV

AGE-RELATED INCREASES IN OXIDATIVELY DAMAGED PROTEINS OF MOUSE KIDNEY MITOCHONDRIAL ELECTRON TRANSPORT CHAIN COMPLEXES

SUMMARY

In this study we analyzed isolated mitochondria from young, middle-aged and old mouse kidneys to determine the enzyme activities of ETC complexes I-V during aging. In addition, we tested the hypothesis to identify whether specific proteins of ETC complexes I-V are susceptible to oxidative damage and whether the levels of these modifications increase with age. The activities of all ETC complexes were measured to determine whether there were any functional changes during aging and to determine if the levels of oxidative modification to these complexes correlate with changes in enzyme activities. The kidney was examined because its high urea levels cause high levels of endogenous oxidative stress in kidney (Zhang et al., 2004), and it is prone to relatively high levels of HNE adduct formation in mitochondrial proteins after iron overload (Zainal et al., 1999). We employed blue-native polyacrylamide gel electrophoresis (BN-PAGE) to further resolve intact ETC complexes (Schagger, 1995) followed by second dimension denaturing SDS-PAGE to resolve individual complex subunits. Protein abundance of each complex was measured using both BN-PAGE gels and complex-specific antibodies to determine the sensitivity and reproducibility of this technique for mouse tissues, and to quantify any age-related changes (Venkatraman et al., 2004). Using immunoblotting with antibodies recognizing specific types of oxidative damage, we detected proteins that were modified by HNE, MDA, and nitration. Proteins shown to be differentially oxidatively damaged were identified by MALDI-TOF mass spectrometry. These studies identify specific protein subunits of the mitochondrial ETC complexes that during aging are susceptible to oxidative damage caused by ROS-mediated protein modification. Finally, there is a direct correlation between increased protein modification and decreased

enzyme function for complexes I, II, IV, and V suggesting a progressive increase in endogenous oxidative stress during aging due to mitochondrial dysfunction. In conclusion, our studies further support the Mitochondrial Theory of Aging as indicated by the deleterious effects of oxidatively modified ETC proteins.

RESULTS

Inhibitor-sensitive enzyme activities

To evaluate the physiological effects of normal aging on kidney mitochondrial ETC complexes, we compared the enzymatic activities of all five complexes including the coupled activity of CI-III and CII-III for all three ages (Figures 4.1 and 4.2). Rotenone-sensitive CI activity did not change significantly between young and middle age but decreased 15-20% by 20-22 months (Figure 4.1A). In contrast, there was a 35% increase in CI-III coupled activity in both middle and old age compared to young age (Figure 4.2A). Malonate-sensitive CII activity decreased by 10% in middle age and by 30% in old age (Figure 4.1B). The coupled CII-III activity also showed an age-associated decline in activity (Figure 4.1B), *i.e.*, 15% decline in middle age and 30% decline in old age (Figure 4.2B). Antimycin A-sensitive CIII activity did not change with age (Figure 4.1C) despite the observed oxidative modifications to its subunits. KCN-sensitive CIV activity did not change significantly from young to middle age but decreased by 18% in old age (Figure 4.1D). Though not statistically significant, oligomycin-sensitive CV activity may increase slightly from young to middle age; however, there was a decrease of CV activity in old age (Figure 4.1E). Compared to young, CV activity decreased by 26% in 20-22 months and, compared to middle age, CV activity decreased by 38% in old age.

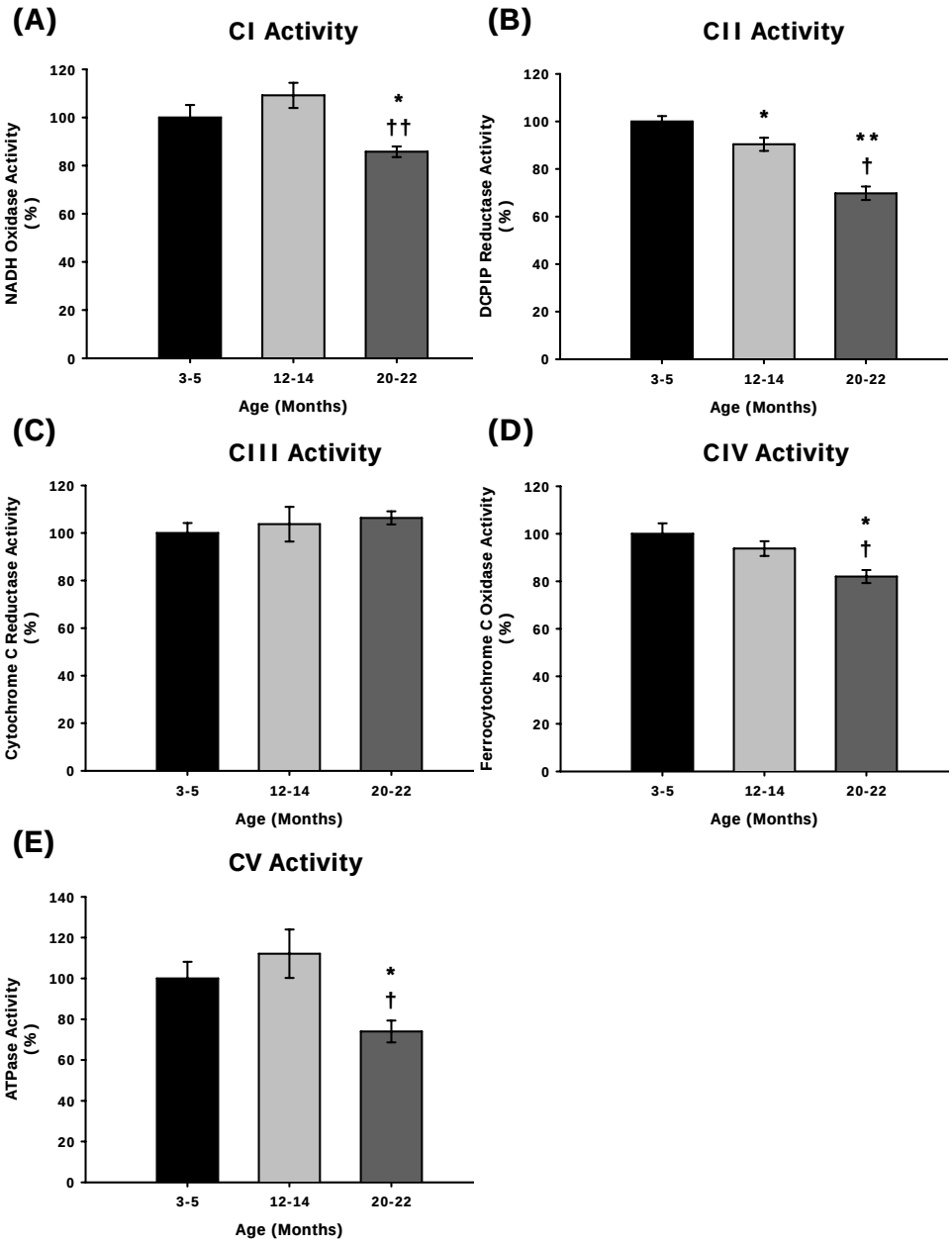


FIGURE 4.1. MEASUREMENT OF ETC COMPLEX ACTIVITIES FROM 3-5, 12-14, AND 20-22 MONTH-OLD MOUSE KIDNEY MITOCHONDRIA

Individual complex enzyme activities were measured spectrophotometrically as described in Experimental Procedures. All activity results are averages of 4 assays from the pooled sample for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (3-5 months), middle-aged (12-14 months), and old (20-22 months) kidney ETC CI-CV are plotted as a percentage of the young

enzyme activity (100%). (A) Kidney CI activity with aging. Young CI activity was 195.6 nmole/min/mg and coefficients of variance were 10.6 % (young), 9.6% (middle-age), and 5.1% (old), respectively. (B) Kidney CII activity with aging. Young CII activity was 352 nmole/min/mg and coefficients of variance were 4.4 % (young), 6.1% (middle-age), and 8.2% (old), respectively. (C) Kidney CIII activity with aging. Young CIII activity was 1648.2 nmole/min/mg and coefficients of variance were 8.3 % (young), 14% (middle-age), and 5.2% (old), respectively. (D) Kidney CIV activity with aging. Young CIV activity was 2731.2 nmole/min/mg and coefficients of variance were 8.8 % (young), 6.6% (middle-age), and 6.6% (old), respectively. (E) Kidney CV activity with aging. Young CV activity was 222.8 nmole/min/mg and coefficients of variance were 16.3 % (young), 21.2% (middle-age), and 14.5% (old), respectively. *- $p<0.05$ compared to young, **- $p<0.001$ compared to young, †- $p<0.05$ compared to middle-aged, ††-$p<0.001$ compared to middle-aged.

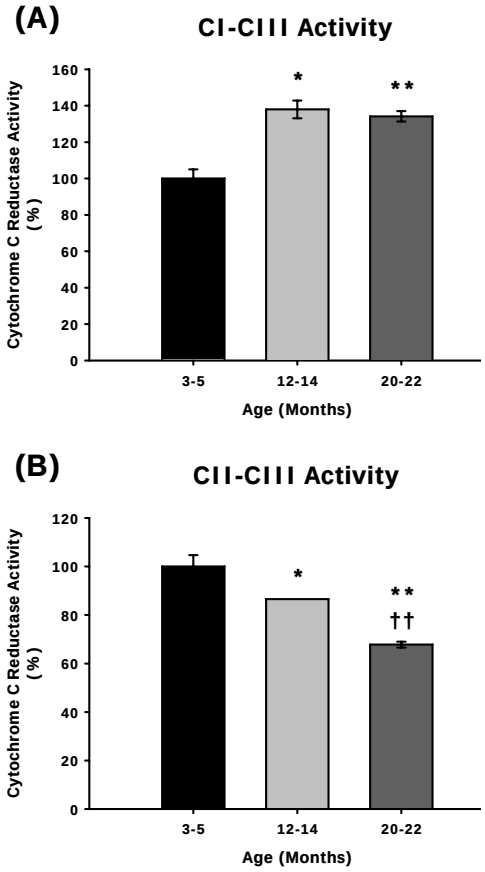


FIGURE 4.2. MEASUREMENT OF COUPLED MITOCHONDRIAL ETC COMPLEX ACTIVITIES FROM 3-5, 12-14, AND 20-22 MONTH-OLD MOUSE KIDNEY MITOCHONDRIA.

CI-III and CII-III coupled enzyme activities were measured spectrophotometrically as described in Experimental Procedures. All activity results are averages of 4 assays from the pooled sample for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (3-5 months), middle-aged (12-14 months), and old (20-22 months) kidney ETC C I-III and CII-III are plotted as a percentage using coupled enzyme activity of the young as 100%. (A) Kidney coupled CI-CIII activity with aging. Young CI-CIII activity was 289.5 nmole/min/mg and coefficients of variance were 9.9% (young), 7.1% (middle-age), and 4.2% (old), respectively. (B) Kidney coupled CII-CIII activity with aging. Young CII-CIII activity was 128.3 nmole/min/mg and coefficients of variance were 10.3% (young), 0% (middle-age), and 3.4% (old), respectively. *- $p<0.05$ compared to young, **- $p<0.001$ compared to young, ††-$p<0.001$ compared to middle-aged.

Coenzyme Q levels in mouse kidney mitochondria

To determine if changes in coenzyme Q (CoQ) levels are a factor in the age-associated loss of enzyme function, we measured the kidney mitochondrial CoQ levels of all three ages (Table 4.1). Although the CoQ_9 and CoQ_{10} homologues make up about

Table 4.1. Coenzyme Q Levels in Mouse Kidney Mitochondria with Aging

Age	CoQ_9 Total (nmol/mg)	CoQ_{10} Total (nmol/mg)	$CoQ_9 + CoQ_{10}$ (nmol/mg)
Young	86.9 ± 1.3	30.2 ± 0.3	117.1 ± 1.3
Middle-Age	118.2 ± 0.5	37.3 ± 1.1	$155.5 \pm 1.3^{**}$
Old	105.5 ± 0.6	33.8 ± 1.5	$139.2 \pm 1.6^{**, \dagger}$

Pooled kidney mitochondrial samples were used for each age group. Coenzyme Q was extracted and quantified as described in Materials and Methods. Data are an average of three experiments ± SEM. Coefficients of variance for $CoQ_9 + CoQ_{10}$ are 7.1% (young), 3.9% (middle-age), and 12.6% (old), respectively. **- $p<0.001$ compared to young, †- $p<0.05$ compared to middle-aged.

95% of the pool present in mitochondria, CoQ_9 is the predominant form in mouse tissues (Boitier et al., 1998; Duncan et al., 2005). Thus, we measured both CoQ_9 and CoQ_{10} levels and used these values as total CoQ for mouse kidney mitochondria. Our results in Table 1 show that there is a significant increase in CoQ levels in both middle-age and old

kidney mitochondria. Compared to the young mice, there is ~ 33% increase in CoQ levels at middle age and ~ 19% increase at old age which indicates that aging does not have any effect on the CoQ substrate availability for ETC complexes. In fact the data suggest that there is more CoQ available for these complexes in middle and old ages.

Abundance of ETC complexes in young, middle-aged and old kidney mitochondria

Mouse kidney mitochondria were isolated, solubilized and subjected to BN-PAGE to resolve intact complexes I-V. To determine if the loss of enzyme activities is due to change in their abundance with age, we first measured the levels of ETC complexes using both Coomassie staining and immunoblotting with complex-specific antibodies. These data were used to determine whether there are age-related quantitative differences in individual complexes (Venkatraman et al., 2004). In addition, these results also served to determine the accuracy of complex-specific antibody data used to normalize loading in second dimension immunoblotting. Therefore, BN-PAGE was performed with kidney mitochondria from young animals using a 2-fold dilution series of 1 X, ½ X, and ¼ X. The Coomassie-stained ETC complexes after first dimensional electrophoresis for these dilutions are shown in Figure 4.3A. Duplicate gels were run and transferred to PVDF membranes to identify individual complexes by immunoblotting using antibodies specific for ETC complex components (Figure 4.3B). The densities of the individual complex bands were measured and compared to the 1 X sample set as 100%. The results clearly show that both Coomassie staining and immunoblotting procedures could detect a two- or four-fold difference in complex abundance. Furthermore, the coefficients of variance of both methodologies for each complex at 1 X sample amount (used for all further experiments) were less than 20%. Thus, complex-specific antibodies were used to normalize loading in immunoblotting done for specific modifications. Having established the validity of Coomassie staining and immunoblotting for BN-PAGE and due to limitation of material obtained from tissues we felt that duplicate gels were sufficient to further test our hypotheses. Thus, we proceeded to use

these techniques to examine potential changes in ETC complex abundance in aged kidney mitochondria. We resolved mitochondrial complexes isolated from young, middle-aged, and old kidneys to determine the levels of ETC complexes in all three age groups (Figure 4.4). No age-related changes in protein levels of complexes in kidney mitochondria were detected by these methods (Figure 4.4).

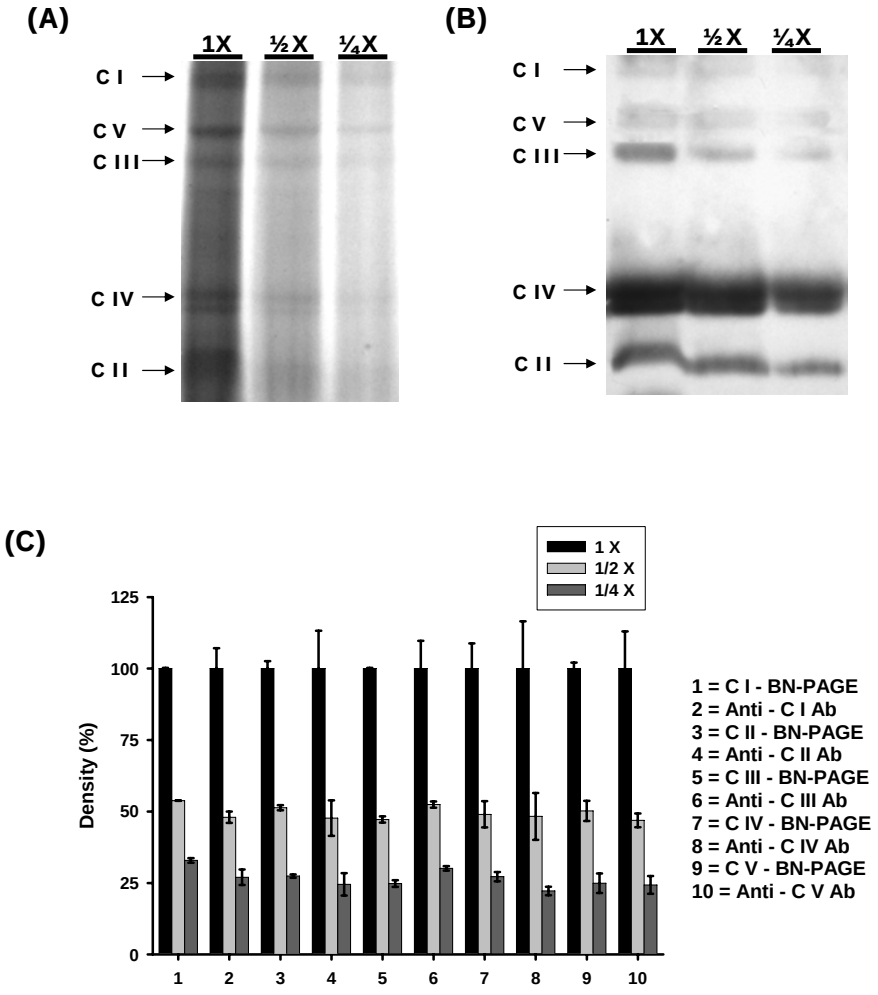


Figure 4.3. Two-fold dilution series of young kidney mitochondrial ETC complexes
Young kidney mitochondria were solubilized and the ETC complexes were separated by BN-PAGE as described in Experimental Procedures. (A) Coomassie-stained first dimension BN-PAGE gel. Lanes 1, 2, and 3 contained 160 μg (1X), 80 μg (1/2X), and 40

μg (1/4X), respectively, of solubilized young kidney mitochondrial ETC complexes. (B) Immunoblotting was performed in parallel using complex-specific antibodies on a duplicate gel transferred to a PVDF membrane. Both (A) and (B) are representative figures of three separate experiments. (C) Density values of each ETC complex band are plotted as a percentage of 160 μg (1X) lane. The bar graph shows the standard deviations of the average of three separate analyses using the same pooled sample for young age group. The coefficients of variance for CI at 1X amount in BN-PAGE and immunoblot were 0.2% and 7.1%, respectively. The coefficients of variance for CII at 1X amount in BN-PAGE and immunoblot were 2.6% and 13.3%, respectively. The coefficients of variance for CIII at 1X amount in BN-PAGE and immunoblot were 0.2% and 9.7%, respectively. The coefficients of variance for CIV at 1X amount in BN-PAGE and immunoblot were 8.8% and 16.5%, respectively. The coefficients of variance for CV at 1X amount in BN-PAGE and immunoblot were 2.1% and 13%, respectively.

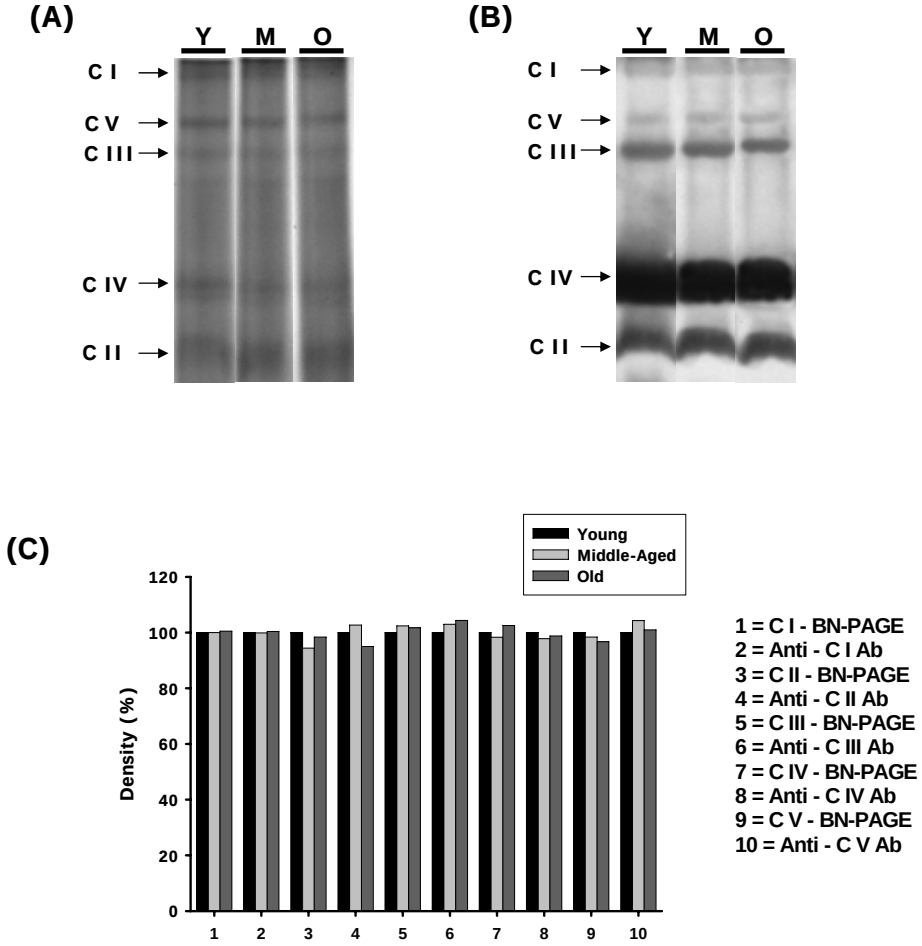


Figure 4.4. Protein abundance of ETC complexes in young middle-aged and old kidney mitochondria

Young, middle-aged and old kidney mitochondria (160 μg) were solubilized and the ETC complexes were separated on a BN-PAGE as described in Experimental Procedures. (A) The gel was stained with Coomassie G-250 stain. Lane 1, 2 and 3 represent young, middle-aged and old kidney mitochondrial ETC complexes, respectively. (B) Immunoblotting was performed using complex-specific antibodies on a duplicate gel transferred to a PVDF membrane. (C) Density values of each ETC complex band are plotted as a percentage of young kidney mitochondrial ETC complexes. Y = young kidney mitochondria, M = middle-age kidney mitochondria and O = old kidney mitochondria.

However, since all ETC complexes are multi-protein complexes, our data do not detect possible subtle changes in other components of these complexes.

Oxidative modification of ETC complex subunits as a function of aging

To identify the oxidatively modified ETC complex proteins, immunoblotting of gels after second dimension electrophoresis was performed to detect individual proteins containing MDA (Figure 4.5A), HNE adducts (Figure 4.6A) and nitrotyrosine (Figure 4.7A). The corresponding change in percent density for protein modification is expressed relative to young protein density and is shown in Figures 4.5B, 4.5C, 4.6B, 4.6C, and 4.7B. Duplicate second dimension gels were run simultaneously for each immunoblot and used for identification of modified proteins by MALDI-TOF-TOF summarized in Table 4.2. Anti-DNP immunoblots to detect carbonylated proteins showed no age-associated differences in modification levels (data not shown). All experiments were performed twice to check for accuracy of results and showed nearly identical results. This along with results from Figures 4.3 and 4.4 confirmed that the results we obtained from immunoblots to detect oxidative modifications were valid and led us to make further conclusions as discussed below.

Oxidatively modified proteins of Complex I

CI proteins that contained MDA adducts are shown in Figure 4.5A and include Fe-S subunit 1 (NDUFS1, band 1), Fe-S subunit 2 (NDUFS2, band 4), 18 kDa IP subunit (NDUFS4, band 5), and 14.5 kDa subunit (NDUFC2, band 6). With respect to age, all

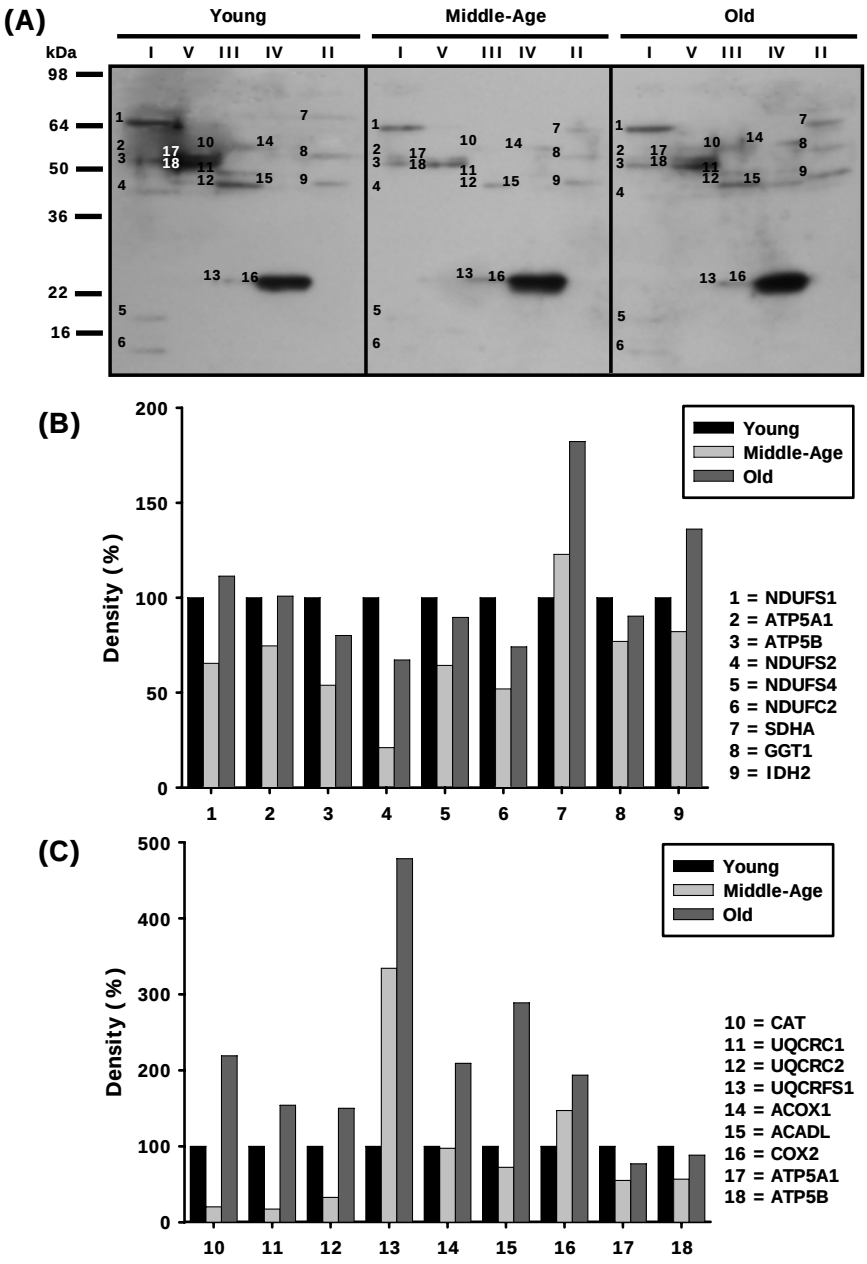


Figure 4.5. Identification of MDA-modified proteins of young, middle-aged and old kidney mitochondrial ETC complex subunits

Kidney mitochondrial ETC complexes were resolved into individual subunits as described in Experimental Procedures followed by immunoblotting. (A) Immunoblot of young, middle-aged and old kidney mitochondrial ETC complex subunits using anti-

MDA antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Experimental Procedures. Normalized density values of each individual protein modified by MDA are plotted as a percentage of the young kidney protein density for all five ETC complex subunits. (B) Densitometry for modified proteins found in CI (1-6) and CII (7-9). (C) Densitometry for modified proteins found in CIII (10-12), CIV (13-16) and CV (17 & 18). Identification of each numbered band is summarized in Table 4.2.

CI proteins bearing MDA adducts show similar profiles: MDA modification is decreased at 12-14 months and show equal or slightly lower modification than young in 20-22 month kidneys (#1, #4, #5, #6 – Figure 4.5B). In addition, NDUFS1 also contained HNE adducts which increased five-fold by middle age and eight-fold by old age (band 1 – Figure 4.6A and #1 – Figure 4.6B). Except for the 14.5 kDa subunit, all of the modified CI proteins are part of the iron-sulfur protein (IP) region. It is of particular interest to note, however, that the α chain and β chain of CV also co-migrated with CI. The CI-associated α chain contained both lipid peroxidation adducts, MDA and HNE (ATP5A1, band 2 – Figures 4.5A & 4.6A), while the CI-associated β chain was modified by MDA and nitration (ATP5B, band 3 – Figure 5A, band 1 – Figure 4.7A).

Oxidatively modified proteins of Complex II

Complex II proteins containing MDA and HNE adducts include subunit 1 (SDHA, band 7 – Figure 4.5A) and subunit 2 (SDHB, band 5 – Figure 4.6A), respectively. Compared to young protein, the modification of SDHA by MDA increased by 23% at middle age and by 82% in old age (#7 – Figure 4.5B). In contrast, the extent of modification of SDHB subunit decreased with aging (#5 – Figure 4.6B). SDHA spans through the inner mitochondrial matrix and more than half of the protein is exposed to the matrix where it houses the FAD co-factor and the active site for substrate binding. However, SDHB is mostly embedded in the inner mitochondrial membrane.

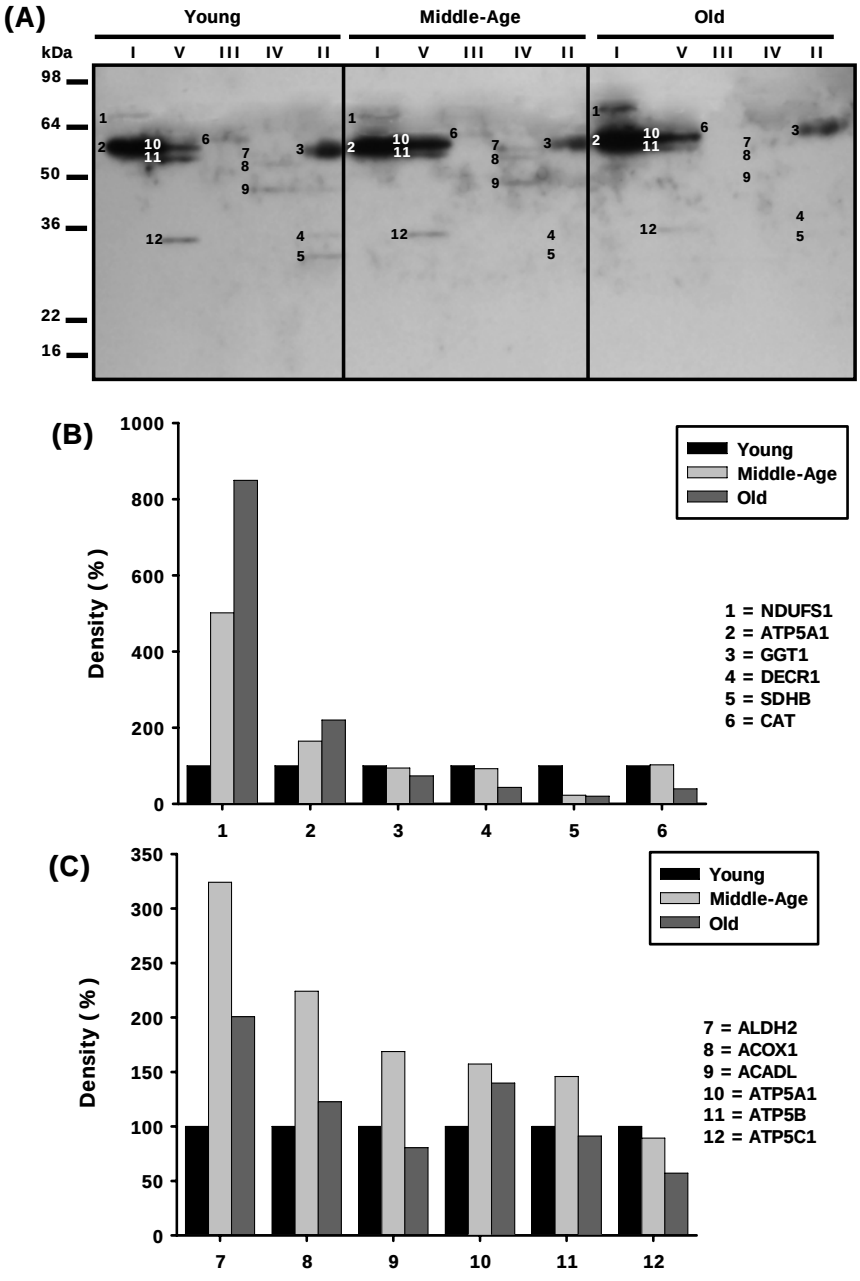


Figure 4.6. Identification of HNE-modified proteins of young, middle-aged and old kidney mitochondrial ETC complex subunits

Kidney mitochondrial ETC complexes were resolved into individual subunits as described in Experimental Procedures followed by immunoblotting. (A) Immunoblot of young, middle-aged and old kidney mitochondrial ETC complex subunits using anti-HNE antibody. Modified proteins were numbered according to their complex localization

followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Experimental Procedures. Normalized density values of each individual protein modified by HNE are plotted as a percentage of the young kidney protein density for all five ETC complex subunits. (B) Densitometry for modified proteins found in CI (1 & 2), CII (3-5), and CIII (6). (C) Densitometry for modified proteins found in CIV (7-9 and CV (10-12). Identification of each numbered band is summarized in Table 4.2.

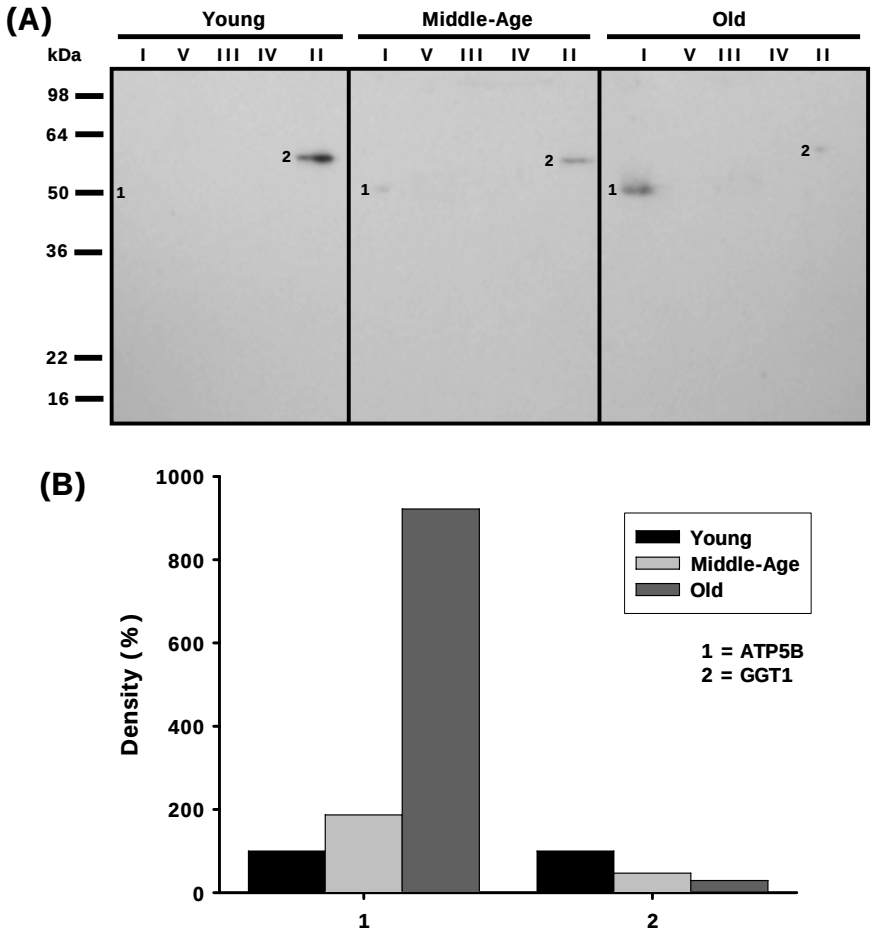


Figure 4.7. Identification of nitrotyrosine-modified proteins of young, middle-aged and old kidney mitochondrial ETC complex subunits

Kidney mitochondrial ETC complexes were resolved into individual subunits as described in Experimental Procedures followed by immunoblotting. (A) Immunoblot of young, middle-aged and old kidney mitochondrial ETC complex subunits using anti-nitrotyrosine antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins.

Protein loading was normalized using complex-specific antibodies as described in Experimental Procedures. Normalized density values of each individual protein modified by nitrotyrosine are plotted as a percentage of the young kidney protein density for all five ETC complex subunits. (B) Densitometry for modified proteins found in CI (1) and CII (2). Identification of both bands is summarized in Table 4.2.

Thus, this topographical arrangement may influence the differential profile of modification of CII subunits. It is interesting that several other modified proteins were found to co-migrate with CII. These include gamma-glutamyltransferase (GGT1), isocitrate dehydrogenase 2 (IDH2), and 2, 4-dienoyl CoA reductase 1 (DECR1). Although no age-related increase was seen, GGT1 showed anti-MDA, anti-HNE and anti-nitrotyrosine immunoreactivity (band 8 – Figure 4.5A and #8 – Figure 5B; band 3 – Figure 4.6A and #3 – Figure 4.6B; and band 2 – Figure 4.7A and #2 – Figure 4.7B, respectively); whereas IDH2 showed an increase in MDA modification (band 9 – Figure 4.5A and #9 – Figure 4.5B) at old age compared to young and DECR1 showed a decrease in HNE modification (band 4 – Figure 4.6A and #4 – Figure 4.6B) at old age compared to young. Except for GGT1, CII-associated proteins detected by immunoreactive blots are located inside the mitochondrial matrix and are associated with the inner mitochondrial membrane.

Oxidatively modified proteins of Complex III

The MDA-modified CIII proteins are shown in Figure 4.5A and include Core 1 (UQCRC1, band 11), Core 2 (UQCRC2, band 12), and the Rieske Iron-Sulfur Protein (ISP or UQCRFS1, band 13). While Core 1 and Core 2 show similar profiles of modification (compared to young age they show a 4-fold decrease in middle age and an increase of 50% by old age, #11 & #12 – Figure 4.5C), ISP shows a three-fold increase in middle age and a five-fold increase by old age compared to young kidney (#13 – Figure 4.5C). Both Core 1 and Core 2 proteins are anchored to the inner mitochondrial membrane with most of the protein exposed to the matrix side.

Table 4.2. MDA, HNE, and nitrotyrosine-modified protein subunits of mouse kidney mitochondrial electron transport chain complexes

#	Gene	ProFound Protein ID (MW)	Z score[a]	Localization
MDA-Modified (Figure 4.5A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	2.38	Complex I
2	ATP5A1	α Chain (59.7 kDa)	2.39	Complex V
3	ATP5B	β Chain (56.3 kDa)	2.41	Complex V
4	NDUFS2	Fe-S Subunit 2 (53.3 kDa)	2.40	Complex I
5	NDUFS4	18 kDa IP Subunit (18.5 kDa)	2.31	Complex I
6	NDUFC2	14.5b Subunit (14.3 kDa)	2.20	Complex I
7	SDHA	Succinate Dehydrogenase 1 (72.3 kDa)	2.38	Complex II
8	GGT1	Gamma-glutamyltransferase 1 (61.5 kDa)	2.41	Cytosol
9	IDH2	Isocitrate Dehydrogenase 2 (50.9 kDa)	2.38	Mitochondria
10	CAT	Catalase (59.8 kDa)	2.41	Peroxisome
11	UQCRC1	Core 1 (52.7 kDa)	2.40	Complex III
12	UQCRC2	Core 2 (48.2 kDa)	2.40	Complex III
13	UQCRFS1	Rieske Iron-Sulfur Protein (29.3 kDa)	2.31	Complex III
14	ACOX1	Acyl-CoA Oxidase 1 (59.9 kDa)	2.42	Peroxisome
15	ACADL	Long Chain (47.9 kDa)	2.37	Matrix
16	COX2	Subunit 2 (25.9 kDa)	2.29	Complex IV
17	ATP5A1	α Chain (59.7 kDa)	2.39	Complex V
18	ATP5B	β Chain (56.3 kDa)	2.41	Complex V
HNE-Modified (Figure 4.6A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	2.42	Complex I
2	ATP5A1	α Chain (59.7 kDa)	2.37	Complex V
3	GGT1	Gamma-glutamyltransferase 1 (61.5 kDa)	2.41	Cytosol
4	DECR1	2,4-Dienoyl CoA Reductase 1 (36.2 kDa)	2.41	Matrix
5	SDHB	Succinate Dehydrogenase 2 (31.8 kDa)	2.34	Complex II
6	CAT	Catalase (59.8 kDa)	2.41	Peroxisome
7	ALDH2	Aldehyde Dehydrogenase 2 (56.5 kDa)	2.38	Matrix
8	ACOX1	Acyl-CoA Oxidase 1 (59.9 kDa)	2.42	Peroxisome
9	ACADL	Long Chain (47.9 kDa)	2.37	Matrix
10	ATP5A1	α Chain (59.7 kDa)	2.39	Complex V
11	ATP5B	β Chain (56.3 kDa)	2.41	Complex V
12	ATP5C1	γ Polypeptide (30.2 kDa)	2.38	Complex V
Nitrotyrosine-Modified (Figure 4.7A)				
1	ATP5B	β Chain (56.3 kDa)	2.41	Complex V
2	GGT1	Gamma-glutamyltransferase 1 (61.5 kDa)	2.41	Cytosol

[a] – Z score is a ProFound database score that describes the probability and quality of the search result. For instance, a Z score of 1.65 for a search means that the search is in the 95th percentile.

ISP is completely embedded in the inner mitochondrial membrane and near the heme b_L of cytochrome b subunit where the second CoQ is thought to associate for single electron transfer (Chen et al., 2003; Han et al., 2001). The topographical arrangement of these CIII proteins and the proximity to electron transfer site may explain the differential profiles of modification for these proteins. In addition, catalase (peroxisomal) was found to co-migrate with CIII and was immunoreactive to anti-MDA showing an increase in modification at old age (band 10 – Figure 4.5A and #10 – Figure 4.5C) and to anti-HNE showing a decrease at old age (band 6 – Figure 4.6A and #6 – Figure 4.6B).

Oxidatively modified proteins of Complex IV

Subunit 2 (COX2) was the only CIV protein found to be modified by MDA (band 16 – Figure 4.5A). Modification of this subunit compared to young increased 50% at 12-14 months and further increased nearly two-fold by 20-22 months (#16 – Figure 4.5C). COX2 is an inner membrane protein that is partially localized in the matrix. Interestingly, several other proteins with oxidative modification were also found to co-migrate with CIV. These include acyl-CoA oxidase 1 (ACOX1 – peroxisomal), acetyl-CoA dehydrogenase long chain (ACADL – mitochondrial matrix), and aldehyde dehydrogenase 2 (ALDH2 – mitochondrial matrix). ACOX1 and ACADL showed an increase in anti-MDA immunoreactivity at middle age (bands 14 & 15 – Figure 4.5A and #14 & #15 – Figure 4.5C) and showed an increase in anti-HNE immunoreactivity at old age (bands 8 & 9 – Figure 4.6A and #8 & #9 – Figure 4.6C), while ALDH2 only contained HNE adducts and showed a very high increase at middle age that lowered at old age but was still higher than young age (band 7 – Figure 4.6A and #7 – Figure 4.6C).

Oxidatively modified proteins of Complex V

CV proteins that contained MDA adducts include the α and β chain (ATP5A1 & ATP5B, bands 17 & 18 – Figure 4.5A). Both proteins show a slight decrease in MDA modification in middle age compared to young but show similar levels of modification by

old age (#17 & #18 – Figure 4.5C). The α and β chains were also HNE- modified (ATP5A1 & ATP5B, bands 10 & 11 – Figure 4.6A) and HNE modification levels increased by 50% in middle age compared to young and remained unchanged (α chain, #10 – Figure 4.6C) or returned to the basal level (β chain, #11 – Figure 4.6C) by old age. In addition, the γ polypeptide of CV (ATP5C1, band 12 – Figure 4.6A) was also modified by HNE and the level of modification decreased with age (#12 – Figure 4.6C).

Nearly all oxidized proteins detected in these studies are either known to associate with, or are embedded in the inner mitochondrial membrane. With the exception of the Rieske iron-sulfur protein, all of the oxidatively modified ETC complex subunits are partly or fully exposed to the mitochondrial matrix. The location of these oxidized subunits is consistent with the possibility that ROS generated by dysfunctional ETC complexes may react with the surrounding mitochondrial membranes thereby causing increased lipid peroxidation, and that these lipid peroxidation products then modify neighboring membrane-bound or membrane- associated subunits of various complexes. In addition, these studies demonstrate the differential targeting susceptibility of specific ETC subunits to oxidative damage of the ETC complexes resulting in unfavorable physiological consequences to the enzyme activities. Furthermore, the decline in enzyme function with aging and no change in substrate availability such as CoQ show that these deficiencies in complex activities are most likely due to increase in oxidative damage of its components. Ultimately, these modifications may lead to further increases in ROS production, thus initiating a vicious cycle of increasing oxidative damage and further deterioration in normal mitochondrial function.

DISCUSSION

Our studies identified oxidatively modified mouse kidney mitochondrial ETC proteins. The levels of their oxidative modification were found to increase with age and in many cases the extent of damage was accompanied by a decrease in complex activity as is predicted by the Free Radical Theory of Aging (Wei et al., 1998; Wei, 1998).

Interestingly, these modifications did not affect the relative protein abundance of each complex whose composition remained stable across all age groups. To further understand the molecular basis of age-associated mitochondrial dysfunction, we examined whether: (a) there are age-related changes in the enzyme activities of ETC complexes; (b) specific subunits of the ETC complexes are more prone to protein oxidation by virtue of their proximity to sites of free radical production; (c) oxidative modification to ETC proteins increases with age, as would be predicted by the Free Radical Theory of Aging; and, (d) there is a correlation between the loss of enzyme function to increased oxidative modification of specific subunits of the ETC complexes.

Our inhibitor-sensitive enzyme activities show that there are age-related alterations in CI, CII, CIV and CV activities. In all cases, there was an age-related decrease in enzyme function of these ETC complexes which is consistent with our hypothesis that mitochondrial dysfunction may be due to the accumulation of oxidatively modified proteins. A similar decline in enzyme activity for CI and CII was not observed by Kwong and Sohal (Kwong and Sohal, 2000). However, those investigators measured enzyme activities at 30^{o}C compared to our assays were performed at room temperature. In addition, these investigators normalized their enzyme activities to total protein which may not be sufficient for these assays. Instead, we have presented our data as the product of the ratio of citrate synthase activity in young/middle aged, as well as in young/old age times each ETC enzyme activity, which normalizes for mitochondrial protein content. This crucial normalization replaces the normalization based solely on total protein. Our results differ, therefore, from those of Kwong and Sohal (Kwong and Sohal, 2000) in that we observed statistically significant age-associated differences in CI, CII, CIV and CV activities as opposed to their report of a decline solely in CIV activity. Numerous studies using other tissues such as brain have shown a similar decline in complex activities and thus, our combined observations support the occurrence of these mitochondrial physiological aging characteristics in many tissues (Benzi et al., 1992; Sandhu and Kaur, 2003).

Although CI activity decreases at old age, the coupled enzyme activity of CI-CIII, which increases with age, is not consistent with the decline in CI function and compared to this inconsistency, both the coupled CII-CIII activity and CII enzyme activity decline with age. Thus, the coupled CI-CIII activity may have the potential to compensate for the loss of individual CI enzyme activity with age. Such a mechanism is unique and requires further exploration. Here we propose that the increase in the coupled rate between CI and CIII in middle and old age kidney may be a compensatory mechanism that enables the cell to balance the decline in CI activity by increasing the efficiency of electron transfer between CI and CIII. Thus, the age-associated increase in CoQ levels may adjust the integrated CI-CIII coupled activity in response to the higher levels of CoQ thereby ameliorating the individual CI dysfunction. Since a ubisemiquinone intermediate is formed during the electron transfer process in CI and since this intermediate has a propensity to donate its electron to molecular oxygen to produce superoxide anion (Johnson, Jr. et al., 2003; Lenaz et al., 2002; Yano et al., 2000), the age-associated increase in CoQ levels may lead to an increase in ROS production that results in an increase in oxidative protein modification. Since the modified CI subunits are in direct proximity to the site of ROS generation, it is not surprising that these subunits are specifically targeted and increased amounts of oxidative modification leads to a decline in enzyme function.

The fact that the CII-CIII coupled activity shows no indication that it can compensate for the loss of its individual enzyme activity in CII, supports our proposal that the effect of age on CII-CIII coupled activity may be the consequence of altered structure of the modified CII subunits. Furthermore, the fact that the loss of individual CII activity is independent of CoQ levels and that there are no significant changes in complex levels suggests that the defect is upstream of the CoQ segment of the ETC pathway, i.e., possibly due to the altered structural change(s) incurred by the modification of the subunits. In addition, 1st dimension BN-PAGE did not show any significant changes in ETC complex levels by two different methods. The combined results of UQ

levels and complex levels suggest that the possible mechanism for the loss of enzyme function is not merely due to lack of substrate availability and lower level of complex present in the mitochondrial membranes.

Two of the four CII subunits were also differentially modified during aging. Perhaps, the localization of these subunits to the inner mitochondrial membrane affects their propensity to acquire lipid peroxidation adducts. The location of SDHA to the hydrophilic environment of the matrix may favor its modification by ROS-induced intermediates whereas SDHB, which is mostly embedded in the mitochondrial membrane, is less exposed to the hydrophilic environment. These differences in localization may lead to the differential susceptibility of particular subunits to oxidative modification, which is consistent with the fact that SDHC and SDHD, which are both fully embedded in the mitochondrial membrane, are not modified. Our data, therefore, suggest that accessibility may play a key role in the ROS-mediated modification of these subunits. The continuous decline of inhibitor-sensitive activity of CII as well as the coupled CII-CIII activity reflects the pattern of continuous increase in modification of SDHA with aging. The positive correlation between the increase in oxidative modification and the decrease in coupled and overall complex activity suggests that this modification affects either the substrate binding or the electron transfer ability of SDHA, or both. This is consistent with the observation that deficiency of and mutation in SDH subunits are known to cause severe diseases in humans and may thus support the argument that modifications in SDHA can lead to structural and functional changes similar to those caused by genetic mutations (Hall et al., 1993; Rustin et al., 2002). Thus, we show for the first time that the increase of in vivo oxidative modification of SDHA subunit directly correlates to an age-associated functional deficiency in CII.

Surprisingly, in spite of a five-fold increase in HNE modification of NDUFS1 between young and middle age there is no change in CI activity. Perhaps, the particular type and/or site of protein modification determines whether enzyme activity is affected, rather than the presence of adducts per se. Further understanding of the mechanisms

affecting activity must await localization of modified amino acid residues, and how they affect protein structure and function. The decrease (15-20%) in CI activity, which occurs between middle and old age, parallels the increase in both lipid peroxidation modifications to specific subunits of CI suggesting that either a threshold in the number of sites modified has been exceeded, or structural changes may expose additional sites for further modification.

The decline in enzyme functions of ETC complexes may play a key role in establishing the physiological properties of mitochondrial dysfunction in the aged kidney. Oxidative damage to the ETC complexes in vitro leads to a decline in enzyme function (Bautista et al., 2000; Chen et al., 1998; Chen et al., 2001; Lashin et al., 2006; Murray et al., 2003; Picklo et al., 1999), and this correlates with the amount of damaging adduct such as HNE. These observations further support our hypothesis that the loss of complex function in the aging kidney mitochondria is in part due to the increase in oxidative modification of subunits of individual ETC complexes. The failure of the aged kidney to turn over these modified proteins, thus, leads to mitochondrial dysfunction of the aged kidney. Our studies suggest, therefore, that the altered ability to replace oxidatively damaged proteins points to a defect in turnover, and results in accumulation of these proteins leading to mitochondrial dysfunction.

The same ETC complex subunits are differentially modified in three age groups, suggesting that these proteins are particularly susceptible to oxidative damage. CI is the rate-limiting enzyme in oxidative phosphorylation, and thus modification of its subunits may have a direct impact on the overall energy state of the cell. Three CI subunits that were oxidatively modified are components of the Iron Protein (IP) region that are located in the mitochondrial matrix. Interestingly, since the CI iron-sulfur clusters donate electrons to molecular oxygen in vitro (Johnson, Jr. et al., 2003; Lenaz et al., 2002), we propose that modifications of these proteins may cause ROS generation via electron leakage at these sites. Furthermore, their proximity to the site of ROS production makes them prime targets for oxidative damage. The increase of HNE-modified NDUFS1 with

age suggests a specificity of this subunit to lipid peroxidation. We conclude, therefore, that oxidative modification may exhibit specificity for the reactants and for proteins targeted.

CIII, a key ETC enzyme, is also a major site of ROS production in vitro (Han et al., 2001; Zhang et al., 1998). Indeed, our studies have shown a strong modification of Rieske Iron-Sulfur Protein (ISP) that is likely due to its close proximity to the predicted site of ROS generation in CIII, *i.e.*, the heme b_L of the cytochrome b subunit (Zhang et al., 1998). Surprisingly, the oxidative modification of CIII subunits has no effect on its overall activity. This raises the question of why this protein can tolerate oxidative modification. Identification and localization of oxidatively modified residues may lead to further understanding of their effects on the structure and function of ISP. Furthermore, the increase in CoQ levels has no effect on CIII activity suggesting that substrate availability does not seem to affect CIII function. It is also possible that the increase in CoQ levels may favor increased ROS production since ubiquinone has been known to form ubisemiquinone in CIII (Chen et al., 2003; Zhang et al., 1998). Furthermore, Core 1 and Core 2, members of the Mitochondrial Processing Peptidase (MPP) family, were also found to be oxidatively modified. These subunits play a dual functional role by providing structural stability to CIII subunits and by processing and folding of proteins imported into the matrix (Deng et al., 2001). Thus, a decrease of MPP activity could result in improper protein folding and mitochondrial dysfunction in kidney with aging.

The decreased enzyme activity during middle and old age in CIV also parallels the increased oxidative modification of COX2, which is the first subunit in the electron transfer pathway of CIV. COX2 contains a copper center (CuA) that mediates electron transfer between reduced cytochrome c and heme a in COX1 (Michel et al., 1998). Therefore, we propose that the loss of activity caused by oxidative damage to COX2 leads to a decline in enzyme activity which may contribute to the increased mitochondrial dysfunction in aged kidneys. This conclusion is consistent with the reports that mutations in COX2 causing loss of its activity also result in several catastrophic human genetic

diseases (Campos et al., 2001; Clark et al., 1999; Davis et al., 1997; Rahman et al., 1999; Wong et al., 2001).

F1F0-ATP synthase or CV is not coupled to the ETC processes. However, its proximity to these enzymes and its location within the matrix, as well as its abundance make it a prime candidate for oxidative modification. Although oxidative modification of the α and β chains and the γ polypeptide increased in the middle age animals, the enzyme activity was not affected. Paradoxically, in aged kidney, the CV activity decreased significantly compared to young and middle age. Since this decline does not correspond to changes in the level of oxidative damage, additional mechanisms are responsible for the decline in CV function. Surprisingly, both α and β chains co-migrated with CI during BN-PAGE which suggests that oxidative modification may facilitate their dissociation from the active complex and association with CI. Thus, the misfolding of these oxidatively modified proteins may enhance their binding to CI, which in turn may play a role in increased ROS production from CI and further damage of ETC complexes. We therefore propose that the displacement of modified α and β subunits from CV to CI may be a consequence of oxidative damage and a basic cause for the loss of activity. Additionally, the decline in CV activity may lead to decrease in ATP production, a hallmark of increasing mitochondrial dysfunction with aging.

Many of the ETC complex subunits appear to be specific targets of ROS-mediated oxidative modifications. We propose that these modifications lead to mitochondrial dysfunction and an increased state of oxidative stress in aged tissue. This is consistent with the observation that a direct correlation between increased oxidative damage and decreased enzyme function was observed for CI, CII and CIV. Although CV shows age-associated decrease in activity that cannot be attributed to oxidative damage of its subunits, it points to another causative factor for this decline in function, such as dissociation of modified subunits from the active complex and other types of oxidative modifications (e.g. S-nitrosylation, tyrosine-hydroxylation) not tested in this study. Similarly, although the function of CIII does not change a possible decline in Core 1 and

Core 2 MPP due to their modification may elicit mitochondrial dysfunction. In addition, the increase in CoQ levels with age suggests that substrate availability is not the cause of loss of enzyme function with aging and this increase in CoQ may lead to increase in ROS production from CI and CIII. We, therefore, propose that the overall effect of increased oxidative modification of components of the ETC complexes may be an underlying mechanism to the development of age-associated state-of-chronic stress and cause increased mitochondrial dysfunction leading to aging (Figure 4.8). Our study provides important insight into physiological effects of oxidative modifications on mitochondrial function and their role in aging.

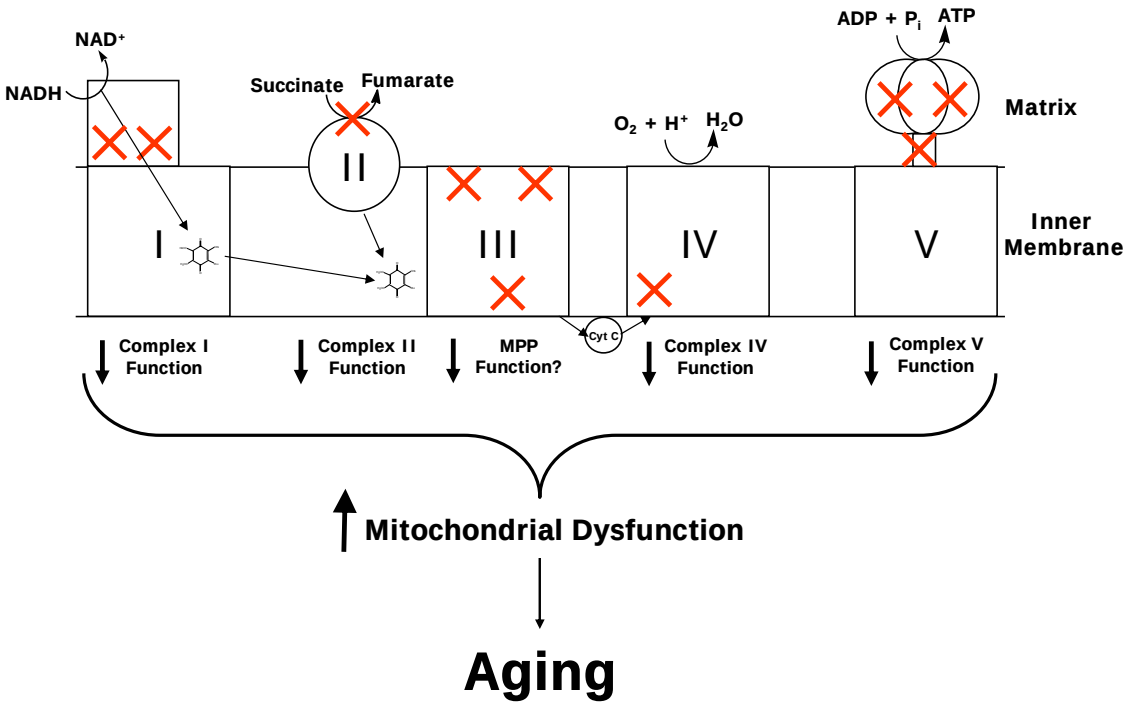


Figure 4.8. Schematic of kidney mitochondrial dysfunction and aging

The model shows the collective effects of oxidative modifications to kidney ETC complex activities leading to increasing mitochondrial dysfunction and aging. Loss of CI, CII, CIV and CV activity was observed at old age compared to both young and middle-aged complexes. Although no change in activity of CIII was noted, the mitochondrial processing peptidase (MPP) activity may be decreased with aging. X – Sites containing subunits with oxidative lesions.

CHAPTER V

AGE-RELATED ALTERATIONS IN OXIDATIVELY DAMAGED PROTEINS OF MOUSE SKELETAL MUSCLE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN COMPLEXES

SUMMARY

In this study we analyzed isolated mitochondria from young, middle-aged and old mouse skeletal muscles to determine the enzyme activities of ETC complexes I-V during aging. We chose two different skeletal muscles, pectoralis which is primarily red muscle and highly aerobic, and quadriceps which is primarily white muscle and primarily glycolytic. We chose these muscles to test the hypothesis that highly aerobic muscle is more affected by aging compared to the muscle that is primarily anaerobic during strenuous exercise. In addition, we tested the hypothesis that specific proteins of ETC complexes I-V are susceptible to oxidative damage and the levels of these modifications increase with age. The activities of all ETC complexes were measured and compared between the two muscles to determine whether there were any functional changes during aging. In addition, we identified oxidatively modified subunits of ETC complexes to compare whether the levels of oxidative modification to these complexes correlate with changes in enzyme activities. We employed blue-native polyacrylamide gel electrophoresis (BN-PAGE) to further resolve intact ETC complexes (Schagger, 1995) followed by second dimension denaturing SDS-PAGE to resolve individual complex subunits. Protein abundance of each complex was measured using complex-specific antibodies to quantify any age-related changes (Choksi et al., 2007; Venkatraman et al., 2004). Using immunoblotting with antibodies recognizing specific types of oxidative damage, we detected proteins that were carbonylated as well as modified by HNE, MDA, and nitration. Proteins shown to be differentially oxidatively damaged were identified by MALDI-TOF-TOF mass spectrometry. These studies identify specific protein subunits of the mitochondrial ETC complexes that during aging are susceptible to oxidative damage

caused by ROS-mediated protein modification. In addition, these studies show that indeed the highly aerobic skeletal muscle ETC complexes are more susceptible to functional changes compared to the skeletal muscle that is more anaerobic. Finally, there is a direct correlation between increased protein modification and decreased enzyme function for all complexes in pectoralis suggesting a progressive increase in endogenous oxidative stress during aging due to mitochondrial dysfunction in pectoralis. In contrast, although many proteins are modified in quadriceps and in some cases to higher degree compared to pectoralis, very little ETC functional changes with aging are noted suggesting that specificity of oxidative modification may play a key role in causing detrimental effects on the protein structure/function. In addition, primarily glycolytic nature of quadriceps may also play a role in possible adaptation to oxidative damage in ETC function in this skeletal muscle. In conclusion, our studies show that different types of skeletal muscles have different effects during aging whereas data from pectoralis, a primarily red muscle, support the Mitochondrial Theory of Aging as indicated by the deleterious effects of oxidatively modified ETC proteins and data from quadriceps, a primarily white muscle shows minimal changes in enzyme function with oxidative damage suggesting a more adaptive nature of this type of muscle.

RESULTS

Inhibitor-sensitive enzyme activities from pectoralis and quadriceps

To evaluate the physiological effects of normal aging on skeletal muscle mitochondrial ETC complexes, we chose two different muscles, pectoralis (~red) and quadriceps (~white) and compared the enzymatic activities of all five complexes as well as the coupled activity of CI-III and CII-III for all three ages (Figures 5.1 and 5.2).

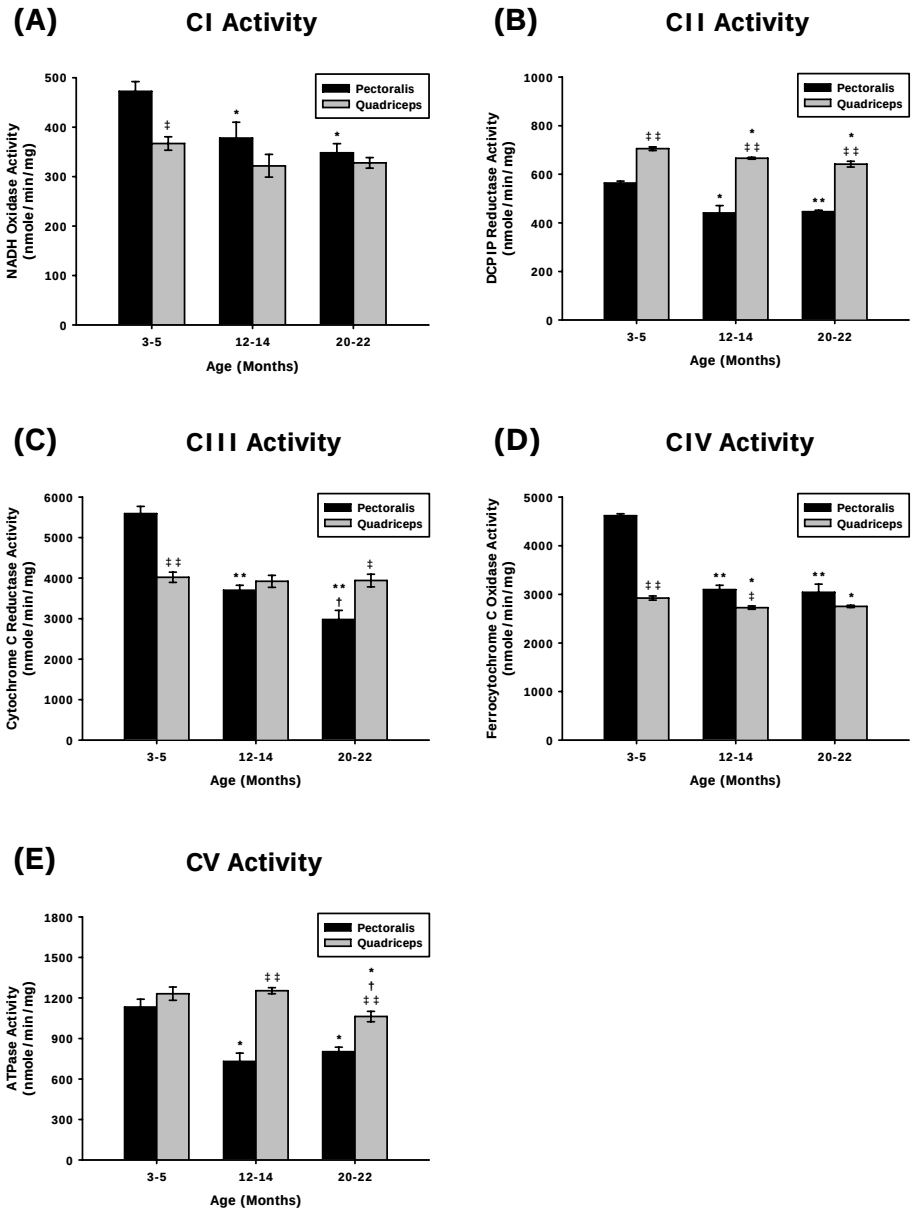


Figure 5.1. Measurement of ETC complex activities from 3-5, 12-14, and 20-22 month-old mouse skeletal muscle mitochondria

Individual complex enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the pooled sample $\pm$ SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (3-5 months), middle-aged (12-14 months), and old (20-22 months) pectoralis and quadriceps ETC CI-CV are plotted as following. (A) CI activity with aging. Coefficients of variance for pectoralis and quadriceps were

4.2% and 3.8% (young), 6.8% and 6.2% (middle-age), and 3.8% and 2.9% (old), respectively. (B) CII activity with aging. Coefficients of variance for pectoralis and quadriceps were 1.3% and 1.1% (young), 5.3% and 0.6% (middle-age), and 1% and 2.9% (old), respectively. (C) CIII activity with aging. Coefficients of variance for pectoralis and quadriceps were 3.2% and 3.1% (young), 2.2% and 3.7% (middle-age), and 4% and 3.9% (old), respectively. (D) CIV activity with aging. Coefficients of variance for pectoralis and quadriceps were 0.8% and 1.5% (young), 1.9% and 1.2% (middle-age), and 3.6% and 0.8% (old), respectively. (E) CV activity with aging. Coefficients of variance for pectoralis and quadriceps were 5.2% and 4% (young), 5.5% and 1.8% (middle-age), and 2.9% and 3.1% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, † - $p<0.05$ compared to middle-aged, †† -$p<0.001$ compared to middle-aged, ‡ - $p<0.05$ compared to pectoralis, and ‡‡ - $p<0.001$ compared to pectoralis.

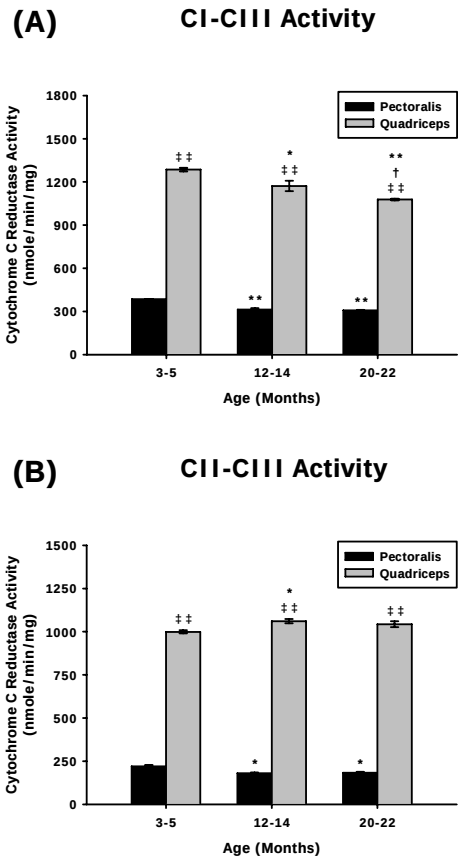


Figure 5.2. Measurement of coupled mitochondrial ETC complex activities from 3-5, 12-14, and 20-22 month-old mouse skeletal muscle mitochondria

CI-III and CII-III coupled enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the

pooled sample ± SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (3-5 months), middle-aged (12-14 months), and old (20-22 months) pectoralis and quadriceps ETC CI-III and CII-III are plotted as following. (A) CI-CIII coupled activity with aging. Coefficients of variance for pectoralis and quadriceps were 0.5% and 1% (young), 2.7% and 2.8% (middle-age), and 1% and 0.5% (old), respectively. (B) CII-CIII coupled activity with aging. Coefficients of variance for pectoralis and quadriceps were 4.7% and 0.9% (young), 1.9% and 1.3% (middle-age), and 2.8% and 1.7% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, † - $p<0.05$ compared to middle-aged, ‡ - $p<0.05$ compared to pectoralis, and ‡‡ - $p<0.001$ compared to pectoralis.

As evident from the data, in all cases the enzyme activity in pectoralis decreased from young to middle age and in some cases further decline was noted from middle-age to aged mice. In contrast, very few changes were seen in quadriceps enzyme activity with aging. Rotenone-sensitive CI activity decreased by 20% from young to middle-age in pectoralis and only slightly decreased from middle-aged to old (Figure 5.1A). Compared to pectoralis, CI activity in quadriceps was ~22% lower at young age and though there was a slight decline of this activity with age it was not significant (Figure 5.1A). Similar to CI activity, there was a 20% decrease in CI-III coupled activity in pectoralis from young to middle-age and there was no further decline from middle-aged to old mice (Figure 5.2A). In contrast, CI-CIII coupled activity decreased continuously with aging in quadriceps though the percent decline (~9%) was not as large from young to middle-age seen in pectoralis (Figure 5.2A). In comparison, the quadriceps has more than 3-fold higher CI-CIII coupled activity suggesting a much tighter coupling between these complexes. Malonate-sensitive CII activity showed similar decline in pectoralis as CI activity such that it decreased by ~22% in middle age and no further decline was noted in old age (Figure 5.1B). In contrast, there was only ~5% decline in CII activity from quadriceps from young to middle-age and very little further decrease by old age (Figure 5.1B). In comparison, CII enzyme activity was significantly higher (~20-35%) at all ages in quadriceps compared to pectoralis. The coupled CII-III activity of pectoralis also showed an age-associated decline in activity, *i.e.*, 18% decline in middle age and no

further decline in old age (Figure 5.2B). In contrast, a slight increase (~4-5%) in coupled CII-CIII activity was seen in quadriceps with aging. When comparing the coupled CII-CIII activity between these two muscles it was evident that quadriceps demonstrated much better coupling between these complexes since this activity was ~5-fold higher at any age compared to pectoralis (Figure 5.2B). Antimycin A-sensitive CIII activity from pectoralis showed a continuous age-associated decline, *i.e.*, ~34% decline in middle age and ~47% decline by old age (Figure 5.1C) while there was no change in CIII activity in quadriceps with aging. Furthermore, in comparison, pectoralis showed a significantly higher activity of CIII at young age (~28%) and by middle age this difference disappeared. KCN-sensitive CIV activity decreased by ~34% by middle age in pectoralis and no further decline was noted in old animals (Figure 5.1D). CIV activity of quadriceps only decreased slightly (~6%) with aging. Similar pattern of higher activity at young age (~36%) was observed in this enzyme's activity in pectoralis compared to quadriceps. Oligomycin-sensitive CV activity in pectoralis again showed similar pattern as all other enzyme activities, *i.e.*, ~35% decrease in middle age and no further decline in old age (Figure 5.1E). Although no change was noted in CV activity in quadriceps in middle age compared to young age, there was ~14% decline in this enzyme's activity by old age (Figure 5.1E). In comparison, pectoralis showed a much drastic decline in CV function with age compared to quadriceps.

Overall, all enzyme activities show and age-associated decline from young to middle age in pectoralis and very little further decline is noted in all enzyme activities by old age. This is not the same in another skeletal muscle like quadriceps and it shows very little change in almost all enzyme activities with the exception of CV activity decreasing at old age and continual decline in CI-CIII coupled activity with aging. In addition, CI, CIII, and CIV activities at young age are at least 30% higher in pectoralis compared to quadriceps and level out by middle age as opposed to CII activity being higher in all ages in quadriceps compared to pectoralis. In comparison, CI-CIII and CII-CIII coupled activities are much higher in quadriceps then in pectoralis which may demonstrate a

better coupling between these complexes and may suggest an adaptive nature of this muscle type.

Coenzyme Q levels in pectoralis and quadriceps mitochondria

To determine if changes in coenzyme Q (CoQ) levels are a factor in the age-associated loss of enzyme function or better coupling seen by much higher activities in one muscle compared to the other, we measured the skeletal muscle mitochondrial CoQ levels of all three ages (Table 1). Although the CoQ_9 and CoQ_{10} homologues make up about 95% of pool present in mitochondria, CoQ_9 is the predominant form in mouse tissues (Boitier et al., 1998; Duncan et al., 2005). Thus, we measured CoQ_9 levels and used these values as a predictor of total CoQ for muscle mitochondria. Our results in Table 1 show that there is very little age-related change in CoQ_9 levels from either muscle with aging. In addition, pectoralis has 3 times more CoQ_9 per mg of mitochondria compared to quadriceps. Thus, no change in CoQ_9 levels with age suggests that the loss of enzyme activities of CI, CII, CIII and CI-CIII plus CII-CIII coupled activities in pectoralis is not due to just decreased substrate availability. Furthermore, the much enhanced coupling seen in quadriceps in not explained by changes in substrate availability, since quadriceps has 3 fold lower levels of CoQ_9. These data actually suggest that even with lower levels of CoQ_9 the coupling is better in quadriceps and supports the notion that there may be some adaptation that has occurred in this primarily glycolytic muscle type to have very little changes in its enzyme activities compared to the primarily aerobic pectoralis muscle.

Abundance of ETC complexes in young, middle-aged and old kidney mitochondria

To determine if the changes in enzyme function with aging are due to changes in enzyme levels, skeletal muscle mitochondria were isolated, solubilized and subjected to BN-PAGE to resolve intact complexes I-V. We measured the levels of ETC complexes

Table 5.1. Coenzyme Q_9 Levels in Mouse Skeletal Muscle Mitochondria with Aging

Age	Pectoralis CoQ_9 (nmol/mg)	Quadriceps CoQ_9 (nmol/mg)
Young	65.7 ± 1.9	21.4 ± 0.5 ‡‡
Middle-Age	62.9 ± 0.1	22.0 ± 0.1 ‡‡
Old	60.5 ± 1.3	20.1 ± 1.0 ‡‡

Pooled skeletal muscle mitochondrial samples were used for each age group. Coenzyme Q was extracted and quantified as described in Materials and Methods. Data are an average of three experiments ± SEM. Coefficients of variance for pectoralis and quadriceps CoQ_9 were 5.0% and 4.4% (young), 0.4% and 0.7% (middle-age), and 3.9% and 8.9% (old), respectively. ‡ ‡ - $p<0.001$ compared to pectoralis.

using immunoblotting with complex-specific antibodies. As previously described by our laboratory, the use of complex-specific antibody is a viable method to determine complex abundance and, thus, these data were used to determine whether there are age-related quantitative differences in individual complexes (Choksi et al., 2007; Venkatraman et al., 2004). Since it was previously established that immunoblotting can be used for BN-PAGE and due to fact that there is limited amount of material obtained from tissues we felt that duplicate gels were sufficient to further test our hypotheses (Choksi et al., 2007). Our results show that very few age-related changes in complex levels in both skeletal muscles mitochondria were detected by these methods (Figure 5.3). CI and CIII levels from pectoralis show only a moderate 15-25% increase in middle age (Figure 5.3A and 5.3C) which does not seem to help the dramatic decrease in enzyme activities of both individual complexes as well as their coupled activity. Though there is a decrease in immunoreactive signal in CI of quadriceps by old age, the background subtracted values do not show any differences when compared to young age. Similar result of very little change was obtained with immunoblotting for CI alone (data not shown) and thus the data shown here in Figure 5.3D was confirmed by a duplicate gel. Furthermore, since all ETC complexes are multi-protein complexes, our data do not detect possible subtle changes in other components of these complexes.

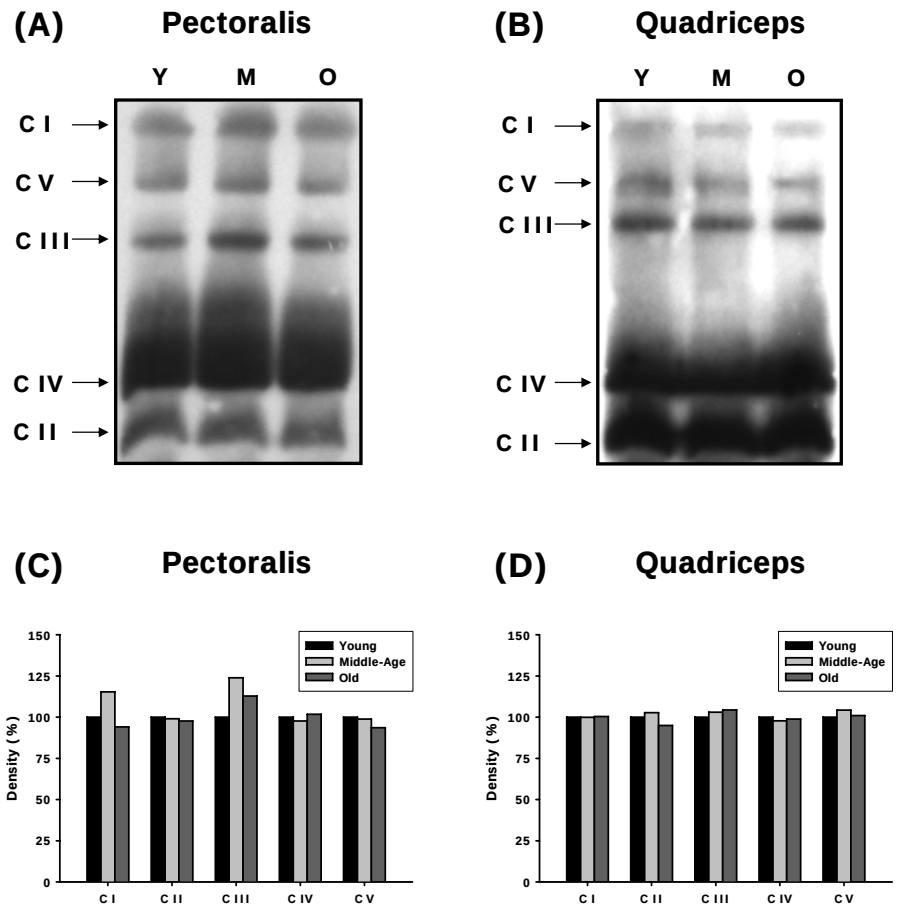


Figure 5.3. Protein abundance of ETC complexes in young, middle-aged and old skeletal muscle mitochondria

Young, middle-aged and old skeletal muscle mitochondria (160 μg) were solubilized and the ETC complexes were separated on a BN-PAGE as described in Materials and Methods. (A) Immunoblot of pectoralis BN-PAGE using complex-specific antibodies. Lane 1, 2 and 3 represent young, middle-aged and old pectoralis mitochondrial ETC complexes, respectively. (B) Density values of each pectoralis ETC complex band are plotted as a percentage of young complexes. (C) Immunoblot of quadriceps BN-PAGE using complex-specific antibodies. Lane 1, 2 and 3 represent young, middle-aged and old quadriceps mitochondrial ETC complexes, respectively. (D) Density values of each quadriceps ETC complex band are plotted as a percentage of young complexes. Y = young mitochondria, M = middle-age mitochondria and O = old mitochondria.

Oxidative modification of ETC complex subunits with aging

To identify the oxidatively modified ETC complex proteins from pectoralis and quadriceps, immunoblotting of second dimension gels was performed to detect individual proteins with carbonylation (Figures 5.4A and 5.5A, respectively), HNE (Figures 5.6A and 5.7A, respectively), nitrotyrosine (Figures 5.8A and 5.9A, respectively) and MDA (Figures 5.10A and 5.11A, respectively) adducts. The corresponding change in percent density for protein modification is expressed relative to young protein density and is shown in Figures 5.4B, 5.4C, 5.5B, 5.6B, 5.7B, 5.8B, 5.9B, 5.10B and 5.11B. Duplicate second dimension gels were run simultaneously for each immunoblot and used for identification of modified proteins by MALDI-TOF-TOF summarized in Table 5.2 from pectoralis and Table 5.3 from quadriceps. All experiments were performed twice to check for accuracy of results and showed almost identical results. This along with results from Figure 3 confirmed that the results we obtained from immunoblots to detect oxidative modifications were valid and led us to make further conclusions as discussed below.

Oxidatively modified proteins of Complex I

CI proteins from pectoralis that contained carbonyl adducts is shown in Figure 5.4A and was identified as the Fe-S subunit 1 (NDUFS1, band 1). With respect to age, this protein shows an increase in carbonylation at 12-14 months and decrease to below young levels by old age (#1 – Figure 5.4B). In addition, NDUFS1 also contained HNE adducts which increased four-fold by middle age and declined back to young levels by old age (band 1 – Figure 5.6A and #1 – Figure 5.6B). Furthermore, NDUFS1 also was shown to be modified by MDA adducts which showed similar pattern of increased levels to three-fold in middle age and decline to young levels by old age. This subunit is part of the iron-sulfur protein (IP) region and thus bears significance that it is differentially modified with age. It is also of particular interest to note, however, that the α chain of CV also co-migrated with CI and was modified with carbonylation (band 2 – Figure 5.4A) and contained HNE (band 2 – Figure 5.6A) and nitrotyrosine (band 1 – Figure 5.8A)

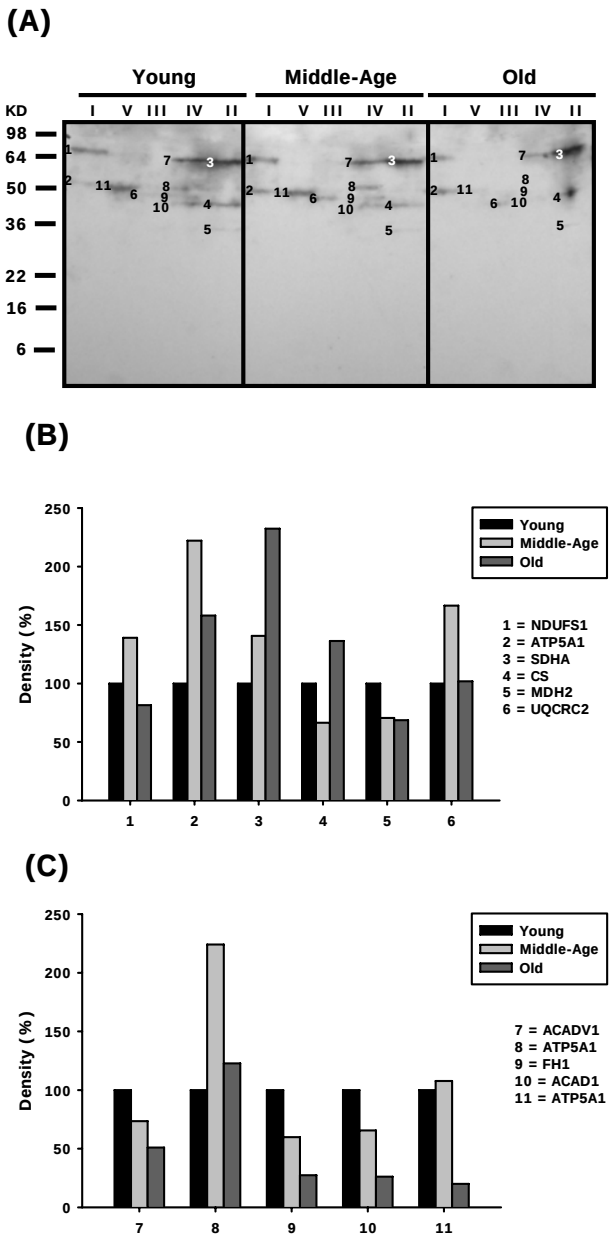


Figure 5.4. Identification of carbonylated proteins of young, middle-aged and old pectoralis mitochondrial ETC complex subunits

Pectoralis mitochondrial ETC complexes were resolved into individual subunits and DNP-derivatized after transfer to PVDF membrane as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old pectoralis mitochondrial ETC complex subunits using anti-DNP antibody. Modified

proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods. Normalized density values of each individual carbonylated protein are plotted as a percentage of the young pectoralis protein density for all five ETC complex subunits. (B) Densitometry for modified proteins found in CI (1 & 2), CII (3-5) and CIII (6). (C) Densitometry for modified proteins found in CIV (7-10) and CV (11). Identification of each numbered band is summarized in Table 5.2.

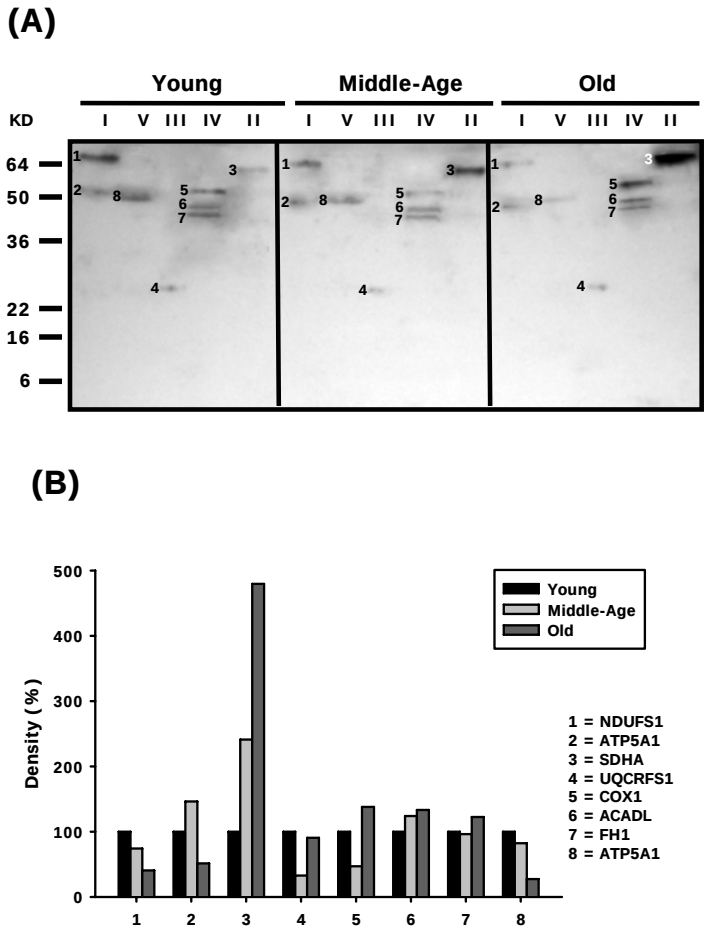


Figure 5.5. Identification of carbonylated proteins of young, middle-aged and old quadriceps mitochondrial ETC complex subunits

Quadriceps mitochondrial ETC complexes were resolved into individual subunits and DNP-derivatized after transfer to PVDF membrane as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old Quadriceps mitochondrial ETC complex subunits using anti-DNP antibody. Modified

proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods. Normalized density values of each individual carbonylated protein are plotted as a percentage of the young quadriceps protein density for all five ETC complex subunits. (B) Densitometry for modified proteins found in CI (1 & 2), CII (3), CIII (4), CIV (5-7) and CV (8). Identification of each numbered band is summarized in Table 5.3.

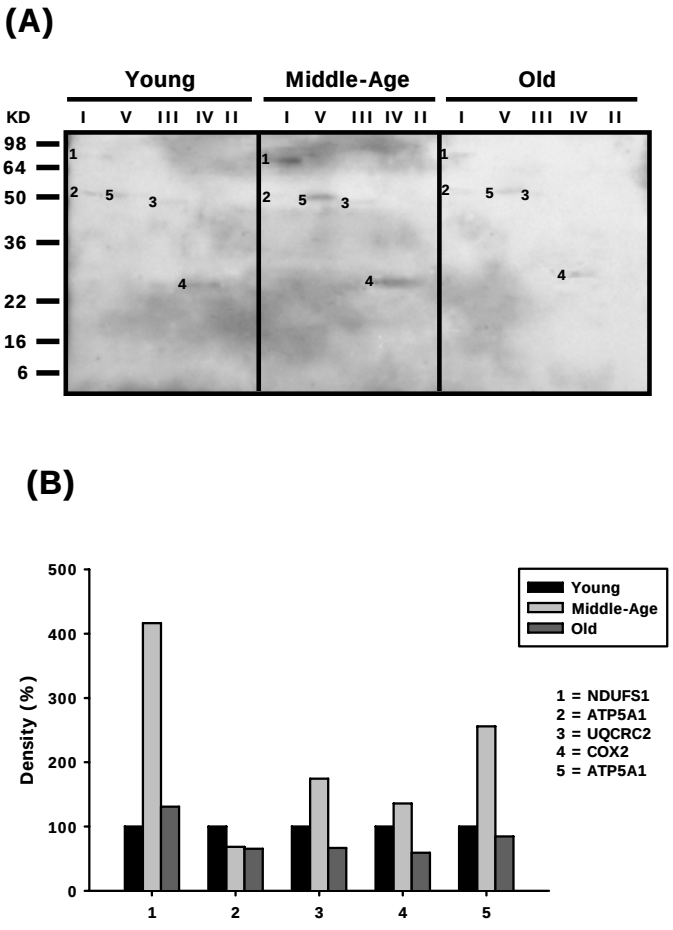


Figure 5.6. Identification of HNE-modified proteins of young, middle-aged and old pectoralis mitochondrial ETC complex subunits

Pectoralis mitochondrial ETC complexes were resolved into individual subunits as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old pectoralis mitochondrial ETC complex subunits using anti-HNE antibody. Modified proteins were numbered according to their complex localization

followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods. Normalized density values of each individual protein modified by HNE are plotted as a percentage of the young pectoralis protein density for each specific complex subunit. (B) Densitometry for modified proteins found in CI (1 & 2), CIII (3), CIV (4) and CV (5). Identification of each numbered band is summarized in Table 5.2.

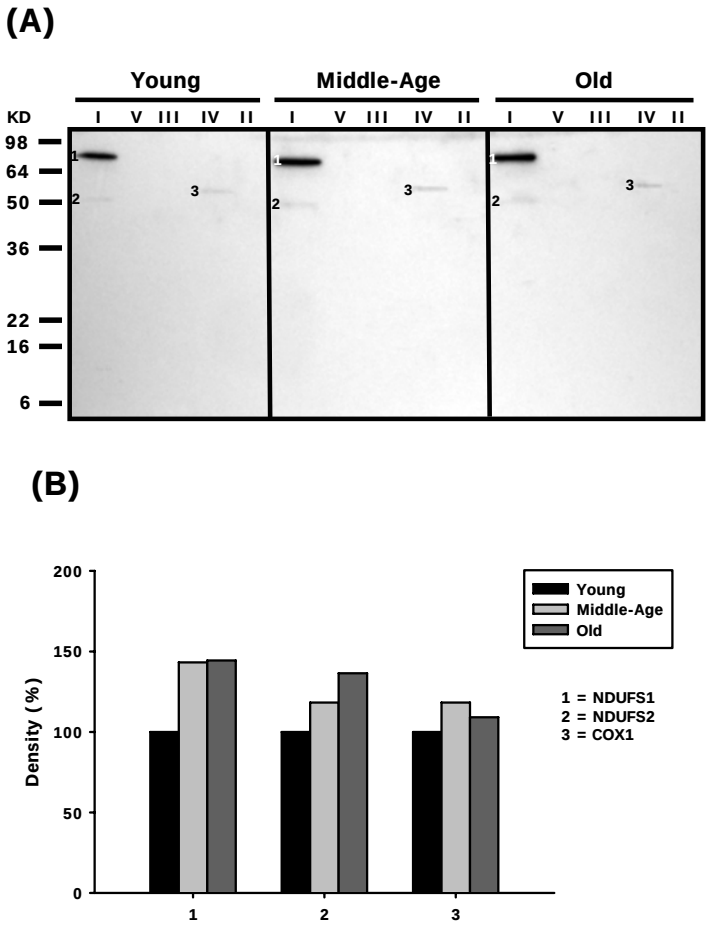


Figure 5.7. Identification of HNE-modified proteins of young, middle-aged and old quadriceps mitochondrial ETC complex subunits

Quadriceps mitochondrial ETC complexes were resolved into individual subunits as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old quadriceps mitochondrial ETC complex subunits using anti-HNE antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Materials and

Methods. Normalized density values of each individual protein modified by HNE are plotted as a percentage of the young quadriceps protein density for each specific complex subunit. (B) Densitometry for modified proteins found in CI (1 & 2) and CIV (3). Identification of each numbered band is summarized in Table 5.3.

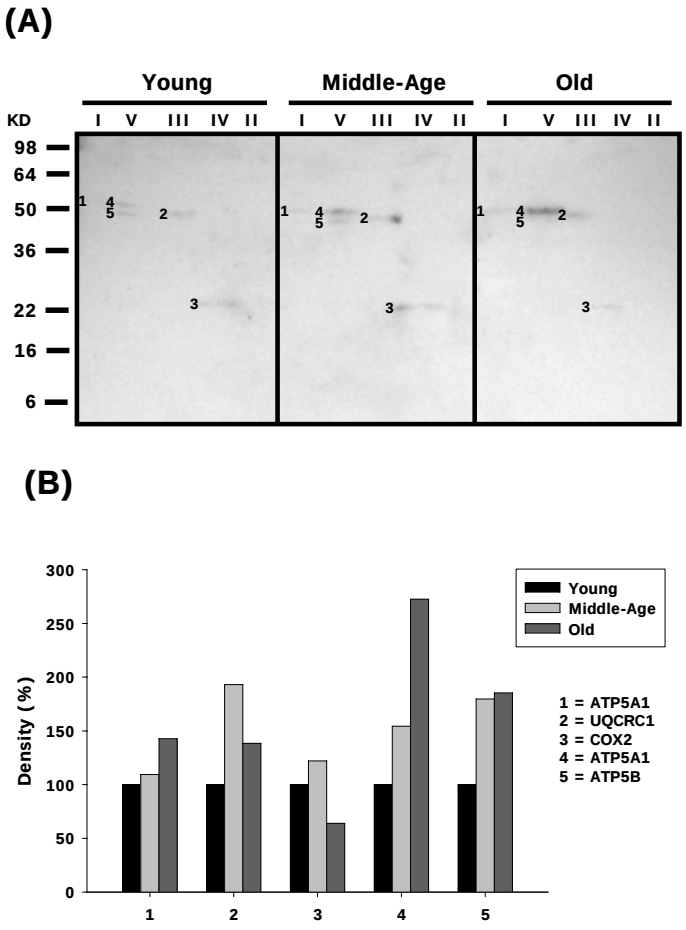


Figure 5.8. Identification of nitrotyrosine-modified proteins of young, middle-aged and old pectoralis mitochondrial ETC complex subunits

Pectoralis mitochondrial ETC complexes were resolved into individual subunits as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old pectoralis mitochondrial ETC complex subunits using anti-nitrotyrosine antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in

Materials and Methods. Normalized density values of each individual protein modified by nitrotyrosine are plotted as a percentage of the young pectoralis protein density for each specific complex subunit. (B) Densitometry for modified proteins found in CI (1), CIII (2), CIV(3) and CV (4 & 5). Identification of both bands is summarized in Table 5.2.

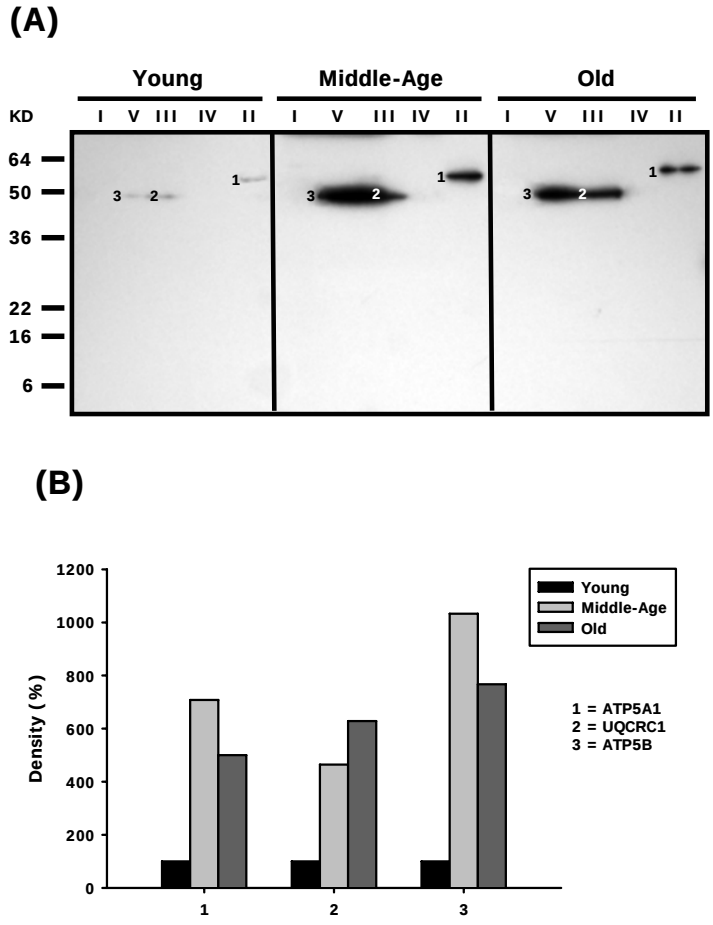


Figure 5.9. Identification of nitrotyrosine-modified proteins of young, middle-aged and old quadriceps mitochondrial ETC complex subunits

Quadriceps mitochondrial ETC complexes were resolved into individual subunits as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old quadriceps mitochondrial ETC complex subunits using anti-nitrotyrosine antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods. Normalized density values of each individual protein modified

by nitrotyrosine are plotted as a percentage of the young quadriceps protein density for each specific complex subunit. (B) Densitometry for modified proteins found in CII (1), CIII (2) and CV (3). Identification of both bands is summarized in Table 5.3.

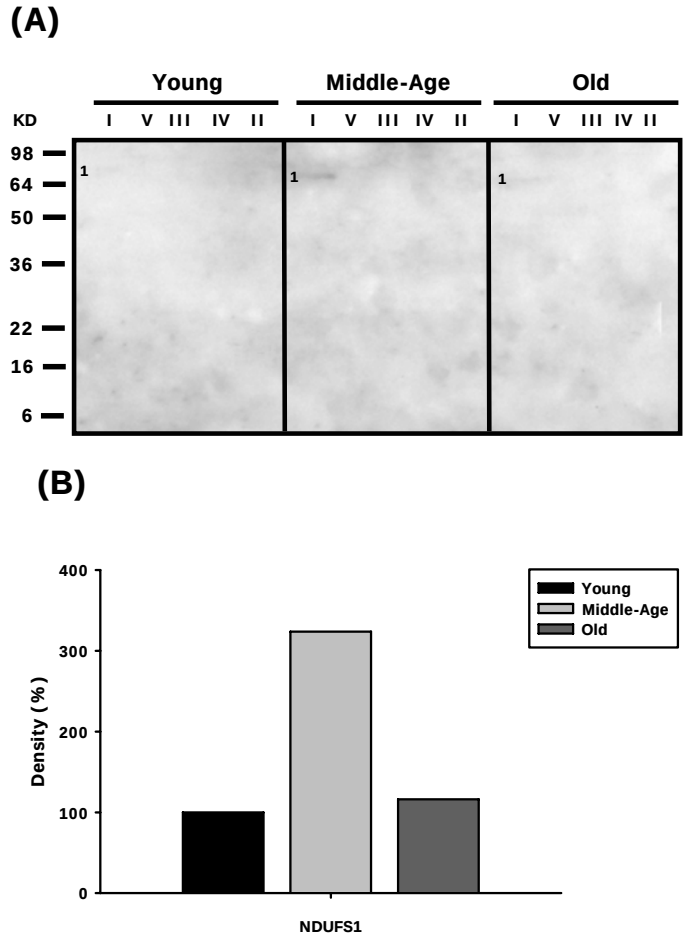


Figure 5.10. Identification of MDA-modified proteins of young, middle-aged and old pectoralis mitochondrial ETC complex subunits

Pectoralis mitochondrial ETC complexes were resolved into individual subunits as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old pectoralis mitochondrial ETC complex subunits using anti-MDA antibody. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods. Normalized density values of the individual protein modified by MDA are plotted as a percentage of the young pectoralis protein density for CI subunit. (B) Densitometry for modified protein found in CI. Identification of the CI band is summarized in Table 5.2.

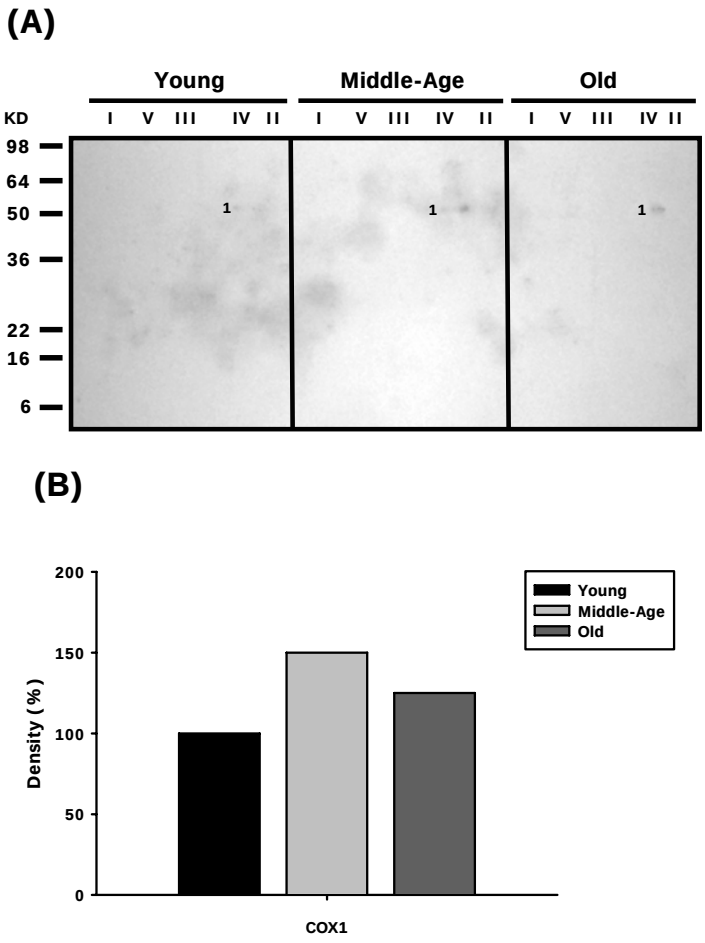


Figure 5.11. Identification of MDA-modified proteins of young, middle-aged and old quadriceps mitochondrial ETC complex subunits

Quadriceps mitochondrial ETC complexes were resolved into individual subunits as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old quadriceps mitochondrial ETC complex subunits using anti-MDA antibody. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods. Normalized density values of the individual protein modified by MDA are plotted as a percentage of the young quadriceps protein density for CIV subunit. (B) Densitometry for modified protein found in CIV. Identification of the CIV band is summarized in Table 5.3.

Table 5.2. Carbonylated and HNE, nitrotyrosine and MDA-modified protein subunits of mouse pectoralis mitochondrial electron transport chain complexes

#	Gene	ProFound Protein ID (MW)	E-value [a]	Localization
Carbonylated (Figure 5.4A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	2.5×10^{-59}	Complex I
2	ATP5A1	α Chain (59.7 kDa)	5.0×10^{-64}	Complex V
3	SDHA	Succinate Dehydrogenase 1 (72.3 kDa)	1.3×10^{-9}	Complex II
4	CS	Citrate Synthase (51.7 kDa)	4.0×10^{-21}	Mitochondria
5	MDH2	Malate Dehydrogenase 2 (35.6 kDa)	5.0×10^{-31}	Mitochondria
6	UQCRC2	Core 2 (48.2 kDa)	6.3×10^{-52}	Complex III
7	ACADV1	Acyl-CoA Dehydrogenase V1 (70.8 kDa)	3.2×10^{-9}	Mitochondria
8	ATP5A1	α Chain (59.7 kDa)	2.5×10^{-26}	Complex V
9	FH1	Fumarate Hydratase 1(50.9 kDa)	1.0×10^{-20}	Mitochondria
10	ACAD1	Acyl-CoA Dehydrogenase 1 (47.9 kDa)	1.3×10^{-17}	Mitochondria
11	ATP5A1	α Chain (59.7 kDa)	1.0×10^{-76}	Complex V
HNE-Modified (Figure 5.6A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	2.5×10^{-59}	Complex I
2	ATP5A1	α Chain (59.7 kDa)	5.0×10^{-64}	Complex V
3	UQCRC2	Core 2 (48.2 kDa)	6.3×10^{-52}	Complex III
4	COX2	Subunit 2 (25.9 kDa)	3.3×10^{-9}	Complex IV
5	ATP5A1	α Chain (59.7 kDa)	1.0×10^{-76}	Complex V
Nitrotyrosine-Modified (Figure 5.8A)				
1	ATP5A1	α Chain (59.7 kDa)	5.0×10^{-64}	Complex V
2	UQCRC1	Core 1 (52.7 kDa)	8.0×10^{-35}	Complex III
3	COX2	Subunit 2 (25.9 kDa)	3.3×10^{-9}	Complex IV
4	ATP5A1	α Chain (59.7 kDa)	1.0×10^{-76}	Complex V
5	ATP5B	β Chain (56.3 kDa)	1.3×10^{-62}	Complex V
MDA-Modified (Figure 5.10A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	2.5×10^{-59}	Complex I

[a] – Protein E-value or expectation value assigned by the Mascot database is the number of matches with equal or better scores that are expected to occur by chance alone. In each MALDI-TOF-TOF run the significance threshold was set at a more stringent value of 1.0×10^{-3} compared to the default value of 0.05. Thus, any number below the threshold was considered significant.

Table 5.3. Carbonylated and HNE, nitrotyrosine and MDA-modified protein subunits of mouse quadriceps mitochondrial electron transport chain complexes

#	Gene	ProFound Protein ID (MW)	E-value [a]	Localization
Carbonylated (Figure 5.5A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	2.9×10^{-34}	Complex I
2	ATP5A1	α Chain (59.7 kDa)	1.5×10^{-51}	Complex V
3	SDHA	Succinate Dehydrogenase 1 (72.3 kDa)	5.8×10^{-24}	Complex II
4	UQCRFS1	Rieske Iron-Sulfur Protein (29.3 kDa)	1.2×10^{-10}	Complex III
5	COX1	Subunit 1 (56.9 kDa)	9.2×10^{-17}	Complex IV
6	ACADL	Long Chain (47.9 kDa)	9.2×10^{-9}	Mitochondria
7	FH1	Fumarate Hydratase 1(50.9 kDa)	8.3×10^{-8}	Mitochondria
8	ATP5A1	α Chain (59.7 kDa)	2.9×10^{-42}	Complex V
HNE-Modified (Figure 5.7A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	2.9×10^{-34}	Complex I
2	NDUFS2	Fe-S Subunit 2 (52.6 kDa)	4.6×10^{-28}	Complex I
3	COX1	Subunit 1 (56.9 kDa)	9.2×10^{-17}	Complex IV
Nitrotyrosine-Modified (Figure 5.9A)				
1	ATP5A1	α Chain (59.7 kDa)	1.5×10^{-51}	Complex V
2	UQCRC1	Core 1 (52.7 kDa)	1.2×10^{-12}	Complex III
3	ATP5B	β Chain (56.3 kDa)	1.5×10^{-58}	Complex V
MDA-Modified (Figure 5.11A)				
1	COX1	Subunit 1 (56.9 kDa)	9.2×10^{-17}	Complex IV

[a] – Protein E-value or expectation value assigned by the Mascot database is the number of matches with equal or better scores that are expected to occur by chance alone. In each MALDI-TOF-TOF run the significance threshold was set at a more stringent value of 1.0×10^{-3} compared to the default value of 0.05. Thus, any number below the threshold was considered significant.

adducts. The CI-associated α chain also showed an-age associated increase in carbonylation levels (#2 – Figure 5.4B) and nitration levels (#1 – Figure 5.8B) but no increase in HNE levels (#2 – Figure 5.6B).

CI proteins from quadriceps that contained carbonyl adducts is shown in Figure 5.5A and was also identified as the Fe-S subunit 1 (NDUFS1, band 1). Although this protein is modified in all age groups, the level of carbonylation decreased continuously with aging (#1 – Figure 5.5B). Furthermore, NDUFS1 also contained HNE adducts (band 1 – Figure 5.7A) which showed a 50% increase in signal by middle age and no further increase in old age (#1 – Figure 5.7B). In addition, Fe-S subunit 2 (NDUFS2) was also shown to contain HNE adducts (band 2 – Figure 5.7A) and this modification increased by 25% at both the middle age and old age. No nitrotyrosine or MDA adducts were detected in CI from quadriceps. It is also of particular interest to note that similar to pectoralis the α chain of CV also co-migrated with CI and was modified with carbonylation (band 2 – Figure 5.4A). The CI-associated α chain also showed an-age associated increase in carbonylation levels at middle age compared to young and declined below young levels by old age (#2 – Figure 5.4B).

Oxidatively modified proteins of Complex II

Subunit 1 (SDHA, band 3 – Figure 5.4A) is the only protein of Complex II in pectoralis that was oxidatively modified and contained carbonylation. The modification of SDA was seen in all ages and there was an age-related increase (~40% at middle age and ~2.5-fold at old age) in carbonylation compared to young mice. SDHA is the core subunit of CII and carries out the conversion of succinate to fumarate coupled with generation of $FADH_2$ and thus is very important protein of this complex. No lipid peroxidation adducts (HNE and MDA) or nitration was found in CII. It is also interesting that two other modified proteins were found to co-migrate with CII. These include citrate synthase (CS) and Malate dehydrogenase 2 (MDH2), both the enzymes of citric acid cycle that are located inside the mitochondrial matrix and are associated with the inner mitochondrial membrane. Both proteins were carbonylated and showed differential profile such that CS showed an increase in modification in old age by ~45% compared to young mice while MDH2 showed an age-related decrease in modification.

In quadriceps, SDHA was also modified by carbonylation (band 3 – Figure 5.5A) and showed a dramatic increase to 2.5-fold and almost 5-fold higher modification in middle age and old age compared to young, respectively (#3 – Figure 5.5B). No additional modifications, such as lipid peroxidation adducts (HNE and MDA) or nitration, were seen in CII proteins from quadriceps. However, it was found that the α subunit of CV co-migrated with CII (band 1 – Figure 5.9A). The α subunit was nitrotyrosine-modified and showed a dramatic increase in modification to over 6-fold in middle age and ~5-fold in old age compared to young mice (#1 – Figure 5.9B).

Oxidatively modified proteins of Complex III

The oxidatively-modified CIII proteins are shown in Figure 5.4A, 5.6A and 5.8A and include Core 1 (UQCRC1, band 2 – Figure 5.8A) and Core 2 (UQCRC2, band 6 – Figure 5.4A and band 3 – Figure 5.6A). While Core 1 is modified by nitration, Core 2 is carbonylated and contains HNE adducts. Both proteins show similar profiles of modification (compared to young age they show a 2-fold increase in middle age and a decrease to basal levels by old age, #2 – Figure 5.8B and #6 – Figure 5.4B as well as #3 – Figure 5.6B for Core 1 and Core 2, respectively). Both Core 1 and Core 2 proteins are anchored to the inner mitochondrial membrane with most of the protein exposed to the matrix side. Since CIII is a putative ROS-generating sites in mitochondria, the topographical arrangement of these CIII proteins and the proximity to electron transfer sites may explain the differential modifications for these proteins. No MDA adducts were found in CIII from pectoralis.

CIII proteins that are oxidatively damaged in quadriceps include the Rieske Fe-S subunit (ISP) and Core 1 subunit. The ISP was modified by carbonylation (UQCRFS1, band 4 – Figure 5.5A) and the Core 1 protein was found to be nitrated (UQCRC1, band 2 – Figure 5.9A). Both proteins showed very different profiles of modifications such that ISP showed a 50% decrease in modification in middle age compared to young and no change in old age whereas Core 1 showed a dramatic increase in nitration to ~5-fold in

middle age and ~6-fold by old age compared to young mice. No lipid peroxidation adducts (HNE and MDA) were seen in CIII from quadriceps.

Oxidatively modified proteins of Complex IV

Subunit 2 (COX2) was the only CIV protein in pectoralis found to be modified by both HNE (band 4 – Figure 5.6A) and nitrotyrosine (band 3 – Figure 5.8A) adducts. Each modification of this subunit showed a similar pattern, such that compared to young, the modification increased ~50% in middle age and was decreased just below basal levels by old age (#4 – Figure 5.6B and #3 – Figure 5.8B, respectively). No carbonylation or MDA adducts were found in CIV from pectoralis. Interestingly, several other proteins with oxidative modification were also found to co-migrate with CIV. These include acyl-CoA dehydrogenase very long chain 1 (ACADV1, band 7 – Figure 5.4A), α chain of CV (ATP5A1, band 8 – Figure 5.4A), fumarate hydratase 1 (FH1, band 9 – Figure 5.4A) and acetyl-CoA dehydrogenase 1 (ACAD1, band 10 – Figure 5.4A). Except for the α chain, all proteins showed an age-related decrease in carbonylation levels (#7, #9, and #10 – Figure 5.4B). Compared to young age, the α chain showed an increase in modification by ~2.5-fold in middle age which was lowered to just above basal levels by old age (#8 – Figure 5.4B).

Subunit 1 (COX1) was the only CIV protein in quadriceps found to be modified by carbonylation (band 5 – Figure 5.5A) and contained HNE (band 3 – Figure 5.7A) and MDA (band 1 – Figure 5.11A) adducts. Both lipid peroxidation adducts showed a modest increase only in middle age compared to young (#3 – Figure 7B and Figure 5.11B) while carbonylation levels were lower in middle age and slightly higher in old age compared to young mice (#5 – Figure 5.5B). No nitration was found in CIV from quadriceps. Interestingly, similar to pectoralis, additional proteins were found to co-migrate with CIV and were carbonylated. These proteins include acetyl-CoA dehydrogenase long chain (ACADL, band 6 – Figure 5.5A) and fumarate hydratase 1 (FH1, band 7 – Figure 5.5A)

and show very little changes in levels of modification with aging (#6 and #7 – Figure 5B, respectively).

Oxidatively modified proteins of Complex V

Both α and β chain of CV were found to be differentially oxidatively modified in pectoralis with aging. The α chain was carbonylated (ATP5A1, band 11 – Figure 5.4A) and contained HNE (band 5 – Figure 5.6A) and nitrotyrosine (band 4 – Figure 5.8A) adducts while the β chain was only found to be nitrated (band 5 – Figure 5.8A). All modifications of the α chain show different patterns such that there is no change in carbonylation level of α chain in middle age and lower levels are seen in old age (#11 – Figure 5.4B) whereas levels of HNE increase to ~2.5-fold in middle age and come back down to basal levels by old age (#5 – Figure 5.6B) while nitrotyrosine levels continue to rise with aging and by old age are ~3-fold higher (#4 – Figure 5.8B) compared to young age. Perhaps the differential profile of modifications found in α chain may be due to the fact that this protein in its modified form is also found to co-migrate with other ETC complexes. The β chain, on the other hand, shows a 2-fold increase in nitration and there is no further oxidative damage by old age compared to young mice (#5 – Figure 5.8B). No MDA adducts were found in quadriceps muscle ETC complexes.

Similar to pectoralis, both α and β chain of CV were found to be differentially oxidatively modified in quadriceps with aging. The α chain was carbonylated (band 8 – Figure 5.5A) and showed an age-related decrease in oxidative modification (#8 – Figure 5B). In contrast, the β chain is heavily nitrated with aging (band 3 – Figure 5.9A) and shows a ~11-fold increase in middle age and ~8-fold increase by old age (#3 – Figure 5.9B) compared to young mice. No lipid peroxidation adducts (HNE and MDA) were found in CV from quadriceps.

Nearly all oxidized proteins detected in these studies are either known to associate with, or are embedded in the inner mitochondrial membrane. With the exception of the Rieske iron-sulfur protein, all of the oxidatively modified ETC complex subunits are

partly or fully exposed to the mitochondrial matrix. The location of these oxidized subunits is consistent with the possibility that ROS generated by dysfunctional ETC complexes may react with the surrounding mitochondrial membranes thereby causing increased lipid peroxidation, and that these lipid peroxidation products then modify neighboring membrane-bound or membrane-associated subunits of various complexes. In addition, these studies demonstrate the differential targeting susceptibility of specific ETC subunits to oxidative damage of the ETC complexes from two different muscles and resulting in either unfavorable physiological consequence to the enzyme activities or no deleterious effects to the enzyme function due to an adaptive mechanism. Clearly, two very different profiles seem to emerge from pectoralis and quadriceps where most proteins are increasingly modified mainly at middle age in pectoralis and with the exception of nitration and SDHA carbonylation very little change in levels of modification are noted in quadriceps with aging. Furthermore, the decline in enzyme function with aging seen in pectoralis and no change in substrate availability such as CoQ show that these deficiencies in complex activities are most likely due to increase in oxidative damage of its components. Ultimately, these modifications may lead to further increases in ROS production, thus initiating a vicious cycle of increasing oxidative damage and further deterioration in normal mitochondrial function.

DISCUSSION

Our studies identified oxidatively modified mouse skeletal muscle mitochondrial ETC proteins. For this study we chose two different groups of skeletal muscles found in mice. These include the primarily red muscles, such as pectoralis, which contain high levels of myoglobin and hence the high oxygen carrying capacity and highly aerobic respiration compared to primarily white muscles, such as quadriceps, which contain considerably less myoglobin and tend to be anaerobic in respiratory state. We found that these two distinctly different muscles show completely different patterns for mitochondrial function with aging. Similarly, the levels of their oxidatively modified

proteins were also found to be very different. Thus, to further understand the molecular basis of age-associated mitochondrial dysfunction we examined whether: (a) there are age-related changes in the enzyme activities of ETC complexes; (b) specific subunits of the ETC complexes are more prone to protein oxidation by virtue of their proximity to sites of free radical production; (c) oxidative modification to ETC proteins increases with age, as would be predicted by the Free Radical Theory of Aging; (d) there is a correlation between the loss of enzyme function to increased oxidative modification of specific subunits of the ETC complexes; and (e) whether there are any differences in ETC function and oxidative damage seen in two distinctly different skeletal muscles found in mice. We found that, in case of pectoralis, there was an increase in level of oxidative damage with age, particularly in middle age, and extent of damage was accompanied by a decrease in complex activity as is predicted by the Free Radical Theory of Aging (Wei et al., 1998; Wei, 1998). In contrast, similar proteins were modified in quadriceps with much lower levels of modifications with few exceptions and the lower level of damage correlated with very little change in enzyme function of ETC complexes with aging. Interestingly, these modifications did not affect the relative protein abundance of each complex in either muscle whose composition remained stable across all age groups.

Our inhibitor-sensitive enzyme activities show completely different profiles for each muscle type where in pectoralis all enzyme activities of C-V and even the C-III and CII-CIII coupled activities decrease with age; there is a dramatic decline from young to middle age and very little further decline from middle to old age. On the other hand, ETC enzyme functions in quadriceps change only slightly with age. Numerous studies, in both rodents and humans, previously have looked at enzyme function of ETC complexes with aging in skeletal muscle mitochondria (Boffoli et al., 1994; Cooper et al., 1992; Kwong and Sohal, 2000; Lenaz et al., 1997; Rasmussen et al., 2003; Sugiyama et al., 1993; Torii et al., 1992; Trounce et al., 1989). However, most studies (6/7) had major flaws since none or improper markers were used to normalize mitochondrial content. Furthermore, most studies did not take inhibitor-sensitive activities into consideration and the only

study that applied proper controls (Rasmussen et al., 2003) was incomplete since they did not determine CV function as well as the CI-CIII and CII- III coupled activities. Thus, in our study we not only applied the proper methods of controlling for mitochondrial content by using ratios of citrate synthase activity from young and middle age and young and old age as well as two methods of protein determination as described in Materials and Methods. This is a crucial step as just normalizing for total protein may not be sufficient for these types of assays. In addition, the use of two different muscle groups aided our study since these two muscles show completely different profiles of changes in enzyme function with aging. The studies done by Kwong and Sohal do not specify which skeletal muscle they used (Kwong and Sohal, 2000). In addition, due to the fact that they did not normalize their activities with citrate synthase activity ratios as well as the assay conditions being slightly different (the assays were done at 30^{o}C whereas we performed our assays at 25^{o}C) our results differ from these researchers such that we observed much higher specific activities for all enzymes as well as statistically significant decline in all enzyme functions in pectoralis by middle age and very little change in all enzyme activities in quadriceps compared to their observation of an increase in enzyme activities noted in middle age of skeletal muscle (Kwong and Sohal, 2000).

In pectoralis, CI activity as well as coupled enzyme activity of CI-CIII decreases with age and the major change is seen between young and middle age. No further decline is noted from middle age to old age. This loss in enzyme function is not due to CoQ levels since these levels do not change with age. Interestingly, only one protein was found to be modified with multiple types of modifications in CI from pectoralis. This protein was identified as NDUFS1, a component of the Iron Protein (IP) region located in the mitochondrial matrix. Its modification by carbonylation, HNE and MDA showed very similar profile of increased modification in middle age compared to young tissue. In fact, the modification by HNE increased ~4-fold and was lowered back to basal levels by old age. These data corroborate that increased modification to this subunit at middle age causes a dramatic decline in CI function that leads to decrease in both CI activity as well

as CI-CIII coupled activity. Furthermore, the reverting nature of oxidative modification from middle age to old age suggests that either this protein has been further modified or replaced by protein turnover. Further oxidation to carbonyls and lipid peroxidation adducts will lead to generation of carboxylate adducts which would be unrecognizable by the antibodies used in this study and hence the decline in signal seen in old age. Protein turnover is equally possible since a threshold of oxidation may be reached such that it would trigger the replacement of this protein from the active complex for the enzyme to function properly. Further studies will elucidate the mechanism for such changes seen at old age in this protein from CI. However, the increased modification of NDUFS1 and loss of enzyme function at middle age is very well correlated for CI in pectoralis. In contrast, though CI activity in quadriceps is lower at young age compared to pectoralis, no changes are seen in its function with aging. Furthermore, the opposite is true for CI-CIII coupled activity since this activity is ~3-fold higher in quadriceps compared to pectoralis and this activity only diminishes slightly with aging. Interestingly the tighter coupling is not explained by the availability of CoQ since there is 3 times more CoQ_9 in pectoralis compared to quadriceps. Thus, quadriceps may have an adaptive mechanism leading to higher coupling and an efficient electron transfer between ETC complexes. The much higher coupled activity would also minimize the loss of electrons to generate ROS from these complexes since a ubisemiquinone intermediate is formed during the electron transfer process in CI and since this intermediate has a propensity to donate its electron to molecular oxygen to produce superoxide anion (Johnson, Jr. et al., 2003; Lenaz et al., 2002; Yano et al., 2000) , the tighter coupling as well as lower levels of CoQ_9 may lead to a decrease in ROS production in quadriceps. However, it remains to be seen whether there is actually a decrease in ROS production due to tighter coupling in quadriceps compared to pectoralis. Surprisingly, we found that NDUFS1 in CI was also modified in quadriceps by carbonylation and contained HNE adducts. However, in case of carbonylation there was age-related decline in the modification and only a modest increase of 50% in HNE adducts was noted. Though the levels of HNE adducts seem

high by looking at the signal intensities in quadriceps compared to pectoralis, the particular site of modification may be much more important than the sheer amount since there is no effect of such modification on CI function in quadriceps. In addition to NDUFS1, another protein identified as NDUFS2 which is also a component of the IP region was found to have HNE adducts and showed very little increase with age that does not seem to affect the enzyme function of CI. Perhaps, once the sites of modification are identified it may become clear why same proteins modified differentially in these two muscles would behave completely differently. Oxidative damage to the ETC complexes *in vitro* leads to a decline in enzyme function (Bautista et al., 2000; Chen et al., 1998; Chen et al., 2001; Lashin et al., 2006; Murray et al., 2003; Picklo et al., 1999), and this correlates with the amount of damaging adduct such as HNE. These observations further support our hypothesis that the loss of complex function in the aging mitochondria from a primarily aerobic skeletal muscle such as pectoralis is in part due to the increase in oxidative modification of subunits of individual ETC complexes.

Similar to CI activity, CII function in pectoralis decreases dramatically at middle age and no further decline is seen in old age. The CII-CIII coupled activity also bears same profile in pectoralis and thus supports our proposal that the loss of enzyme function in both CII and CII-CIII coupling with aging may be the consequence of altered structure of the modified CII subunits. Furthermore, the fact that the loss of individual CII activity is independent of CoQ levels and that there are no significant changes in complex levels suggests that the defect is upstream of the CoQ segment of the ETC pathway, i.e., possibly due to the structural change(s) incurred by the modification of the subunits. In addition, 1st dimension BN-PAGE did not show any significant changes in ETC complex levels. The combined results of CoQ levels and complex levels suggest that the possible mechanism for the loss of enzyme function is not merely due to lack of substrate availability and lower level of complex present in the mitochondrial membranes. In contrast, CII activity in quadriceps shows very little decline and the CII-CIII coupled activity does not decrease at all with aging. When comparing CII activity in these two

muscles, it is clearly evident that quadriceps has much higher activities in the individual complex and the coupled activity is five-fold higher suggesting that there is better coupling of CII and CIII in quadriceps. Similar to CI-CIII coupling this higher rate is not explained simply by the substrate availability since CoQ levels in pectoralis are three times higher than quadriceps. Again, the same argument can be made that the tighter coupling may be an adaptive mechanism for efficient electron transfer and would lead to lower ROS production but cannot be proven at this time. Interestingly, the same protein, SDHA, is modified by carbonylation in both tissues and the modification increases with aging. However, only pectoralis shows functional deficits in the enzyme activities and not quadriceps. In pectoralis, the carbonyl levels only increase by 40% by middle age but the enzyme function suffers drastically by even this much modification and further modification even more so from middle age to old age does not seem to cause any more damage to its enzyme function or to the coupled activity. Previously, our lab has shown that the same protein is increasingly modified in age-dependent manner that correlates well with loss of enzyme function in other tissue and the observation that deficiency in and mutation of SDH subunits are known to cause severe diseases in humans and may thus support the argument that modifications in SDHA can lead to structural and functional changes similar to those caused by genetic mutations (Hall et al., 1993; Rustin et al., 2002). Thus, we show further evidence to support our theory that the increase of *in vivo* oxidative modification of SDHA subunit directly correlates to an age-associated functional deficiency in CII from pectoralis. In quadriceps, we have not tested whether the back flow of electrons from SDHA, by which ROS is generated in CII (Bacsi et al., 2006), is affected. It is possible that though the function of CII is not affected, the increased modification of SDHA may cause increase leakage of electrons and generate higher amounts of ROS compared to pectoralis as suggested by higher levels of oxidative modification of proteins in quadriceps compared to pectoralis. Thus in future once the site of modification is identified the effects of increased oxidative modification in SDHA from quadriceps may become clear. These results lead us to further refine our hypothesis

such that it is the quality of the modification rather than the quantity, *i.e.*, the specificity of sites of modification compared to general overall modification, which may be more important to predict age-associated decline in mitochondrial function. In future, once the modifications sites are identified, the mechanism by which the protein is modified at different sites with drastically different outcomes will become clear and shed light on how specific modifications rather than random oxidative damage may play a key role in age-related tissue dysfunction.

CIII, a key ETC enzyme, is also a major site of ROS production *in vitro* (Han et al., 2001; Zhang et al., 1998). Indeed, our studies have shown that both Core 1 and Core 2 subunits from pectoralis are more highly modified in middle age. Surprisingly, only Core 1 is modified in quadriceps and this modification is dramatically increased in middle age and continues to increase by old age. In addition, the Rieske Iron-Sulfur Protein (ISP) is also differentially modified in quadriceps and the level of modification decreases only at the middle age. Modification of ISP is likely due to its close proximity to the predicted site of ROS generation in CIII, *i.e.*, the heme b_L of the cytochrome b subunit (Zhang et al., 1998). Looking In comparison, the oxidative modification shows direct correlation to increased modification of both Core proteins to loss of CIII function in pectoralis and despite the heavily modified Core 1 no change is noted in CIII function from quadriceps with aging. Core 1 and Core 2 proteins have dual function in CIII, *i.e.*, they play a key role in providing structural stability to the complex as well as they are members of the Mitochondrial Processing Peptidase (MPP) family that are involved in processing and folding of proteins imported into the matrix (Deng et al., 2001). Thus, a decrease of MPP activity could also result in improper protein folding and mitochondrial dysfunction in pectoralis with aging. Since Core 1 is heavily damaged in quadriceps, there may be a decline in MPP function leading to impairment in protein processing and increased mitochondrial dysfunction with aging that is not directly related to ETC function.

The decreased enzyme activity during middle age in CIV also parallels the increased oxidative modification of COX2 in pectoralis. COX2 is the first subunit in the electron transfer pathway of CIV and contains a copper center (Cu_A) that mediates electron transfer between reduced cytochrome c and heme a in COX1 (Michel et al., 1998). Therefore, we propose that the loss of activity caused by oxidative damage to COX2 leads to a decline in enzyme activity which may contribute to the increased mitochondrial dysfunction in the pectoralis muscle with aging. This conclusion is consistent with the reports that mutations in COX2 causing loss of its activity also result in several catastrophic human genetic diseases (Campos et al., 2001; Clark et al., 1999; Davis et al., 1997; Rahman et al., 1999; Wong et al., 2001). Interestingly, COX1, and not COX2, is modified in quadriceps and shows only modest increase in modification with aging. However, there is very little decline in enzyme function of CIV with aging in quadriceps further supporting the hypothesis that the specificity of modification sites may determine the overall outcome of such damage. In addition, these results suggest that quadriceps is more adaptive to such damage compared to pectoralis.

CV or F1F0-ATP synthase is not part of the ETC processes. However, its proximity to ROS production from these enzymes and its location within the matrix, as well as its abundance make it a prime candidate for oxidative modification. In pectoralis, increased oxidative modification of the α and β chains directly correlates to the decline in enzyme function. Additionally, the decline in CV activity may lead to decrease in ATP production, a hallmark of increasing mitochondrial dysfunction with aging. Furthermore, oxidatively modified α chain was also found to co-migrate with CI and CIV suggesting that oxidative modification may facilitate its dissociation from the active complex and association with these complexes. Thus, the misfolding of this oxidatively modified protein may enhance its binding to CI, which in turn may play a role in increased ROS production from CI and further damage of ETC complexes. The modified α chain associated with CIV may also affect the respiration of pectoralis mitochondria, which would in turn cause increased mitochondrial dysfunction. On the other hand, both α and

β chains are also modified in quadriceps but show very different profiles compared to pectoralis. The modification of α chain decreases with age and β chain is heavily nitrated in middle age and the nitration stays high in old age. Despite very dramatic modification of β chain, very little change is seen in CV function. These results yet again provide evidence to the adaptive nature of quadriceps to high amounts of damage compared to pectoralis. In addition, the heavily modified α chain was also found to co-migrate with CI. Although, no functional consequences were seen from this association of α chain to CI, the modified protein may solicit unfolded protein stress response and interfere in overall respiration as well as ROS production from CI in quadriceps.

Many of the ETC complex subunits appear to be specific targets of ROS-mediated oxidative modifications. We propose that these modifications lead to mitochondrial dysfunction and an increased state of oxidative stress in aged tissue. Our studies utilized two different skeletal muscles and our observations in pectoralis, a primarily red or aerobic muscle, show that almost all enzyme activities decrease with age, especially at the middle age. We find consistent increases in oxidatively modified subunits from the ETC complexes that directly correlate with the loss of ETC function and support the mitochondrial theory on aging in pectoralis. However, very little change in ETC function is noted in quadriceps and these observations are consistent with previously published human study (Rasmussen et al., 2003). Although there are age-related increases in oxidative modification of similar proteins in both muscles, quadriceps shows a very adaptive nature to these modifications and is not consistent with the mitochondrial theory on aging. However, loss of MPP function and possible unfolded protein stress response may still cause increase in mitochondrial dysfunction with aging in quadriceps. Our results suggest that the site-specific oxidative modification may play a key role in effects of oxidative damage to these complexes and hence mitochondrial function. Furthermore, our studies show how two different groups of skeletal muscles age completely differently and shed some light on possible mechanisms of muscle aging in humans. Therefore, our

study provides important insight into physiological effects of oxidative modifications on mitochondrial function and their role in aging.

CHAPTER VI

AGE-RELATED ALTERATIONS IN OXIDATIVELY DAMAGED PROTEINS OF MOUSE HEART MITOCHONDRIAL ELECTRON TRANSPORT CHAIN COMPLEXES

SUMMARY

In this study we analyzed isolated mitochondria from young, middle-aged and old mouse hearts to determine the enzyme activities of ETC complexes I-V during aging. We chose hearts to test the hypothesis that post-mitotic tissue such as heart is irreversibly affected by aging leading to tissue dysfunction. In addition, we tested the hypothesis that specific proteins of ETC complexes I-V are susceptible to oxidative damage and the levels of these modifications increase with age. The activities of all ETC complexes were measured to determine whether there were any functional changes during aging. In addition, we identified oxidatively modified subunits of ETC complexes to compare whether the levels of oxidative modification of these complexes correlate with changes in enzyme activities. We employed blue-native polyacrylamide gel electrophoresis (BN-PAGE) to further resolve intact ETC complexes (Schagger, 1995) followed by second dimension denaturing SDS-PAGE to resolve individual complex subunits. Protein abundance of each complex was measured using complex-specific antibodies to quantify any age-related changes (Choksi et al., 2007; Venkatraman et al., 2004). Using immunoblotting with antibodies recognizing specific types of oxidative damage, we detected proteins that were carbonylated as well as modified by HNE and nitration. Proteins shown to be differentially oxidatively damaged were identified by MALDI-TOF-TOF mass spectrometry. These studies identify specific protein subunits of the mitochondrial ETC complexes that during aging are susceptible to oxidative damage caused by ROS-mediated protein modification. In addition, these studies show that indeed heart ETC complexes show functional changes, particularly CI and CV. Finally, there is a direct correlation between increased protein modification and decreased

enzyme function for both complexes suggesting a progressive increase in endogenous oxidative stress during aging due to mitochondrial dysfunction and detrimental effects of oxidative modification on protein structure/function in mouse heart. In conclusion, our studies support the Mitochondrial Theory of Aging as indicated by the deleterious effects of oxidatively modified ETC proteins on enzyme activities leading to increased tissue dysfunction.

RESULTS

Inhibitor-sensitive enzyme activities from heart

To evaluate the physiological effects of normal aging on heart mitochondrial ETC complexes, we measured the enzymatic activities of all five complexes as well as the coupled activity of CI-III and CII-III for all three ages (Figures 6.1 and 6.2). As evident from the data, only CI and CV showed decrease in enzyme activities with aging. In contrast, CII as well as CI-CIII and CII-CIII coupled activities showed an age-related increase in enzyme function. Rotenone-sensitive CI activity decreased by ~9% at middle-age and by ~13% at old age compared to young (Figure 6.1A). In contrast, there was a ~20% increase in CI-III coupled activity in both middle and old age compared to young age (Figure 6.2A). Malonate-sensitive CII activity also showed an increase with age, i.e., CII activity increased by ~12% and ~19% at middle and old age, respectively (Figure 6.1B). Similarly, the CII-CIII coupled activity increased with age and there was ~18% increase at middle age and ~13% increase by old age (Figure 6.2B). Antimycin A-sensitive CIII activity also increased at old age (~14%, Figure 6.1C). KCN-sensitive CIV activity did not show any change with aging (Figure 6.1D). Oligomycin-sensitive CV activity showed the most dramatic decline with aging especially at middle age, *i.e.*, ~25% decrease in middle age and no further decline in old age (Figure 6.1E).

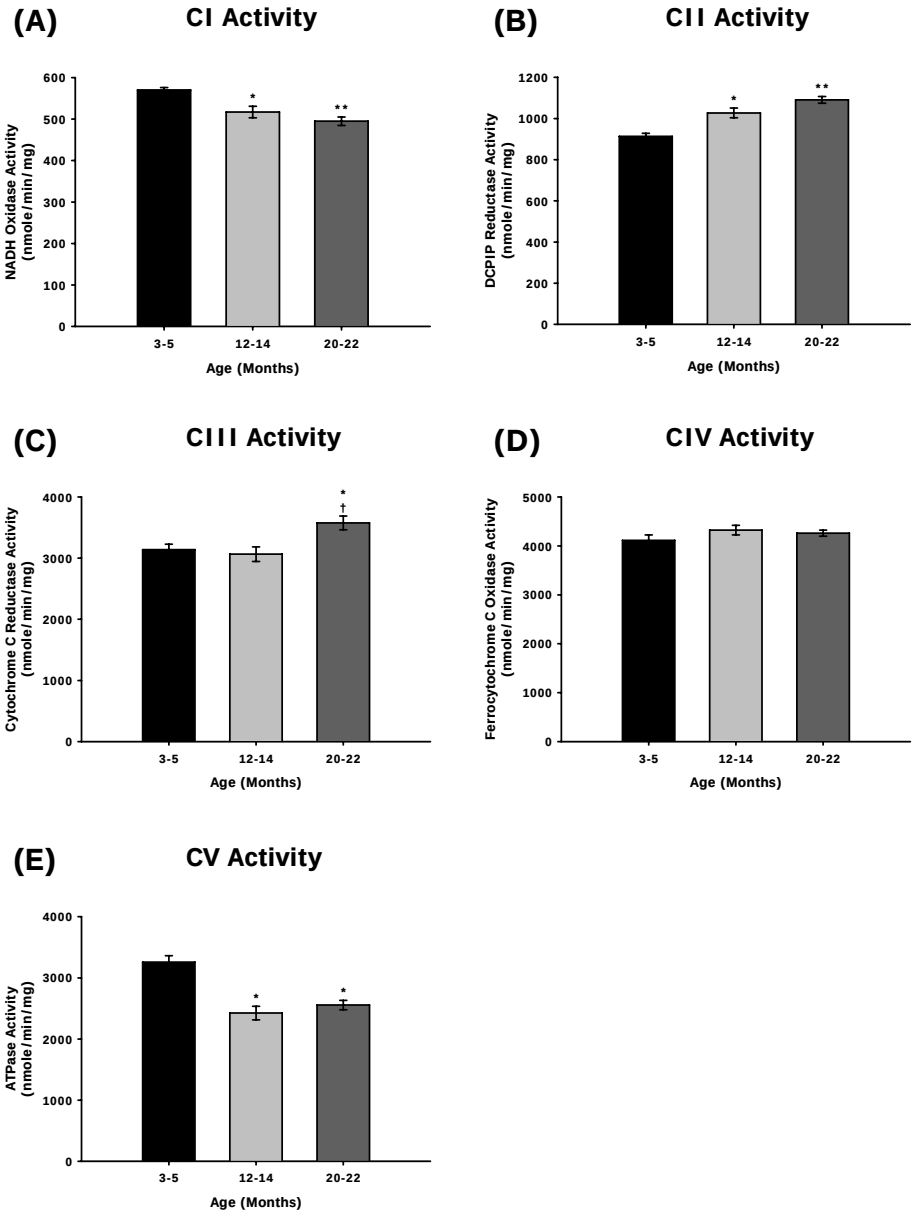


Figure 6.1. Measurement of ETC complex activities from 3-5, 12-14, and 20-22 month-old mouse heart mitochondria.

Individual complex enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the pooled sample $\pm$ SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (3-5 months), middle-aged (12-14 months), and old (20-22 months) heart ETC CI-CV are plotted as following. (A) CI activity with

aging. Coefficients of variance were 2% (young), 5.4% (middle-age), and 4.1% (old), respectively. (B) CII activity with aging. Coefficients of variance were 3.2% (young), 4.6% (middle-age), and 3% (old), respectively. (C) CIII activity with aging. Coefficients of variance were 5.7% (young), 7.8% (middle-age), and 6.3% (old), respectively. (D) CIV activity with aging. Coefficients of variance were 5.4% (young), 4.6% (middle-age), and 2.9% (old), respectively. (E) CV activity with aging. Coefficients of variance were 6.5% (young), 9.1% (middle-age), and 6% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, and † - $p<0.05$ compared to middle-aged.

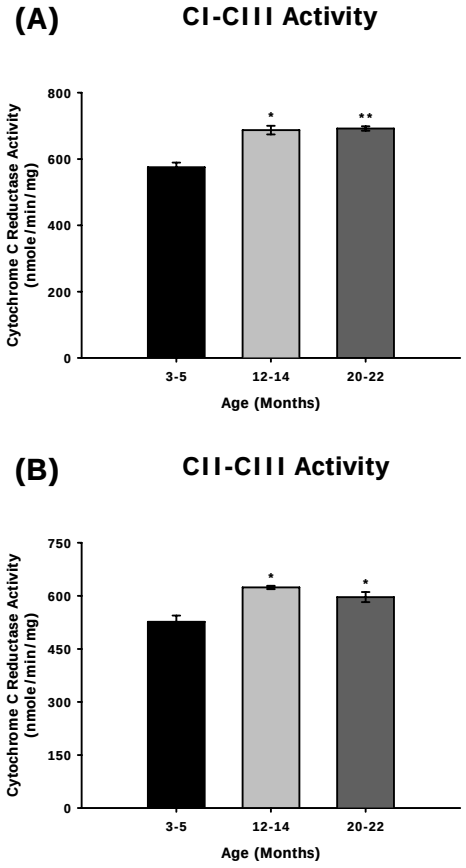


Figure 6.2. Measurement of coupled mitochondrial ETC complex activities from 3-5, 12-14, and 20-22 month-old mouse heart mitochondria

CI-III and CII-III coupled enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the pooled sample $\pm$ SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (3-5 months), middle-aged (12-14 months), and old (20-22 months) heart ETC CI-III and CII-III are plotted as following. (A) CI-CIII coupled activity with aging. Coefficients of variance were 4.9% (young),

3.8% (middle-age), and 1.9% (old), respectively. (B) CII-CIII coupled activity with aging. Coefficients of variance were 6.8% (young), 1.5% (middle-age), and 4.8% (old), respectively. * - $p<0.05$ compared to young and ** - $p<0.001$ compared to young.

Overall, ETC enzyme activities from mouse heart show a differential pattern such that CIV activity does not change with age, CII and CIII as well as CI-CIII and CII-CIII coupled activities increase with age, and CI and CV activities show an age-associated decline in enzyme function.

Abundance of ETC complexes in young, middle-aged and old heart mitochondria

To determine if the changes in enzyme function with aging are due to changes in enzyme levels, heart mitochondria were isolated, solubilized and subjected to BN-PAGE to resolve intact complexes I-V. We measured the levels of ETC complexes using immunoblotting with complex-specific antibodies. As previously described by our laboratory, the use of complex-specific antibody is a viable method to determine complex abundance and, thus, these data were used to determine whether there are age-related quantitative differences in individual complexes (Choksi et al., 2007; Venkatraman et al., 2004). Since it was previously established that immunoblotting can be used for BN-PAGE and due to fact that there is limited amount of material obtained from tissues we felt that triplicate gels were sufficient to further test our hypotheses (Choksi et al., 2007). Our results show that very little age-related changes in complex levels in heart mitochondria were detected by these methods (Figure 6.3). Though CIV and CV levels do show a modest 15-20% increase in middle age (Figure 6.3B) the changes are not statistically significant and the enzyme activities of both individual complexes do not seem to be affected. Furthermore, since all ETC complexes are multi-protein complexes, our data do not detect possible subtle changes in other components of these complexes.

Oxidative modification of ETC complex subunits with aging

To identify the oxidatively modified ETC complex proteins from mouse hearts, immunoblotting of second dimension gels was performed to detect individual proteins

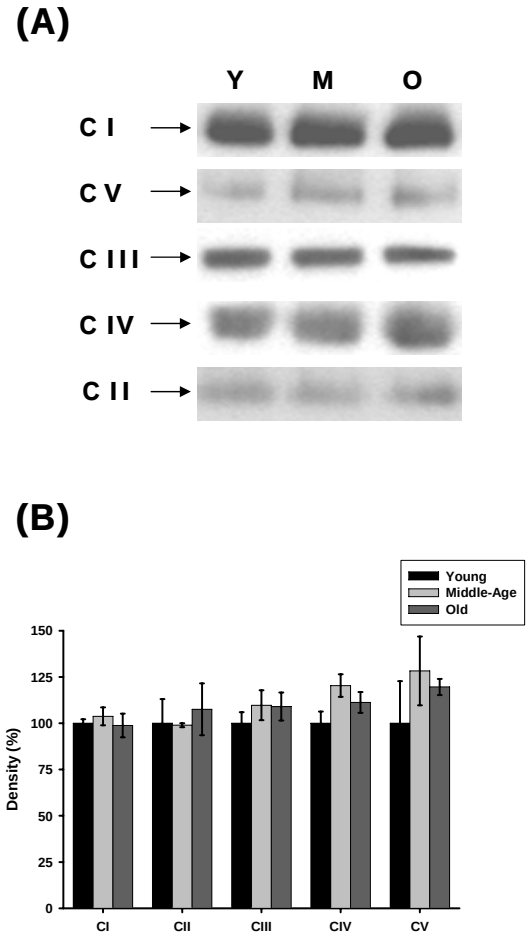


Figure 6.3. Protein abundance of ETC complexes in young, middle-aged and old heart mitochondria

Young, middle-aged and old heart mitochondria (160 μg) were solubilized and the ETC complexes were separated on a BN-PAGE as described in Materials and Methods. (A) Representative immunoblots of heart BN-PAGE using complex-specific antibodies. Lane 1, 2 and 3 represent young, middle-aged and old heart mitochondrial ETC complexes, respectively. (B) Density values of each ETC complex band are plotted as a percentage of young complexes. All results are averages of 3 immunoblot analyses from the pooled sample $\pm$ SEM for each age group. Coefficients of variance for CI were 2.2% (young), 4.6% (middle-age), and 6.5% (old), respectively. Coefficients of variance for CII were 13% (young), 1.1% (middle-age), and 13% (old), respectively. Coefficients of variance for CIII were 6% (young), 7.4% (middle-age), and 7% (old), respectively. Coefficients of variance for CIV were 6.3% (young), 5.1% (middle-age), and 5.1% (old), respectively. Coefficients of variance for CV were 22.7% (young), 14% (middle-age), and 3.6% (old),

respectively. Y = young mitochondria, M = middle-age mitochondria and O = old mitochondria.

with carbonylation (Figure 6.4A), as well as HNE (Figure 6.5A), nitrotyrosine (Figure 6.6A) and MDA (none detected) adducts. The corresponding change in percent density for protein modification is expressed relative to young protein density and is shown in Figures 6.4B, 6.5B, and 6.6B. Duplicate second dimension gels were run simultaneously for each immunoblot and used for identification of modified proteins by MALDI-TOF-TOF summarized in Table 1. All experiments were performed twice to check for accuracy of results and showed almost identical results. This along with results from Figure 3 confirmed that the results we obtained from immunoblots to detect oxidative modifications were valid and led us to make further conclusions as discussed below.

Oxidatively modified proteins of Complex I

CI protein that contained carbonyl and HNE adducts is shown in Figure 4A and Figure 6.5A and was identified as the Fe-S subunit 1 (NDUFS1, band 1). With respect to age, this protein shows a mild decrease in carbonylation (#1 – Figure 6.4B) and an increase in HNE (#1 – Figure 6.5B). Even though the oxidative modification of NDUFS1 shows very little change with age, since this subunit is part of the iron-sulfur protein (IP) region, it bears significance that it is specifically modified at all ages. In contrast, NDUFV1, part of the flavoprotein (FP) region, was heavily modified with nitration in an age-dependent manner (Figure 6.6A). The nitration of NDUFV1 increased to more than 7-fold by middle age and ~14.5-fold at old age. Again, since this protein is a component of FP region, the dramatic modification of this subunit shows that it is specifically targeted by ROS-mediated damage. It is also of particular interest to note that the α chain of CV also co-migrated with CI and was modified with carbonylation (band 2 – Figure 6.4A) and contained HNE (band 2 – Figure 6.5A) adducts. The CI-associated α chain showed an age-associated decrease in carbonylation levels (#2 – Figure 6.4B) and in HNE levels (#2 – Figure 6.5B).

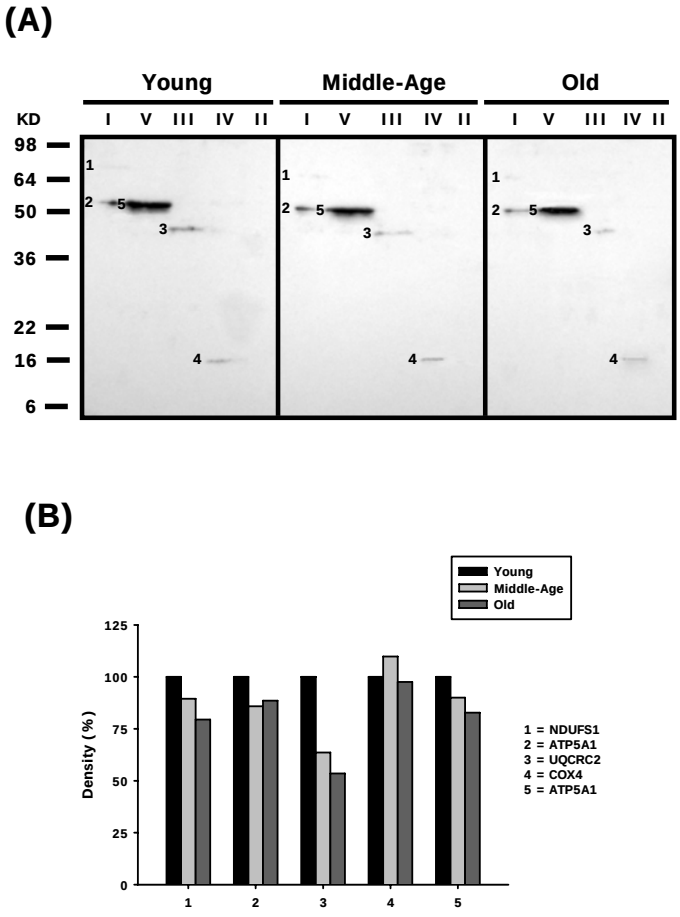


Figure 6.4. Identification of carbonylated proteins of young, middle-aged and old heart mitochondrial ETC complex subunits

Heart mitochondrial ETC complexes were resolved into individual subunits and DNP-derivatized after transfer to PVDF membrane as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old heart mitochondrial ETC complex subunits using anti-DNP antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods. Normalized density values of each individual carbonylated protein are plotted as a percentage of the young heart protein density for all five ETC complex subunits. (B) Densitometry for modified proteins found in CI (1 & 2), CII (3-5) and CIII (6). (C) Densitometry for modified proteins found in CIV (7-10) and CV (11). Identification of each numbered band is summarized in Table 6.1.

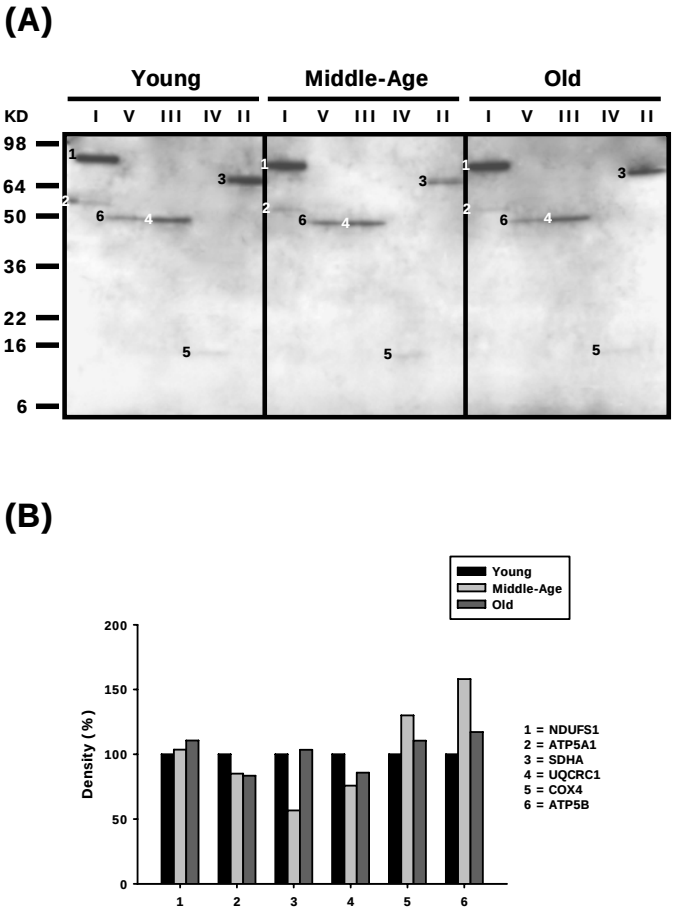


Figure 6.5. Identification of HNE-modified proteins of young, middle-aged and old heart mitochondrial ETC complex subunits

Heart mitochondrial ETC complexes were resolved into individual subunits as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old pectoralis mitochondrial ETC complex subunits using anti-HNE antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods. Normalized density values of each individual protein modified by HNE are plotted as a percentage of the young heart protein density for each specific complex subunit. (B) Densitometry for modified proteins found in CI (1 & 2), CIII (3), CIV (4) and CV (5). Identification of each numbered band is summarized in Table 6.1.

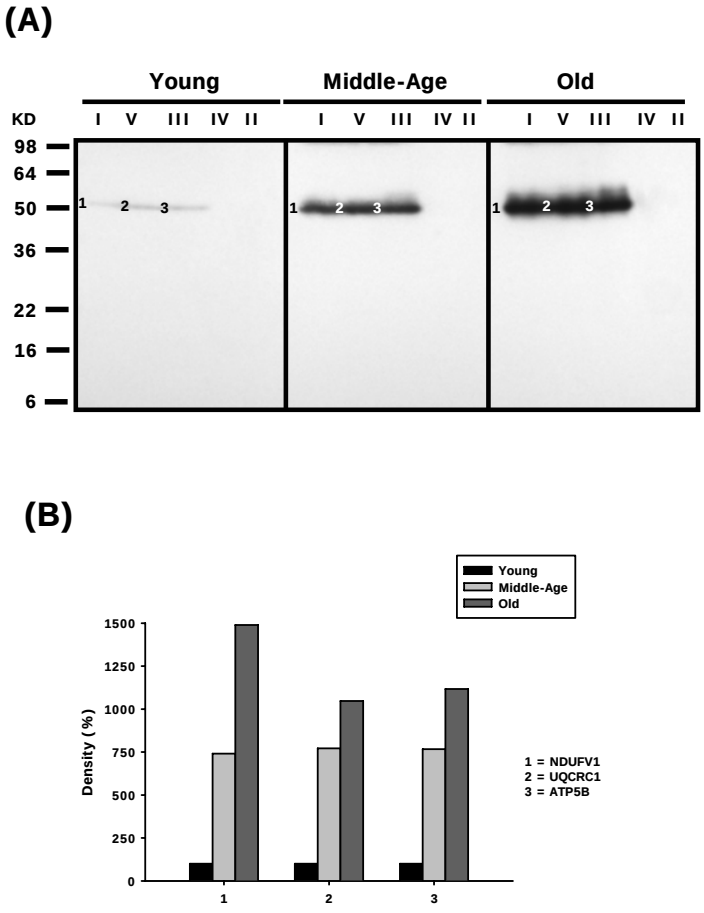


Figure 6.6. Identification of nitrotyrosine-modified proteins of young, middle-aged and old heart mitochondrial ETC complex subunits

Heart mitochondrial ETC complexes were resolved into individual subunits as described in Materials and Methods followed by immunoblotting. (A) Immunoblot of young, middle-aged and old heart mitochondrial ETC complex subunits using anti-nitrotyrosine antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described in Materials and Methods Normalized density values of each individual protein modified by nitrotyrosine are plotted as a percentage of the young heart protein density for each specific complex subunit. (B) Densitometry for modified proteins found in CI (1), CIII (2), CIV(3) and CV (4 & 5). Identification of both bands is summarized in Table 6.1.

Table 6.1. Carbonylated and HNE and Nitrotyrosine-modified protein subunits of mouse heart mitochondrial electron transport chain complexes

#	Gene	ProFound Protein ID (MW)	E-value [a]	Localization
Carbonylated (Figure 4A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	1.1×10^{-26}	Complex I
2	ATP5A1	α Chain (59.7 kDa)	1.7×10^{-56}	Complex V
3	UQCRC1	Core 1 (52.7 kDa)	5.4×10^{-34}	Complex III
4	COX4	Subunit 4 (19.5 kDa)	5.4×10^{-17}	Complex IV
5	ATP5A1	α Chain (59.7 kDa)	4.3×10^{-50}	Complex V
HNE-Modified (Figure 5A)				
1	NDUFS1	Fe-S Subunit 1 (79.7 kDa)	1.1×10^{-26}	Complex I
2	ATP5A1	α Chain (59.7 kDa)	1.7×10^{-56}	Complex V
3	SDHA	Succinate Dehydrogenase 1 (72.3 kDa)	2.2×10^{-15}	Complex II
4	UQCRC1	Core 1 (52.7 kDa)	5.4×10^{-34}	Complex III
5	COX4	Subunit 4 (19.5 kDa)	5.4×10^{-17}	Complex IV
6	ATP5B	β Chain (56.3 kDa)	1.4×10^{-48}	Complex V
Nitrotyrosine-Modified (Figure 6A)				
1	NDUFV1	Flavoprotein 1 (50.1 kDa)	5.0×10^{-64}	Complex I
2	UQCRC1	Core 1 (52.7 kDa)	8.0×10^{-35}	Complex III
3	ATP5B	β Chain (56.3 kDa)	1.4×10^{-48}	Complex V

[a] – Protein E-value or expectation value assigned by the Mascot database is the number of matches with equal or better scores that are expected to occur by chance alone. In each MALDI-TOF-TOF run the significance threshold was set at a more stringent value of 1.0×10^{-3} compared to the default value of 0.05. Thus, any number below the threshold was considered significant.

Oxidatively modified proteins of Complex II

Subunit 1 (SDHA, band 3 – Figure 6.4A) is the only protein of Complex II that was oxidatively modified and contained HNE adducts. Though the modification of SDHA was seen in all ages, there was a decrease in HNE levels at middle age (~44%) and no change in old age (#3 – Figure 6.5B) compared to young mice. Since SDHA extends into the inner mitochondrial matrix and more than half of the protein is exposed

to the matrix where it houses the FAD co-factor and the active site for substrate binding, its differential modification with lipid peroxidation suggests that it is specifically targeted by ROS-mediated damage.

Oxidatively modified proteins of Complex III

The oxidatively-modified CIII proteins are shown in Figure 6.4A, 6.5A and 6.6A and include Core 1 (UQCRC1, band 4 – Figure 6.5A and band 2 – Figure 6.6A) and Core 2 (UQCRC2, band 3 – Figure 6.4A). While Core 1 is modified by nitration and HNE, Core 2 is carbonylated. The modification of Core 2 shows an age-related decrease in carbonylation and by old age it decreases by ~ 46% compared to young mice (#3 – Figure 6.4B). Core 1, on the other hand, shows a differential profile of decreased HNE modification and very high increase in nitration with age. The HNE levels in Core 1 decrease by ~24% in middle age and ~14% at old age compared to young age (#4 – Figure 6.5B). In contrast, Core 1 is heavily nitrated in an age-dependent manner and the nitration increases to more than 7.5-fold in middle age and ~10-fold at old age. Both Core 1 and Core 2 proteins are anchored to the inner mitochondrial membrane with most of the protein exposed to the matrix side. Since CIII is one of the ROS-generating sites in mitochondria, the topographical arrangement of these CIII proteins and the proximity to electron transfer sites may explain the differential modifications for these proteins.

Oxidatively modified proteins of Complex IV

Subunit 4 (COX4) was the only CIV protein found to be modified by both carbonyl (band 4 – Figure 6.4A) and HNE (band 5 – Figure 6.5A) adducts. Each modifications of this subunit showed similar pattern such that compared to young, the modification increased by ~10% and ~30% in middle age and was decreased back to basal levels by old age (#4 – Figure 6.4B and #5 – Figure 6.5B, respectively). COX4 mainly plays a role in stability of this enzyme in the mitochondria inner membrane and is not involved in the physiological activity of CIV (Yoshikawa et al., 1998).

Oxidatively modified proteins of Complex V

Both α and β chain of CV were found to be differentially oxidatively modified with aging. The α chain was carbonylated (ATP5A1, band 5 – Figure 6.4A) while the β chain was found have both HNE (band 6 – Figure 6.5A) and nitrotyrosine (band 3 – Figure 6.6A) adducts. The α chain shows an age-related decrease in carbonylation level by ~10% in middle age and ~18% by old age (#5 – Figure 6.4B). Interestingly, carbonylated α chain was also found to co-migrate with CI and showed similar profile of modification (band 2 – Figure 6.4A and #2 – Figure 6.4B). Although, α chain is modified with HNE, the oxidatively damaged protein is not present in the intact complex and instead it is associated with CI (band 2 – Figure 6.5A). The β chain, on the other hand, shows an age-associated increase in HNE modification (band 6 – Figure 6.5A) and is heavily modified with nitration (band 3 – Figure 6.6A). The HNE levels in β chain increase to ~56% in middle age and by old age this increase is attenuated to only ~17% compared to young mice (#6 – Figure 6.5B). In contrast, the heavily nitrated β chain shows a more than 7.5-fold increase in modification in middle age and by old it further increases to more than 11-fold. Both α and β chains are involved in ATP biosynthesis that is coupled with proton translocation and are not directly proximal to electron transfer sites of ETC. Thus, the differential modification of these subunits with aging suggests that they are specific targets of ROS-mediated damage.

Nearly all oxidized proteins detected in these studies are associated with and/or are embedded in the inner mitochondrial membrane. In addition, all of the oxidatively modified ETC complex subunits are partly or fully exposed to the mitochondrial matrix. The location of these oxidized subunits is consistent with the possibility that ROS generated by dysfunctional ETC complexes may react with the surrounding mitochondrial membranes thereby causing direct oxidation to generate carbonylation, increased lipid peroxidation as well as forming peroxynitrite with the presence of mitochondrial nitric oxide synthase, and that these ROS reactions then modify

neighboring membrane-bound or membrane-associated subunits of various complexes. Furthermore, these studies demonstrate the differential targeting susceptibility of specific ETC subunits to oxidative damage of the ETC complexes from mouse hearts and resulting in either unfavorable physiological consequence to the enzyme activities or no deleterious effects to the enzyme function. Ultimately, these modifications may lead to further increases in ROS production, thus initiating a vicious cycle of increasing oxidative damage and further deterioration in normal mitochondrial function.

DISCUSSION

Our studies identified oxidatively modified mouse heart mitochondrial ETC proteins. The levels of their oxidative modification were found to increase with age and in many cases the extent of damage was accompanied by a decrease in complex activity as is predicted by the Free Radical Theory of Aging (Choksi et al., 2007; Wei et al., 1998; Wei, 1998; Yarian et al., 2005). Interestingly, these modifications did not affect the relative protein abundance of each complex whose composition remained stable across all age groups. Thus, to further understand the molecular basis of age-associated mitochondrial dysfunction we examined whether: (a) there are age-related changes in the enzyme activities of ETC complexes; (b) specific subunits of the ETC complexes are more prone to protein oxidation by virtue of their proximity to sites of free radical production; (c) oxidative modification to ETC proteins increases with age, as would be predicted by the Free Radical Theory of Aging; and (d) whether there is a correlation between the loss of enzyme function to increased oxidative modification of specific subunits of the ETC complexes in mice.

Our inhibitor-sensitive enzyme activities show that there are age-related alterations in CI, CII and CV as well as CI-CIII and CII-CIII coupled activities. In case of CI and CV, there was an age-related decrease in enzyme function of these ETC complexes which is consistent with our hypothesis that mitochondrial dysfunction may be due to the accumulation of oxidatively modified proteins. On the other hand, CII

activity and CI-CIII and CII-CIII coupled activities increased with age suggesting that the decline in CI function may be compensated by better coupling and possible shunting of electrons through a less favorable pathway through CII. Numerous studies, in both rodents and humans, previously have looked at enzyme function of ETC complexes with aging in heart mitochondria (Andreu et al., 1998; Barazzoni et al., 2000; Jarreta et al., 2000; Kwong and Sohal, 2000; Marin-Garcia et al., 1998; Miro et al., 2000; Sugiyama et al., 1993; Torii et al., 1992; Yarian et al., 2005). In humans, all studies show consistently that there is no change in ETC function with aging. However, studies involved in mice and rats do not show any consensus as to which enzyme function changes since some studies show there is a decline in CI, CIV (Sugiyama et al., 1993) and CV (Yarian et al., 2005) function and show no changes at all. This disagreement in results may be due to the fact that the rodent studies had major flaws since none or improper markers were used to normalize mitochondrial content. Furthermore, not all studies took inhibitor-sensitive activities into consideration and they did not determine the CI-CIII and CII- III coupled activities. Thus, in our study we not only applied the proper methods of controlling for mitochondrial content by using ratios of citrate synthase activity from young and middle age and young and old age as well as two methods of protein determination as described in Materials and Methods. This is a crucial step as just normalizing for total protein may not be sufficient for these types of assays. Our study, therefore, is a concise and complete and shows that there is an age-related decline in CI and CV function.

In hearts, although CI activity continuously decreases with age, the coupled enzyme activity of CI-CIII, which increases with age, is not consistent with the decline in CI function. In addition, both CII activity as well as CII-CIII coupled activity show an age-associated increase and the increase is similar in both activities suggesting improved CII function. Thus, these results suggest that tighter coupling between CI and CIII and improved CII function may have the potential to compensate for the loss of individual CI enzyme activity with age. Such a mechanism is unique and requires further consideration. Here we propose that the increase in the coupled rate between CI and CIII and improved

CII function may be a compensatory mechanism that enables the cell to balance the decline in CI activity by increasing the efficiency of electron transfer between CI and CIII and shunting of electrons through CII, a less energetically favorable pathway.

Interestingly, only two proteins were found to be modified with multiple types of modifications in CI. These proteins were identified as NDUFS1, a component of the Iron Protein (IP) region, and NDUFV1, a component of Flavoprotein (FP) region, both located in the mitochondrial matrix. Since both proteins are in the proximity of the electron transfer and the proposed site of ROS generation in CI (Johnson, Jr. et al., 2003; Lenaz et al., 2002; Yano et al., 2000), it is not surprising that these proteins were specifically modified. The same ETC complex subunits are differentially modified in all age groups, suggesting that these proteins are particularly susceptible to oxidative damage, and, since CI is the rate-limiting enzyme in oxidative phosphorylation, the modification of its subunits may have a direct impact on the overall energy state of the cell. The oxidative modification of NDUFS1 by carbonylation and HNE showed very little change with aging. In contrast, NDUFV1 was heavily modified with nitration and this modification increased dramatically with age. Oxidative damage to the ETC complexes *in vitro* leads to a decline in enzyme function (Bautista et al., 2000; Chen et al., 1998; Chen et al., 2001; Lashin et al., 2006; Murray et al., 2003; Picklo et al., 1999), and this correlates with the amount of damaging adducts. However, even though NDUFV1 was modified 7 to 10-fold higher with aging, only a small decline in CI function is noted. This suggests that the possible site(s) of modification will determine whether the oxidative damage will have any functional consequences (Choksi et al., 2007).

In contrast to CI activity, CII function increases with age. The CII-CIII coupled activity also bears same profile and thus supports our proposal that the loss of enzyme function in CI may be compensated by improved CII function with aging. Interestingly, SDHA, a core component of CII, is modified by HNE. However, there is a decrease in this modification with age suggesting that the location of SDHA in the hydrophilic environment of the matrix may favor its modification by ROS-mediated intermediates

because of its proximity to lipid peroxidation products. Since there is an increase in enzyme function of CII with aging and a decrease in oxidative modification with aging, even though SDHA is a specific target of oxidative damage, the lowered modification may prevent the loss of complex activity and this may be a mechanism by which improved CII function produces less tissue dysfunction. Previously, our lab has shown that SDHA is increasingly modified in an age-dependent manner that correlates well with the loss of enzyme function in the kidneys and the observation that deficiency in and mutation of SDH subunits are known to cause severe diseases in humans (Hall et al., 1993; Rustin et al., 2002). These results lead us to further refine our hypothesis such that the specificity of sites of modification compared to general overall modification, which may be more important to predict age-associated decline in mitochondrial function. In future, once the modifications sites are identified the mechanism by which same protein is modified at different sites with drastically different outcomes will become abundantly clear and shed light on how specific modifications rather than random oxidative damage may play a key role in age-related tissue dysfunction.

CIII, a key ETC complex, is also a major site of ROS production *in vitro* (Han et al., 2001; Zhang et al., 1998). Indeed, our studies have shown that both Core 1 and Core 2 subunits are modified in all ages. While carbonylation of Core 2 and HNE modification of Core1 decreased with age, Core 1 was heavily nitrated and this nitration increased dramatically with age. Despite the heavy nitration in both middle and old age, the enzyme function of CIII did not change and in fact it increased slightly at old age. Since, Core 1 and Core 2 span the entire inner mitochondrial membrane and are in the proximity to the predicted site of ROS generation in CIII, *i.e.*, the heme b_L of the cytochrome b subunit (Zhang et al., 1998), it is not surprising that they are both targets of oxidative modifications. The differential modification of Core 1 and Core 2 suggests that the type of modification at a specific location in these proteins would be more important in causing changes to the enzyme function rather than sheer amount of modification. Thus, these results support our hypothesis that the quality of modification rather than quantity,

i.e., the specificity of sites of modification compared to general overall modification, may be more important in predicting age-associated mitochondrial dysfunction. Core 1 and Core 2 proteins have dual functions in CIII, *i.e.*, they play a key role in providing structural stability to the complex as well as being members of the Mitochondrial Processing Peptidase (MPP) family that are involved in processing and folding of proteins imported into the matrix (Deng et al., 2001). Since Core 1 is heavily damaged, a decrease of MPP activity could result in impairment in protein processing and increased mitochondrial dysfunction with aging that is not directly related to ETC function.

Our studies did not show any changes to CIV function with aging. COX4 was the only subunit that was found to be modified with both carbonylation and HNE and showed only a mild increase in modification with aging. Since COX4 mainly plays a role in stability of this complex and is not involved in the physiological activity of CIV (Yoshikawa et al., 1998), its modification does not seem to have any consequences to the function of CIV. Again, no change in CIV activity with increasing oxidative damage to COX4 supports the hypothesis that the specificity of modification sites may determine the overall outcome of such damage.

CV or F1F0-ATP synthase is not part of the ETC processes. However, its proximity to ROS produced by these enzymes and its location within the matrix, as well as its abundance make it a prime candidate for oxidative modification. Our results found that CV activity decreased with aging. Though the α chain is oxidatively damaged, it did not show any increase in modification with aging. In addition, the HNE-modified α chain was only found to co-migrate with CI and not in the intact CV. The removal of oxidatively damaged protein may be a protective mechanism by which the impaired protein is replaced by active protein and lowers the effect on the function of the complex. Since CV is the ATP producing component of mitochondria, it has to function properly or the energy production of the cell would suffer dramatically and lead to decrease in ATP production, a hallmark of increasing mitochondrial dysfunction with aging.

Similarly, Yarian *et al.* found that CV function in mouse heart decreased with age and a mitochondrial 1D SDS-PAGE immunoblot suggested that the β chain may be modified with MDA (Yarian et al., 2005). However, we did not find any MDA modifications in any of the intact complexes. We believe that the MDA-modified β chain may also have been removed from the active complex to reduce the effect of such modification its function. In contrast to α chain, the β chain was heavily nitrated and this nitration dramatically increased with age to more than 10-fold by old age. This increase in oxidative modification directly correlates with the decline in CV function observed with aging. Interestingly, the major decrease in enzyme function occurs at middle age and no further decline was seen with aging even though the β chain was increasingly modified. Thus, these results provide suggest that the specificity of the modifications rather than abundance may be a better determinant of functional consequences to ETC complexes and decline in mitochondrial function.

In conclusion, many of the ETC complex subunits appear to be specific targets of ROS-mediated oxidative modifications. However not all oxidative modifications lead to decline in complex function. In fact, the decreased modification of the specific subunit or removal of modified subunit may reduce the effect of oxidative damage to the enzyme function of ETC complexes. Furthermore, we found that heavily nitrated subunits with aging have only a mild effect on enzyme function. Therefore, we propose that the specificity of modifications is a better predictor of functional consequences in ETC complexes from mouse hearts. We find that an increase in oxidative damage to its subunits correlates to a decrease in enzyme function of CI and CV and supports the Mitochondrial Theory on Aging in mouse hearts. Although the function of CIII does not change, a possible decline in Core 1 and Core 2 MPP function due to Core 1 modification may elicit protein misfolding and lead to lead to mitochondrial dysfunction and an increased state of oxidative stress in aged heart tissue. We, therefore, propose that the overall effect of increased oxidative modification of components of the ETC complexes may be an underlying mechanism to the development of age-associated increase in

mitochondrial dysfunction leading to tissue dysfunction. Our study provides important insight into physiological effects of oxidative modifications on mitochondrial function and their role in aging.

CHAPTER VII

LOWER LEVELS OF F_2-ISOPROSTANES IN SERUM AND LIVERS OF LONG-LIVED AMES DWARF MICE: A POSSIBLE MECHANISM OF LONGEVITY AND RESISTANCE TO OXIDATIVE STRESS

SUMMARY

In these studies we have measured the differences in levels of IsoPs in the young, middle-aged and old Ames DW mutant and WT mice. Our results clearly show that the Ames DW mice have lower levels of IsoPs in both serum and liver at all ages, and provide further evidence in support of the Free Radical Theory on Aging. In addition, the differences in IsoP serum levels between aged DW and WT mice significantly decreased suggesting there is a delay in the development of aging characteristics by Ames DW mice. Thus we propose that Ames DW mice have endogenously lower levels of oxidative stress, that this plays a key role in longevity in these mice.

RESULTS

F_2-isoprostane levels in serum of Ames WT and DW mice

To determine whether there are changes in the levels of IsoPs that correlate with levels of oxidative stress in Ames WT and DW mice, we measured IsoP levels from sera of young, middle and old age mice. The results in Figure 7.1 show that at all ages the IsoP levels are lower in DW mice compared to WT (~20% at young, ~25% at middle and ~13% at old age). The small coefficients of variance clearly show statistically significant differences between WT and DW IsoP levels at these ages. In addition, there is an increase in IsoP levels in both the mutant and normal mice at old age suggesting that the level of oxidative stress is elevated in the aged animals compared to young and middle age. However, the difference between WT and DW IsoP levels diminishes with age suggesting that the development of aging characteristics is delayed in DW mice.

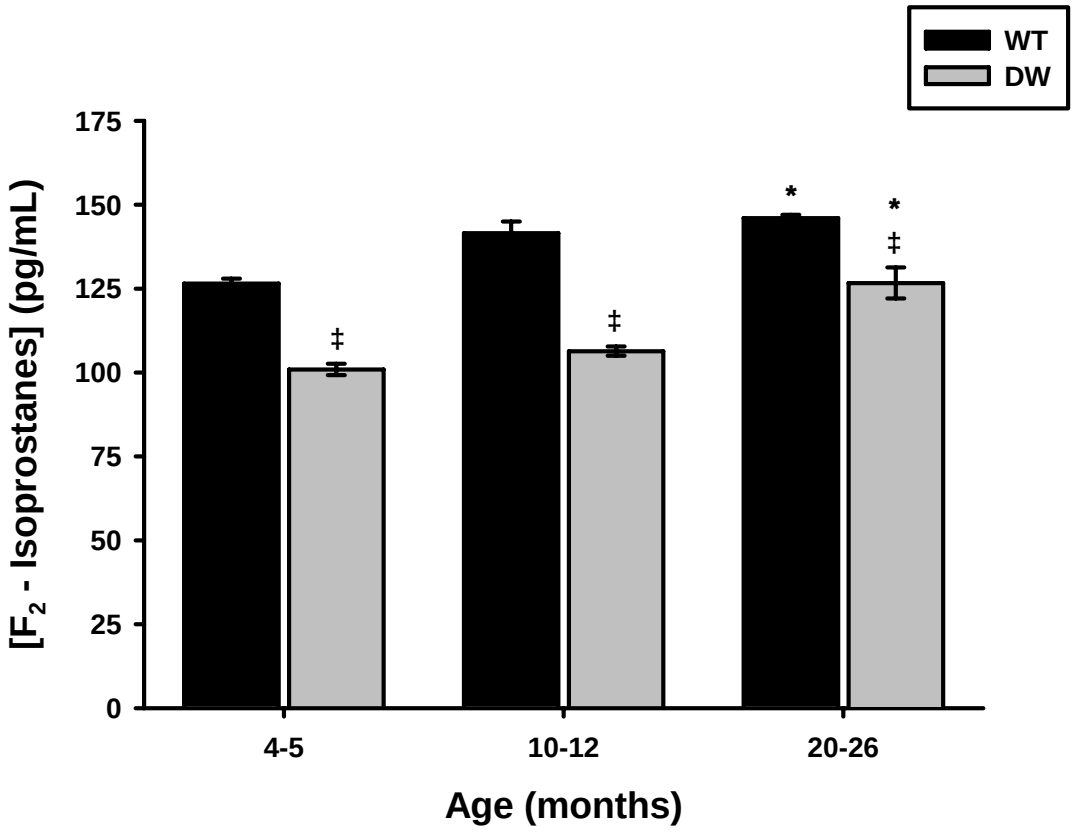


Figure 7.1. Serum F_2-Isoprostane levels from Ames wild-type and dwarf mice

F_2-Isoprostane (IsoP) levels were measured from serums collected from Ames wild-type and dwarf mice as described in Materials and Methods. IsoP levels were determined in young (4-5 months), middle-aged (10-12 months), and old (20-26 months) mice. The data represented are averages of IsoP values obtained for serums from 7 young, 8 middle-aged, and 8 old wild-type and 12 young, 13 middle-aged, and 8 old dwarf mice. Coefficients of variance for wild-type were 1.7% (young), 3.5% (middle-age), and 1% (old), respectively. Coefficients of variance for dwarf were 2.4% (young), 1.8% (middle-age), and 6.5% (old), respectively. * - $p<0.05$ compared to young and ‡ - $p<0.05$ compared to wild-type.

F_2-Isoprostane levels in liver of Ames WT and DW mice

To determine if IsoP levels are changed in WT and DW tissues, we utilized liver samples to measure the amount of IsoPs at young, middle and old age. The results in

Figure 7.2 demonstrate dramatic differences between WT and DW IsoP levels in livers at all ages, *i.e.,* WT mice have significantly higher levels of IsoPs compared to DW (~45% at young age, ~154% at middle age and ~272% at old age). Interestingly, there is an age-related two-fold increase in IsoP levels from young to middle age in WT suggesting that these mice are undergoing significantly elevated levels of oxidative stress whereas the DW mice show a slight but non significant increase suggesting a corresponding lower level of oxidative stress. In contrast, both WT and DW mice show a decrease in IsoP levels from middle to old age where in WT the IsoP levels reverted to those of the young; in DW the levels are significantly lower than both young and middle age. These data suggest that in tissues such as liver both WT and DW revert to a lower level of endogenous oxidative stress at this age compared to middle age.

DISCUSSION

The Ames dwarf mouse model, which exhibits ~40-60% increased life span, has been extensively used to study the molecular mechanisms of longevity (Murakami, 2006). The mutation of the Prop 1 locus in this mouse leads to incomplete development of the anterior pituitary gland and to a deficiency of growth hormone, thyroid stimulating hormone and prolactin (Brown-Borg et al., 1996). Although numerous studies have indicated that the Ames dwarf mouse model as well as the parallel Snell dwarf model are more resistant to oxidative stress generated by extrinsic factors such as UV light, hydrogen peroxide and paraquat (Bartke and Brown-Borg, 2004; Flurkey et al., 2001; Flurkey et al., 2002; Murakami et al., 2003; Salmon et al., 2005), no studies have shown that these mice generate less ROS and hence lower levels of oxidative stress. However, that the Ames DW mice exhibit a lower endogenous level of ROS is suggested by our observations that the oxidative stress induced p38 MAPK stress response pathway is down-regulated in the DW mice compared to WT (Hsieh and Papaconstantinou, 2004; Hsieh and Papaconstantinou, 2006; Madsen et al., 2004; Papaconstantinou et al., 2005).

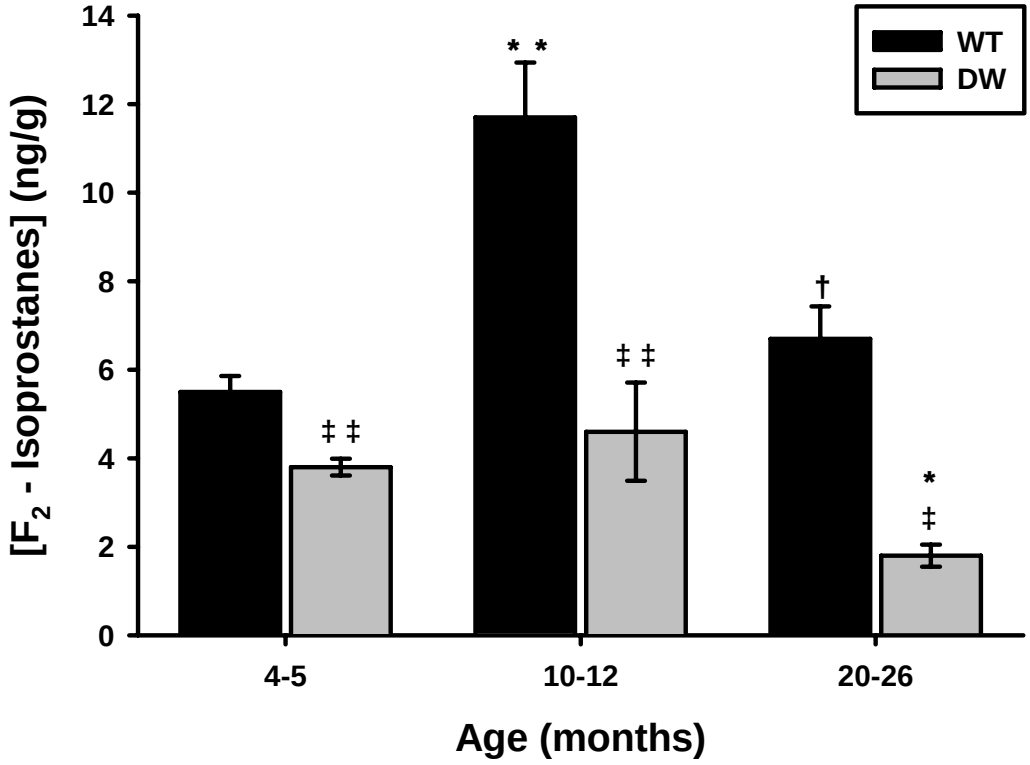


Figure 7.2. Liver F_2-Isoprostane levels from Ames wild-type and dwarf mice

F_2-Isoprostane (IsoP) levels were measured from liver pieces collected from Ames wild-type and dwarf mice as described in Materials and Methods. IsoP levels were determined in young (4-5 months), middle-aged (10-12 months), and old (20-24 months) mice. The data represented are averages of IsoP values obtained for liver pieces from 5 young, 7 middle-aged, and 8 old wild-type and 12 young, 13 middle-aged, and 4 old dwarf mice. Coefficients of variance for wild-type were 6.5% (young), 10.6% (middle-age), and 10.9% (old), respectively. Coefficients of variance for dwarf were 5% (young), 24.2% (middle-age), and 13.9% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.005$ compared to young, † - $p<0.05$ compared to middle-aged, ‡ - $p<0.05$ compared to wild-type and ‡‡ - $p<0.005$ compared to wild-type.

Taken together, these studies suggest that the endogenous ROS levels are significantly lower in the Ames dwarf at all ages.

Our results from the sera of WT and DW mice show that the DW mice have lower levels of IsoPs in young, middle and old age. Thus, the production of IsoPs from non-

enzymatic catalysis of arachidonic acid by free radicals is a more direct marker of decreased levels of endogenous oxidative stress. Furthermore, the lower levels of IsoPs in young, middle and old ages suggest that resistance to intracellular oxidative stress occurs in all tissues and at all ages of these mutant mice. We conclude that the increase in oxidative stress indicated by IsoP levels, with aging, support the Free Radical Theory of Aging.

Our studies show that the differences in levels of IsoP between WT and DW decrease by old age suggesting that the processes that generate higher levels of ROS in aging tissues are delayed in DW mice. We propose that this delay is associated with lower levels of oxidative stress at young and middle age that correlates with resistance to oxidative stress. Thus the biological processes associated with lower endogenous ROS and resistance to oxidative stress are overcome or neutralized in the long-lived mutant but at a delayed rate. Finally we propose that resistance to oxidative stress promotes longevity in this animal.

The levels of liver IsoP show a similar profile to that of serum such that at any age DW mice have lower levels of IsoPs. However, the levels in liver, in contrast to the serum levels, are much higher, especially at the middle and old age. We propose that the dramatic increase in IsoP levels in livers of WT from young to middle age is indicative of a significant increase in oxidative stress in this tissue and that the mild age-related increase observed in DW mice suggests major differences in endogenous oxidative stress between the WT and long-lived DW mouse. Again, these results support the theory that DW mice show characteristics of delayed aging. In contrast, the decrease in IsoP levels from middle to old age suggests a major change in oxidative metabolism at this stage of the life cycle. This is the first time that such a dramatic change in oxidative metabolism has been reported to occur in normal aged tissue. We hypothesize that as the tissues age, they lose the ability to respire efficiently (normally) thereby decreasing the level of mitochondrial ROS production. Based on this hypothesis it is possible that tissue dysfunction may be due to lower levels of mitochondrial function and that the level of

mitochondrial ROS leakage may also slow down. The fact that the decline of IsoP levels is much more dramatic in aged DW mice compared to WT also suggests a possible protective mechanism in response to oxidative stress in the long-lived DW mice which may involve decreased mitochondrial function or population.

In conclusion, we demonstrate that Ames dwarf mice have lower levels of oxidative stress compared to the wild-type mice; they show characteristics of delayed aging; and their resistance to intracellular oxidative stress may play a key role in delaying the aging phenomenon and conferring extended life-span in these dwarf mutants.

CHAPTER VIII

AGE-RELATED CHANGES IN FUNCTION OF MITOCHONDRIAL ELECTRON TRANSPORT CHAIN COMPLEXES FROM SEVERAL TISSUES OF LONG-LIVED AMES DWARF MICE

SUMMARY

In this study we analyzed isolated mitochondria from several tissues of young, middle-aged and old Ames WT and DW mice to determine the enzyme activities of ETC complexes I-V during aging. We chose both post-mitotic (heart and skeletal muscles) and slowly proliferating (kidney) tissues. In addition, we tested the hypothesis that specific proteins of ETC complexes I-V are differentially modified in WT and DW mice. The activities of all ETC complexes were measured and compared between the WT and DW mice to determine whether there were any functional changes during aging and to determine if the long-lived DW mice have improved ETC function compared to WT. In addition, we identified oxidatively modified subunits of ETC complexes to compare whether the levels of oxidative modification to these complexes correlate with changes in enzyme activities. These studies demonstrate that during aging, very little changes in ETC function is noted in post-mitotic tissues. The decreased CIV activity in heart and pectoralis muscle of DW mice may suggest lower levels of respiration in these tissues. In contrast, the slowly proliferating tissue such as kidney shows dramatic improvement in ETC function in DW mice which may help in delaying tissue dysfunction seen with aging and be a factor in longevity. Interestingly, no changes in complex levels or oxidative modification levels were seen between WT and DW mice suggesting that mitochondrial ROS damage may not be the final determinant of extended life-span in the mutants. In conclusion, our studies show that there are age-related changes in ETC function in some tissues of Ames DW mice and these differences despite no changes in levels of oxidative damage to ETC components may be a factor in longevity of Ames DW mice.

RESULTS

Inhibitor-sensitive enzyme activities from heart

To evaluate the physiological effects of dwarfism on heart mitochondrial ETC complexes, we compared the enzymatic activities of CI-CV as well as the coupled activity of CI-III and CII-III for all three ages in WT and DW mice (Figures 8.1 and 8.2). As evident from the data, the individual complex activities in heart decreased with aging in both WT and DW mice. On the other hand, increase in CI-CIII coupled activity was noted with aging in both WT and DW as supposed to no change CII-CIII coupled activity in DW versus decline with aging in WT mice. Rotenone-sensitive CI activity decreased by ~15% and ~19% from young to middle-age and by old age this activity decreased by ~29% and 33% in WT and DW, respectively (Figure 8.1A). No changes between WT and DW were noted for CI activity at any age. Contrast to CI activity, there was an increase of ~15% and ~10% in CI-III coupled activity from young to middle-age and further increase to ~28% and ~37% by old age in WT and DW mice, respectively (Figure 8.2A). In comparison, the DW mice have higher CI-CIII coupled activity at young (~22%), middle (~17%) and old (~30%) age then WT suggesting tighter coupling between these complexes at all ages in DW mice. Malonate-sensitive CII activity showed similar decline in WT as CI activity such that it decreased by ~17% in middle age and ~28% by old age (Figure 8.1B). In contrast, no decline in DW CII activity from young to middle-age was seen and the activity decreased by ~28% in old age (Figure 8.1B). In comparison, CII enzyme activity was higher in DW mice then WT only at the middle age (~28%). The coupled CII-III activity of WT also showed an age-associated decline in activity, *i.e.*, ~15% decline in middle age and ~25% decline by old age (Figure 8.2B). In contrast, no change in coupled CII-CIII activity was seen in DW mice with aging. When comparing the coupled CII-CIII activity between DW and WT, no significant changes were seen between these two groups of mice at any age. Antimycin A-sensitive CIII activity from WT showed a continuous age-associated decline, *i.e.*, ~19% decline in

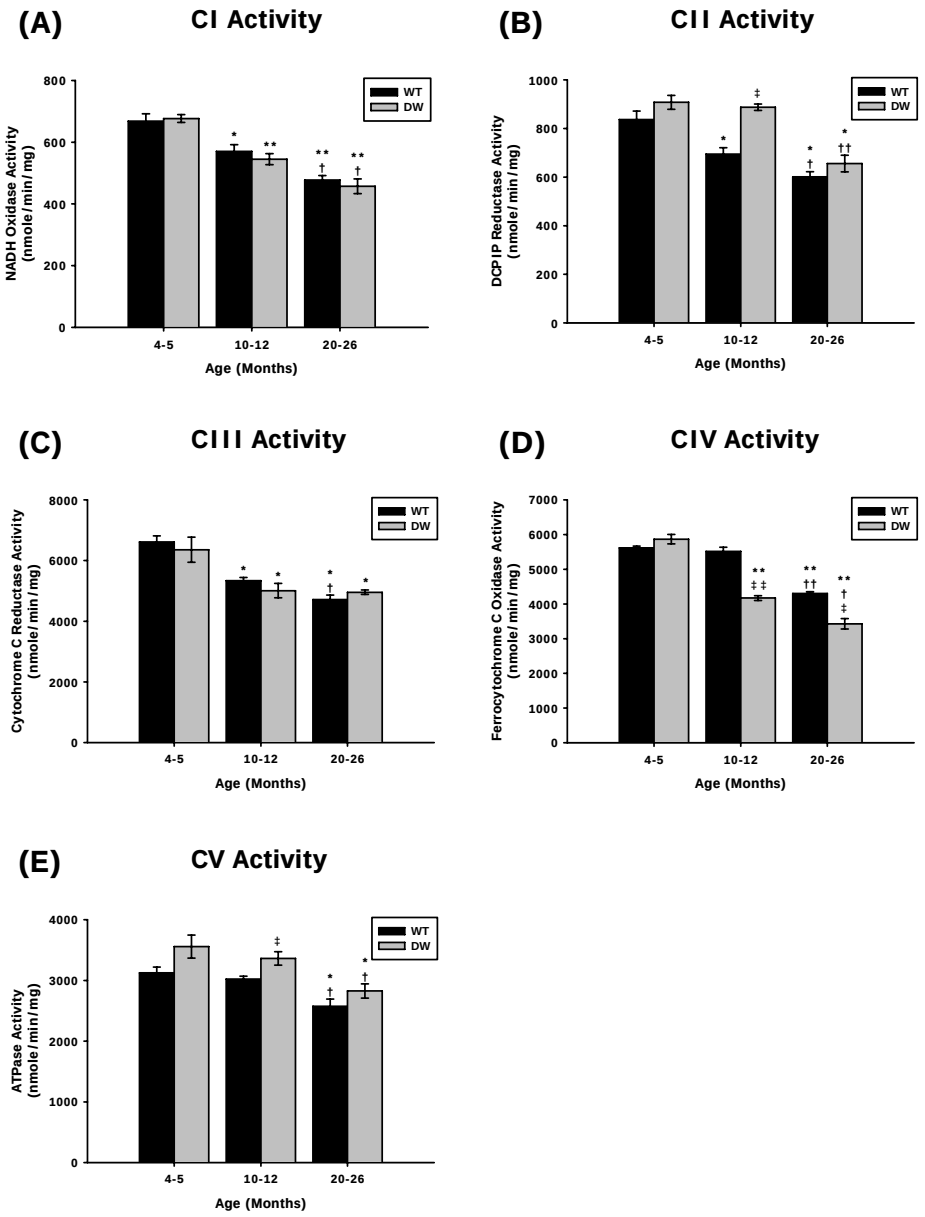


Figure 8.1. Measurement of ETC complex activities from 4-5, 10-12, and 20-26 month-old WT and DW mouse heart mitochondria

Individual complex enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the pooled sample ± SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (4-5 months), middle-aged (10-12 months), and old (20-26 months) WT and DW heart ETC CI-CV are plotted as following. (A) CI

activity with aging. Coefficients of variance for WT and DW were 7.1% and 3.7% (young), 7.7% and 6.5% (middle-age), and 6.1% and 10.5% (old), respectively. (B) CII activity with aging. Coefficients of variance for WT and DW were 8.3% and 6.3% (young), 7.5% and 3% (middle-age), and 6.9% and 10.5% (old), respectively. (C) CIII activity with aging. Coefficients of variance for WT and DW were 6.1% and 13% (young), 3.8% and 9.5% (middle-age), and 6.2% and 3% (old), respectively. (D) CIV activity with aging. Coefficients of variance for WT and DW were 1.8% and 4.6% (young), 4.3% and 3.3% (middle-age), and 2.4% and 8.7% (old), respectively. (E) CV activity with aging. Coefficients of variance for WT and DW were 6.1% and 10.7% (young), 3.2% and 6.6% (middle-age), and 9.3% and 8.3% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, † - $p<0.05$ compared to middle-aged, †† -$p<0.001$ compared to middle-aged, ‡ - $p<0.05$ compared to WT, and ‡‡ - $p<0.001$ compared to WT.

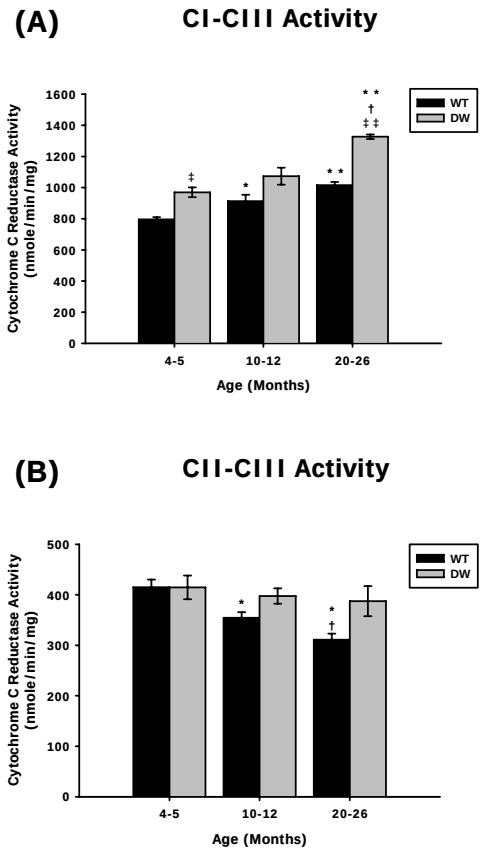


Figure 8.2. Measurement of coupled mitochondrial ETC complex activities from 4-5, 10-12, and 20-26 month-old WT and DW mouse heart mitochondria

CI-III and CII-III coupled enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the

pooled sample ± SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (4-5 months), middle-aged (10-12 months), and old (20-26 months) WT and DW heart ETC CI-III and CII-III are plotted as following. (A) CI-CIII coupled activity with aging. Coefficients of variance for WT and DW were 3.4% and 6.5% (young), 9% and 10.2% (middle-age), and 4.1% and 2.2% (old), respectively. (B) CII-CIII coupled activity with aging. Coefficients of variance for WT and DW were 7.2% and 11.3% (young), 6.3% and 7.7% (middle-age), and 7.7% and 15.4% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, † - $p<0.05$ compared to middle-aged, ‡ - $p<0.05$ compared to WT, and ‡‡ - $p<0.001$ compared to WT.

middle age and ~28% decline by old age, while DW mice only showed a decline by ~21% in middle age and no further decline was seen in old age (Figure 1C). No changes between WT and DW were seen in CIII activity at any age. KCN-sensitive CIV activity in WT decreased by ~24% only in old animals, while DW mice showed a continuous decline with aging, *i.e.*, ~29% decline in middle age and ~42% decline by old age (Figure 8.1D). DW mice showed a marked decrease in CIV activity compared to WT mice suggesting that these mice may have lower rates of oxygen consumption by old age. Oligomycin-sensitive CV activity in both WT and DW decreased only in old age by ~16% and ~21% (Figure 8.1E). In comparison, CV activity in DW was higher then WT at middle age (~12%) suggesting a higher level of ATP production in DW mice at this age.

Overall, most ETC enzyme activities in heart from WT and DW mice show similar patterns of age-associated decline. However, CIV was significantly lower in DW mice at middle and old age suggesting that there are changes in enzyme function with aging in WT and DW mice.

Inhibitor-sensitive enzyme activities from pectoralis and quadriceps

To evaluate the physiological effects of dwarfism on skeletal muscle mitochondrial ETC complexes, we chose two different muscles, pectoralis (~red) and quadriceps (~white) and compared the enzymatic activities of all five complexes as well as the coupled activity of CI-III and CII-III for all three ages in both WT and DW mice

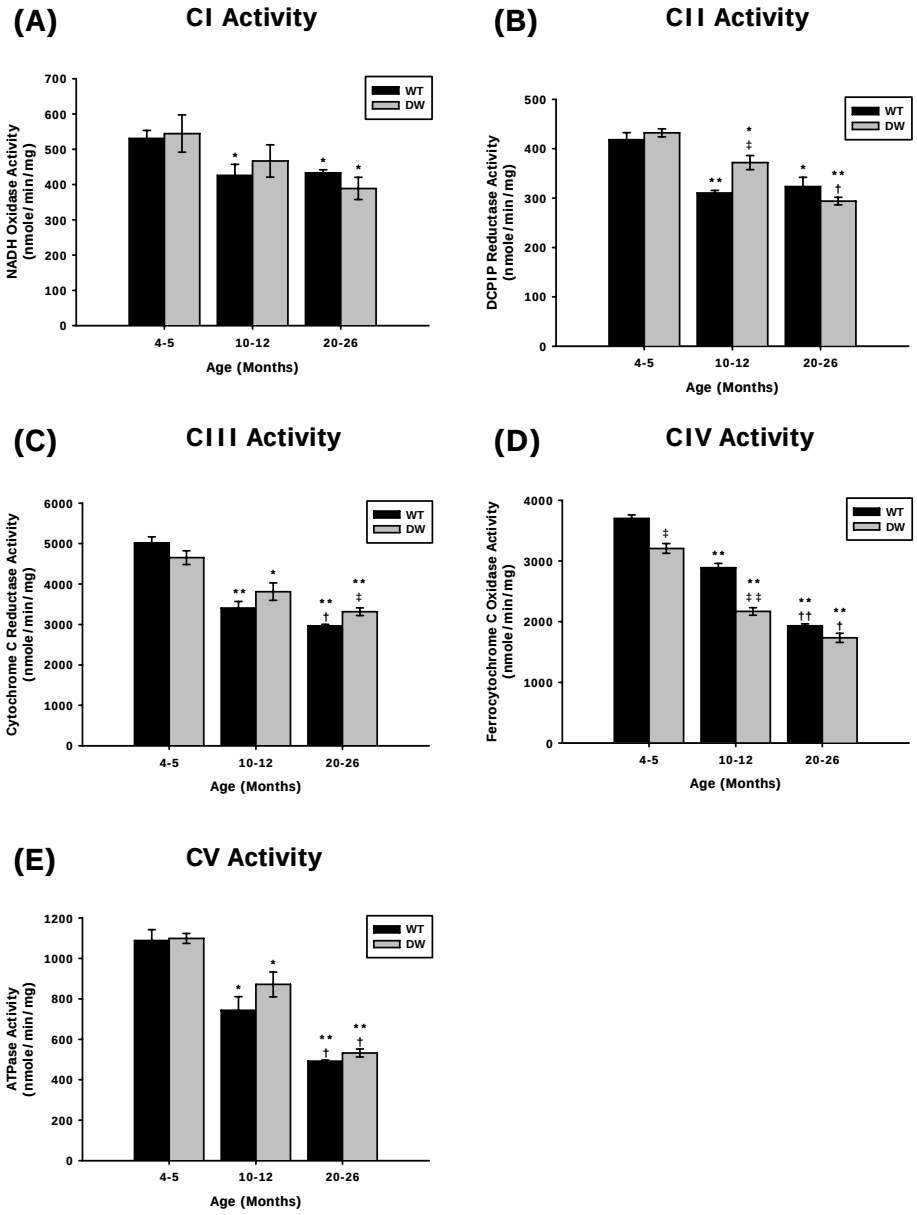


Figure 8.3. Measurement of ETC complex activities from 4-5, 10-12, and 20-26 month-old WT and DW mouse pectoralis muscle mitochondria

Individual complex enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the pooled sample ± SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (4-5 months), middle-aged (10-12 months), and old (20-26 months) WT and DW pectoralis ETC CI-CV are plotted as following. (A)

CI activity with aging. Coefficients of variance for WT and DW were 8.4 % and 19.3% (young), 14.5% and 19.6% (middle-age), and 4.1% and 16.2% (old), respectively. (B) CII activity with aging. Coefficients of variance for WT and DW were 6.7% and 3.7% (young), 3.5% and 7.7% (middle-age), and 11.8% and 5.1% (old), respectively. (C) CIII activity with aging. Coefficients of variance for WT and DW were 6% and 7.3% (young), 9.6% and 11.3% (middle-age), and 2.8% and 5.7% (old), respectively. (D) CIV activity with aging. Coefficients of variance for WT and DW were 3.2% and 5% (young), 4.9% and 5.7% (middle-age), and 3.3% and 8.7% (old), respectively. (E) CV activity with aging. Coefficients of variance for WT and DW were 9.9% and 4.5% (young), 18.2% and 14.1% (middle-age), and 2.2% and 7.4% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, † - $p<0.05$ compared to middle-aged, †† - $p<0.001$ compared to middle-aged, ‡ - $p<0.05$ compared to WT, and ‡‡ - $p<0.001$ compared to WT.

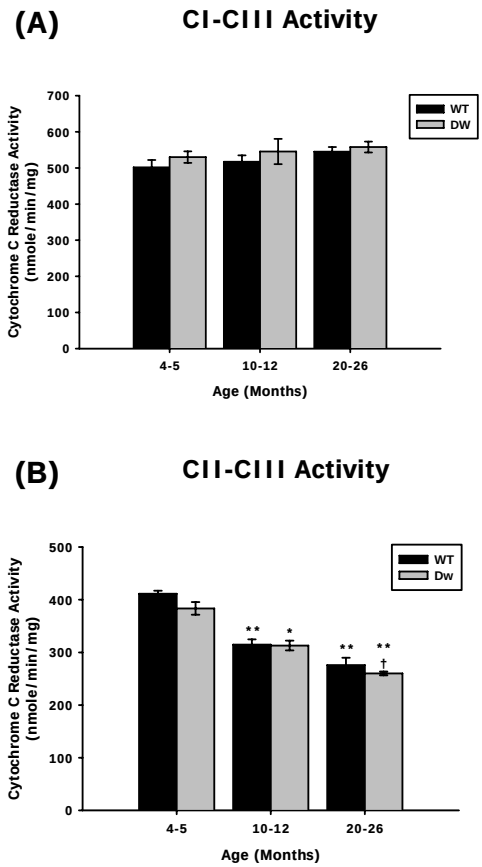


Figure 8.4. Measurement of coupled mitochondrial ETC complex activities from 4-5, 10-12, and 20-26 month-old WT and DW mouse pectoralis muscle mitochondria
CI-III and CII-III coupled enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the

pooled sample ± SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (4-5 months), middle-aged (10-12 months), and old (20-26 months) WT and DW pectoralis ETC CI-III and CII-III are plotted as following. (A) CI-CIII coupled activity with aging. Coefficients of variance for WT and DW were 8.2% and 6.1% (young), 7% and 12.9% (middle-age), and 4.7% and 5.4% (old), respectively. (B) CII-CIII coupled activity with aging. Coefficients of variance for WT and DW were 2.9% and 6.3% (young), 6.2% and 5.9% (middle-age), and 10.2% and 2.9% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, and † - $p<0.05$ compared to middle-aged.

(Figures 8.3-6). As evident from the data, these two muscles showed different profiles of enzyme activity in WT and DW mice. In pectoralis, except for CI-CIII coupled activity, which showed no change in its activity with aging, all other enzyme activities showed an age-related decline and by old age this decline was ~19% and ~28% in CI, ~23% and ~32% in CII, ~41% and 28% in CIII, ~48% and ~46% in CIV, ~55% and ~52% in CV, and ~33% and ~32% in CII-CIII coupled activity from WT and DW mice, respectively (Figure 8.3A-E and Figure 8.4B). Only minor differences were noted between DW and WT in CII and CIII activities. However, in CIV DW had much lower activity at both young and middle age compared to WT (~13% and ~25%, respectively) and by old age these differences were no longer present (Figure 8.3D).

On the other hand, in WT quadriceps only minor decrease in CI, CII and CI-CIII coupled activity were seen in old age (Figure 8.5A, 8.5B, and Figure 8.6A, respectively). In DW quadriceps, not only CI and CI-CIII coupled activity decreased in old age (Figure 8.5A and Figure 8.6A, respectively), there was a continuous decline in CIV activity seen with aging, i.e., ~14% decline in middle age and ~32% decline by old age (Figure 8.5D). In comparison, though there was a difference noted between WT and DW CV activity at young age (Figure 8.5E) and CI-CIII coupled activity at old age (Figure 8.6A), the major differences between WT and DW were seen in CIV activity (Figure 8.5D). At all ages, DW mice had a significantly lower CIV activity compared to WT and since CIV activity in DW decreased with aging and did not change in WT the difference between WT and DW increased with aging (from ~21% at young age to ~38% at old age).

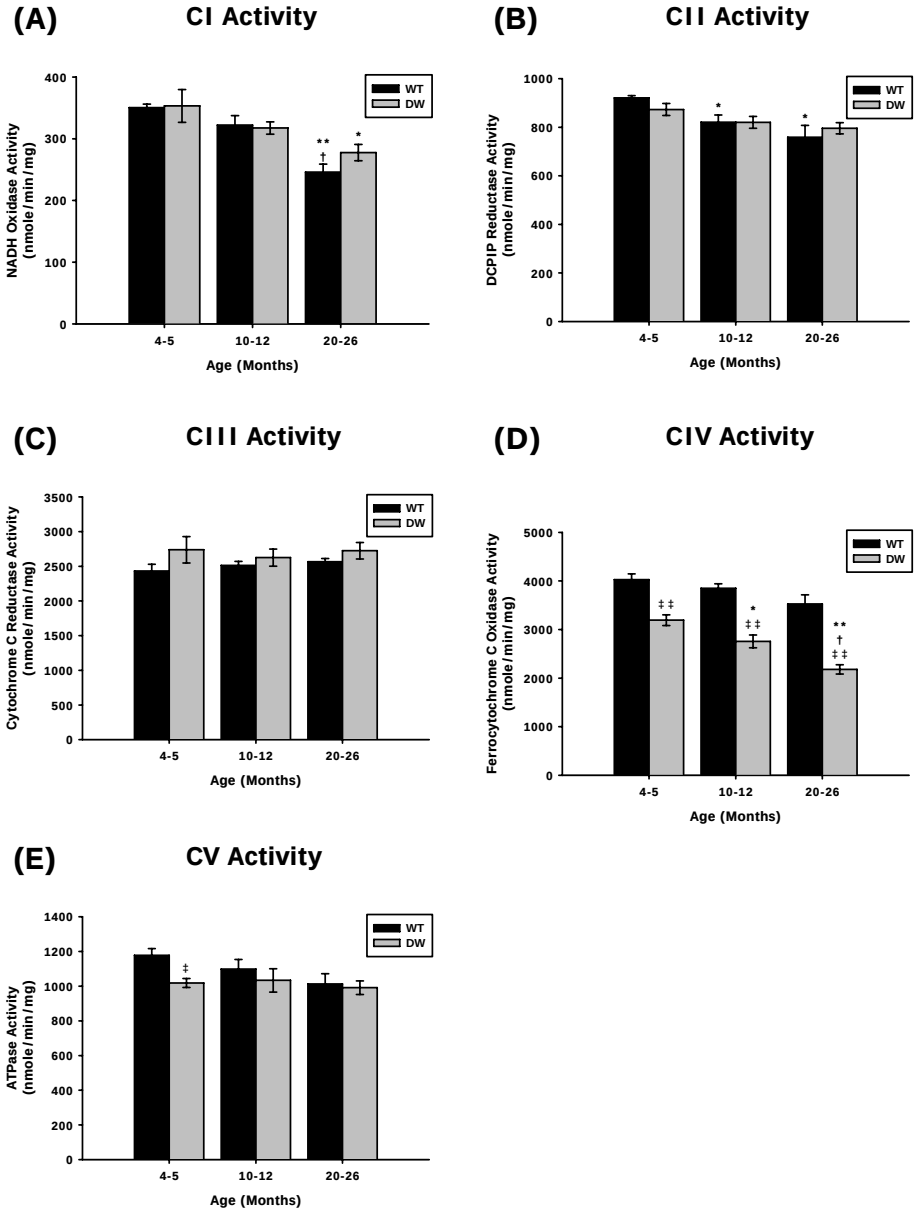


Figure 8.5. Measurement of ETC complex activities from 4-5, 10-12, and 20-26 month-old WT and DW mouse quadriceps muscle mitochondria

Individual complex enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the pooled sample $\pm$ SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (4-5 months), middle-aged (10-12 months),

and old (20-26 months) WT and DW quadriceps ETC CI-CV are plotted as following. (A) CI activity with aging. Coefficients of variance for WT and DW were 3.2 % and 15.1% (young), 9.4% and 6.3% (middle-age), and 10.5% and 9.5% (old), respectively. (B) CII activity with aging. Coefficients of variance for WT and DW were 1.9% and 5.7% (young), 7.2% and 5.9% (middle-age), and 12.9% and 5.8% (old), respectively. (C) CIII activity with aging. Coefficients of variance for WT and DW were 8% and 13.9% (young), 4.6% and 9.4% (middle-age), and 3.6% and 8.7% (old), respectively. (D) CIV activity with aging. Coefficients of variance for WT and DW were 5.9% and 6.9% (young), 4.6% and 9.5% (middle-age), and 10.5% and 8.7% (old), respectively. (E) CV activity with aging. Coefficients of variance for WT and DW were 6.5% and 5.1% (young), 10.2% and 13% (middle-age), and 11.6% and 8% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, † - $p<0.05$ compared to middle-aged, ‡ - $p<0.05$ compared to WT, and ‡‡ - $p<0.001$ compared to WT.

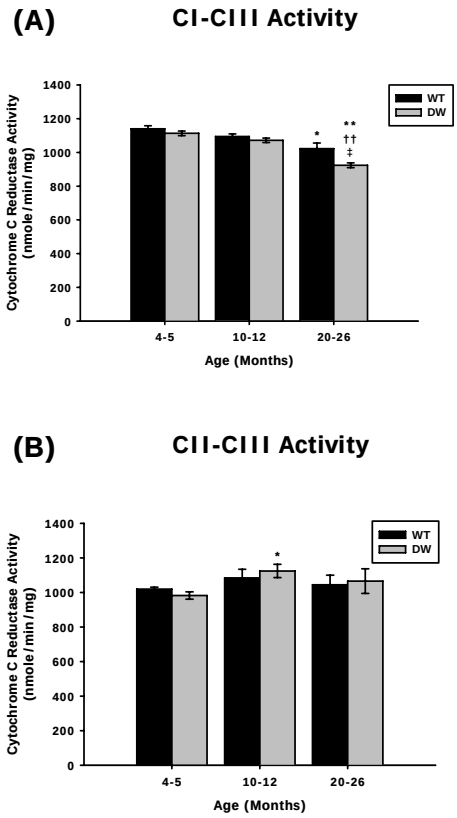


Figure 8.6. Measurement of coupled mitochondrial ETC complex activities from 4-5, 10-12, and 20-26 month-old WT and DW mouse quadriceps muscle mitochondria

CI-III and CII-III coupled enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the

pooled sample ± SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (4-5 months), middle-aged (10-12 months), and old (20-26 months) WT and DW quadriceps ETC CI-III and CII-III are plotted as following. (A) CI-CIII coupled activity with aging. Coefficients of variance for WT and DW were 3.1% and 2.5% (young), 2.8% and 2.5% (middle-age), and 6.5% and 3.1% (old), respectively. (B) CII-CIII coupled activity with aging. Coefficients of variance for WT and DW were 2.2% and 4.3% (young), 9.2% and 6.8% (middle-age), and 10.5% and 13.3% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, †† - $p<0.001$ compared to middle-aged, and ‡ - $p<0.05$ compared to WT.

Overall, two different skeletal muscles showed very different profile of ETC enzyme activities with aging in both WT and DW mice. In pectoralis, a primarily aerobic muscle, the age-associated decline in most enzyme activities was dramatic and except for CI-CIII coupled activity, all activities decreased in both WT and DW mice in similar fashion. Only major difference between WT and DW mice was seen in CIV activity where DW had markedly lower CIV activity in young and middle age. In contrast, quadriceps, a primarily anaerobic muscle, showed very little changes in most enzyme activities with aging in both WT and DW mice. Again, the only major difference between WT and DW mice was found to be in CIV activity where in WT this activity did not change with age and in DW this activity was lower than WT at all ages and showed a continuous age-related decline.

Inhibitor-sensitive enzyme activities from kidney

To evaluate the physiological effects of dwarfism on kidney mitochondrial ETC complexes, we measured the enzymatic activities of CI-CV as well as the coupled activity of CI-III and CII-III for all three ages in WT and DW mice (Figures 8.7 and 8.8). In contrast to heart and skeletal muscle, kidney showed major differences in most enzyme activities between WT and DW mice. In WT kidney, no changes were seen in CI and CIII activities (Figure 8.7A and 8.7C); however, an age-associated increase was seen in CI-CIII coupled activity such that the activity increased to ~26% at old age (Figure 8.8A).

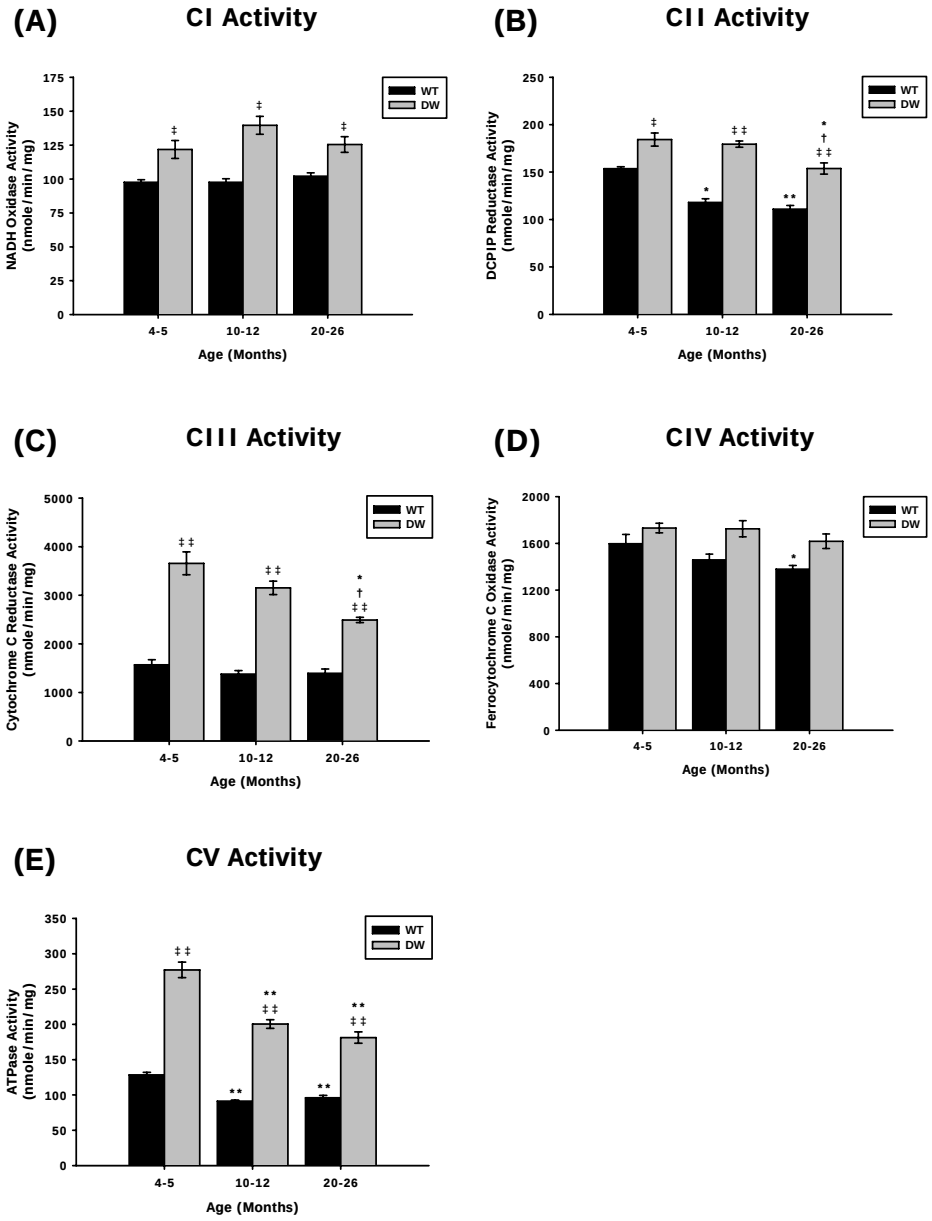


Figure 8.7. Measurement of ETC complex activities from 4-5, 10-12, and 20-26 month-old WT and DW mouse kidney mitochondria

Individual complex enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the pooled sample $\pm$ SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (4-5 months), middle-aged (10-12 months), and old (20-26 months) WT and DW kidney ETC CI-CV are plotted as following. (A) CI

activity with aging. Coefficients of variance for WT and DW were 3.9 % and 10.8% (young), 5.6% and 9.5% (middle-age), and 4.9% and 9.2% (old), respectively. (B) CII activity with aging. Coefficients of variance for WT and DW were 2.6% and 7.5% (young), 6.5% and 3.6% (middle-age), and 6.8% and 7.7% (old), respectively. (C) CIII activity with aging. Coefficients of variance for WT and DW were 13.7% and 12.9% (young), 10.2% and 8.8% (middle-age), and 12.7% and 4.3% (old), respectively. (D) CIV activity with aging. Coefficients of variance for WT and DW were 9.9% and 4.8% (young), 6.8% and 8% (middle-age), and 4.6% and 7.7% (old), respectively. (E) CV activity with aging. Coefficients of variance for WT and DW were 5.5% and 8% (young), 3.4% and 6.1% (middle-age), and 7.5% and 9% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, † - $p<0.05$ compared to middle-aged, ‡ - $p<0.05$ compared to WT, and ‡‡ - $p<0.001$ compared to WT.

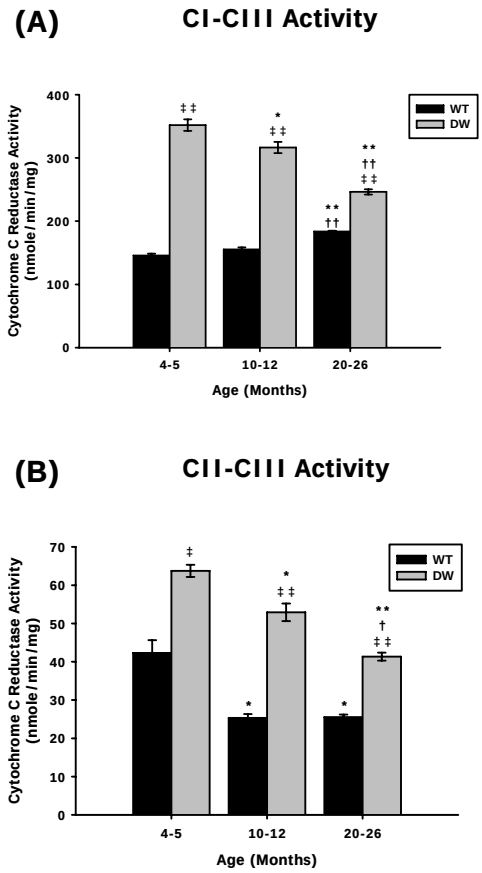


Figure 8.8. Measurement of coupled mitochondrial ETC complex activities from 4-5, 10-12, and 20-26 month-old WT and DW kidney heart mitochondria

CI-III and CII-III coupled enzyme activities were measured spectrophotometrically as described in Materials and Methods. All activity results are averages of 4 assays from the pooled sample $\pm$ SEM for each age group. Citrate synthase assay results were used to

normalize mitochondrial proteins. Activities for young (4-5 months), middle-aged (10-12 months), and old (20-26 months) WT and DW kidney ETC CI-III and CII-III are plotted as following. (A) CI-CIII coupled activity with aging. Coefficients of variance for WT and DW were 4.1% and 5.2% (young), 4.4% and 5.6% (middle-age), and 1.5% and 3.4% (old), respectively. (B) CII-CIII coupled activity with aging. Coefficients of variance for WT and DW were 15.8% and 5% (young), 8% and 8.7% (middle-age), and 5.4% and 5% (old), respectively. * - $p<0.05$ compared to young, ** - $p<0.001$ compared to young, † - $p<0.05$ compared to middle-aged, †† - $p<0.001$ compared to middle-aged, ‡ - $p<0.05$ compared to WT, and ‡‡ - $p<0.001$ compared to WT.

An age-related decline in function was seen in WT CII (~23% in middle age and ~28% by old age; Figure 8.7B), CIV (~14% by old age; Figure 8.7D), and CV (~29% in middle age and no further decline in old age; Figure 8.7E) as well as CII-CIII coupled activity (~40% in middle age and no further decline in old age; Figure 8.8B).

On the other hand, DW kidney showed no age-related changes in CI and CIV activities (Figure 8.7A and 8.7D) and all other enzyme functions declined with age. In particular, CII and CIII function decreased at old age by ~17% and ~32%, respectively (Figure 8.7B and 8.7C). CV activity in DW showed a continuous decline with aging such that the activity decreased by ~28% in middle age and ~35% at old age (Figure 8.7E). Similarly, both CI-CIII and CII-CIII coupled activities declined with aging, where CI-CIII activity decreased by ~10% in middle and ~30% at old age (Figure 8.8A) and CII-CIII activity decreased by ~17% and ~35% at middle and old age, respectively (Figure 8.8B).

In comparison, almost all kidney ETC complexes showed a higher level of enzyme activities at all ages in DW then WT mice. With the exception of CIV activity which did not show any differences in DW and WT, CI, CII, CIII, and CV activities were much higher in DW mice. In fact, in case of CIII and CV these activities were more than two-fold higher in DW at young age then WT. Similarly, there was at least 2.5-fold higher CI-CIII coupled activity in DW compared to WT kidney. Interestingly, with aging the differences between WT and DW activities decreased and suggested that DW kidneys were finally showing similar aging characteristics as WT.

Overall, kidney showed the most significant differences between DW and WT in almost all enzyme activities. Surprisingly, CIV activity did not show any differences between WT and DW. Most enzyme activities in DW were much higher at young age compared to WT and showed an age-related decline in function. Even though, the enzyme function of DW ETC complexes decreased with age, the enzyme activities were similar or higher than young WT.

Coenzyme Q levels in kidney mitochondria

To determine if changes in coenzyme Q (CoQ) levels are a factor in the large differences seen in WT and DW kidney enzyme function, we measured kidney mitochondrial CoQ levels of all three ages in WT and DW mice (Figure 8.9). Although the CoQ_9 and CoQ_{10} homologues make up about 95% of pool present in mitochondria, CoQ_9 is the predominant form in mouse tissues (Boitier et al., 1998; Duncan et al., 2005). Thus, we measured both CoQ_9 and CoQ_{10} levels and used these values as total CoQ (CoQ_{Total}) for mouse kidney mitochondria. Our results in Figure 8.9 show that there is no change in CoQ_{Total} at young age between WT and DW kidney mitochondria. WT shows an increase in CoQ_{Total} by middle age (~88%), and though CoQ_{Total} decreases in old age the level is still higher than young age. Similarly, DW also shows an increase in CoQ_{Total} in middle age (~32%) and declines in old age to young levels. However, in comparison, WT kidney mitochondria contain higher CoQ_{Total} levels then DW in both middle and old age suggesting that the substrate availability may not explain the dramatic increase in ETC function seen in DW kidneys compared to WT.

Abundance of ETC complexes and oxidative modification of their subunits

To determine if the differences in ETC enzyme activities seen between WT and DW mice are due to changes in the ETC complex levels and/or the levels of oxidatively modified complex proteins, we used immunoblotting of 1st dimension BN-PAGE with complex-specific antibodies and second dimension SDS-PAGE to detect carbonylation,

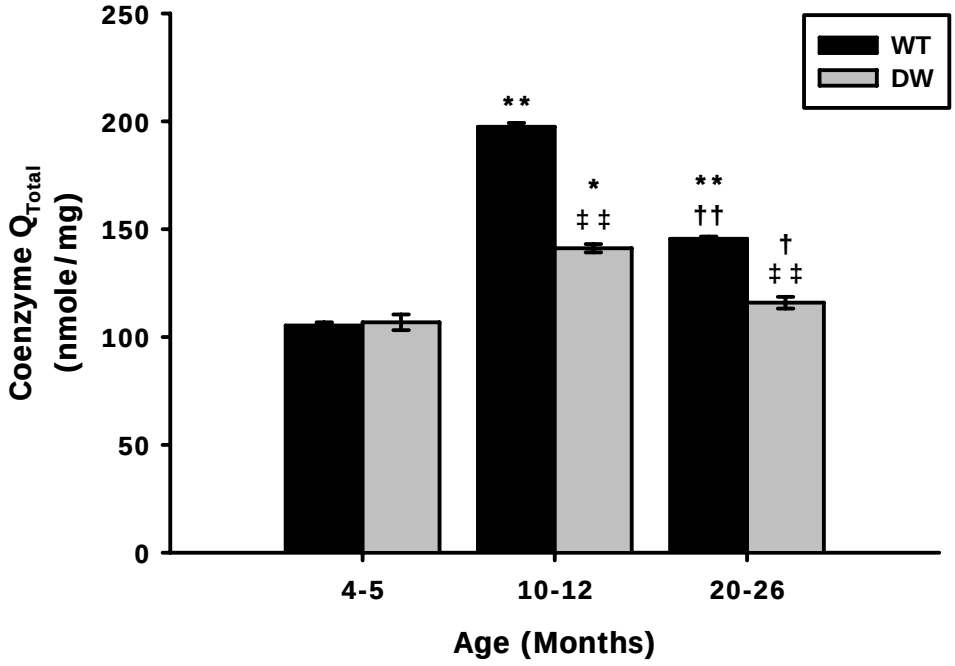


Figure 8.9. Measurement of CoQ levels in Ames WT and DW kidney mitochondria
Pooled kidney mitochondrial samples were used for each age group. Coenzyme Q was extracted and quantified as described in Materials and Methods. Data are an average of three experiments ± SEM. Coefficients of variance for CoQ_{Total} from WT and DW kidney mitochondria are 2.5% and 5.9% (young), 1.6% and 2.3% (middle-age), and 1.2% and 4% (old), respectively. * - $p<0.05$ compared to young, **- $p<0.001$ compared to young, †- $p<0.05$ compared to middle-aged, †† - $p<0.001$ compared to middle-aged, and ‡‡ - $p<0.001$ compared to WT.

HNE, MDA, and nitrotyrosine adducts. No differences in ETC complex levels were found in any tissue at any age (data not shown).

Similarly, very little changes in levels of oxidative modifications were noted between WT and DW mice for all four tissues at any age (data not shown). These data suggest that the differences in CIV enzyme function from heart, pectoralis and quadriceps may be due to deficiency of growth hormone in the mutant and may lead to lower respiration with aging in these tissues of DW mice. Similarly, the dramatic differences in enzyme functions from kidneys may also be due to hormonal deficiencies in the mutant

causing metabolic changes in this tissue such that it may lead to much improved mitochondrial function in this type of tissue in DW mice.

DISCUSSION

The Ames dwarf mouse, which exhibits ~40-60% increased life span relative to the wild-type mouse, has been extensively used to study the molecular mechanisms of longevity (Murakami, 2006). A mutation in the Prop 1 locus in this mouse leads to poor development of the anterior pituitary gland and to a deficiency of growth hormone, thyroid stimulating hormone and prolactin (Brown-Borg et al., 1996). Although numerous studies have indicated that the Ames dwarf mouse as well as the parallel Snell dwarf mouse used as models for increased longevity are more resistant to oxidative stress generated by extrinsic factors such as UV light, hydrogen peroxide and paraquat (Bartke and Brown-Borg, 2004; Flurkey et al., 2001; Flurkey et al., 2002; Murakami et al., 2003; Salmon et al., 2005), no studies have conclusively shown that these mice generate less ROS leading to lower levels of mitochondrial dysfunction. In addition, there is no direct evidence that suggests that these mutant mice have more efficient mitochondrial function than the wild-type mice that would explain their higher longevity. Thus we examined whether: (a) there are age-related changes in the enzyme activities of ETC complexes between DW and WT mice; (b) oxidative modification to ETC proteins is higher in WT mice compared to DW mice; and (c) there is a correlation between lower level of oxidative damage and change in ETC function in DW mice leading to extended life-span. We used two different types of tissues to test our hypothesis and we found that, in case of post-mitotic tissues such as heart and skeletal muscles, there was very little difference in the ETC function with aging between WT and DW mice and in both mice many of the ETC complexes show age-related decline in complex activity as is predicted by the Free Radical Theory of Aging (Wei et al., 1998; Wei, 1998). In contrast, we found that, in the case of slowly proliferating tissues such as kidneys, DW mice had much higher levels of ETC function in most complex activities compared to WT tissues suggesting that these

mice have improved mitochondrial function which may contribute to longevity in the mutant mice.

Our inhibitor-sensitive enzyme activity measurements in heart show three major differences between WT and DW mice. In DW mice the enzyme activity of CII and CI-CIII coupled activity is higher than WT mice. On the other hand, CIV activity in DW mice decreases dramatically by the middle age such that it is much lower than WT and it continues to decline with age. These differences between WT and DW mice may lead to some improved function in CII and tighter coupling in CI-CIII and the decline in CIV may lower respiration in these mice such that this would lead to decreased energy production. The lower energy production in middle and old ages in DW mouse hearts would lead to changes in metabolism and may allow for resistance to oxidative stress. Interestingly, we found very few differences in complex levels and oxidative damage to ETC proteins between WT and DW mice. These results suggest that there are no differences in levels of ROS and oxidative stress between WT and DW hearts and, therefore, changes seen in ETC function may be due to hormonal deficiency in DW mice leading to metabolic changes. Thus, unlike the caloric restriction mouse models that also have extended life-span and there is decreased ROS production and no change in energy metabolism in hearts (Drew et al., 2003), we propose that metabolic changes that confer resistance to exogenous oxidative stress may be the primary factor in longevity in Ames DW mice.

Similar to hearts, CIV activity showed a major difference between DW and WT mouse skeletal muscles. In the case of pectoralis, the complex activity of all ETC complexes decreased with aging and very little differences between WT and DW mice was seen except for the CIV function. In DW mice, CIV activity decreased dramatically by the middle age and this decline was much higher than the decline observed in the WT mice. At the old age, WT and DW mice had similar declines but the differences in the middle age may allow for changes in metabolic activities such that these tissues would become more resistant to oxidative stress. In contrast to pectoralis, there is very little

change in ETC complex activities from quadriceps in both WT and DW mice with aging. However, in WT mice, CIV activity does not change with age compared to DW mice where CIV function is lower in young age and continues to decline dramatically with aging. Since no differences in enzyme levels or oxidative damage were seen in either pectoralis or quadriceps between WT and DW mice, these results suggest that the lower CIV function leading to lower respiration and energy production would cause changes in metabolic rates leading to differences in stress-response pathways and conferring resistance to oxidative stress seen in a similar long-lived mutant mouse (Hsieh and Papaconstantinou, 2006).

On the other hand, kidney, a slowly proliferating tissue, shows the most differences for enzyme activities of all ETC complexes between WT and DW. In DW mice, CI, CII, CIII, CV activities as well as CI-CIII and CII-CIII coupled activities show markedly increased function at all ages and though the enzyme function decreases with age, even at old age the enzyme activity is much higher than that of young WT in most cases. In addition, since substrate availability was shown to be higher in WT at both middle and old age compared to DW as seen by CoQ levels in kidney, these results suggest that there are major physiological changes in ETC complexes in DW mice. Furthermore, no differences in complex levels as well as very little changes in level of oxidative damage to proteins of ETC complexes were seen between WT and DW mice. These results along with dramatic changes in enzyme function suggest that in DW kidneys, improved mitochondrial function leads to decreased tissue dysfunction and may be a contributing factor in longevity of these mice.

In conclusion, there are age-related changes in ETC complex activities from several tissues of Ames WT and DW mice. The differences between WT and DW mice are much higher in slowly proliferating tissue such as kidney compared to post-mitotic tissues such as heart and skeletal muscles. Our studies find that changes in CIV function from post-mitotic tissue in DW mice may lead to decreased energy production and may confer oxidative stress resistance in these tissues. On the other hand, the improved ETC

function in slowly proliferating tissues would lead to decreased tissue dysfunction and contribute to prolonged life-span in the DW mutant. Unlike, the diet-restricted mice that are long-lived than normally fed mice and have lower ROS production, very little change in oxidative damage to ETC proteins between WT and DW mice in all four tissues studied here suggest that the longevity in these DW mice may not be simply lower ROS production and decreased accumulation of damage, rather, metabolic changes leading to differences in stress-response pathways may play a key role in increased resistance to oxidative stress and extended life-span in these animals. Therefore, our study may provide important insight into physiological effects of hormonal deficiencies on mitochondrial ETC function in long-lived Ames dwarf mice.

CHAPTER IX

CONCLUSIONS

The Free Radical Theory on Aging proposes age-dependent increase in ROS production causes macromolecular damage that accumulates leading to tissue dysfunction in aged mammals. In addition, since mitochondrial ETC complexes are the primary production sites of ROS their proximity makes them susceptible to oxidative damage. This accumulation of damage is thought to be the major component leading to increased mitochondrial dysfunction and decline of tissue function with age. Therefore, we hypothesized that the mitochondrial ETC complexes are specific targets of oxidative modifications caused by ROS and that the levels of modification will increase with age. We also propose that the increase in oxidative damage to these complexes will lead to decline in their function with aging.

The recent discovery of oxidative stress markers F_2-isoprostanes have tremendously advanced the field of aging and age-associated diseases. These markers have successfully been used to show levels of chronic oxidative stress in normal aging as well as acute injuries. In addition, numerous mutant mouse models have been developed that are long-lived compared to their normal counterparts. In particular, the Ames dwarf mice live ~40-60% longer than wild-type mice and have been shown to be resistant to exogenous oxidative stress. Since there is no direct evidence that these mice have lower levels of ROS, we hypothesize that the levels of IsoPs, markers of oxidative stress, may be lower in the dwarf mice and the lower levels of ROS may play key role longevity in these mice. Furthermore, we propose that there are age-related changes in the ETC function from tissues of Ames dwarf mice compared to the wild-type such that these changes may be a factor in extended life-span of these animals.

The results from our studies are as follows:

- Bovine heart mitochondrial ETC complexes are specific targets of oxidative modifications.

- ETC proteins are specific targets of oxidative modification in various mouse tissues.
- In many tissues there is an age-associated increase in the level of oxidative modification in these proteins.
- In C57BL/6 mouse kidney, we found increase in oxidative modification of CI, CII and CV proteins that directly correlates with decline in enzyme function with aging.
- In C57BL/6 mouse pectoralis, we found that almost all complexes have a dramatic loss of function by middle age and this decline corresponds to an increase in oxidative modification of their subunits.
- In contrast to pectoralis, C57BL/6 mouse quadriceps muscle showed very little change in ETC function despite increased levels of modification suggesting that the specific site(s) of modification may be more important than general increase in oxidative damage to determine its effects on structure and function of ETC complexes.
- In C57BL/6 mouse heart, we found that only CI and CV function decreases with age and interestingly we find dramatically higher levels of oxidative modifications to its subunits compared to only a minimal loss of function.
- Measurement of IsoPs clearly showed that Ames dwarf mice have lower levels of endogenous oxidative stress and the difference between WT and DW decreases with age suggesting that DW mice may have delayed characteristics of aging.
- Interestingly, very few differences in ETC function between Ames WT and DW mice were seen for all post-mitotic tissues studied.
- On the other hand, a slowly proliferating tissue such as kidney showed higher activities for ETC complexes suggesting improved mitochondrial function that may contribute to the higher longevity of these mutant animals.
- No changes in enzyme levels or oxidative modifications were found in Ames ETC complexes suggesting that these may undergo similar mitochondrial oxidative

stress but may have down-regulated cellular stress pathways due to hormonal imbalance and metabolic changes in mitochondrial function may play a key role in extended life-span in DW mice.

Although we found major changes in both complex activities and oxidative modifications of ETC subunits with age in various mouse tissues, there were several limitations of this study. Since the mitochondrial yield from the selected tissues was very low all experiments in this study were done with pooled tissues and crude mitochondria were used rather than purified mitochondria. Thus, pooled mitochondria may lead to underestimation of the results in this study. The immunoblot results showing oxidative modifications were only verified by running duplicate gels and performing the same analyses on the duplicate gels. Thus, the results in figures depicting oxidative modifications may be a possible artifact due to isolation as well as multiple normalizations that were done to do proper age-related comparisons. In addition, the oxidative modifications that were not tested (i.e. nitrosylation, peptide backbone breaks due to direct oxidation, other lipid peroxidation adducts) may also be important in determining the functional effects of oxidative damage. Finally, since we did not measure ROS levels, mitochondrial respiration, and ATP levels the results from our studies are only correlative and do not show mitochondrial dysfunction with age *in vivo*.

The heart, kidney and pectoralis tissues of C57BL/6 mice showed decrease in complex activities with increase in oxidative modification to their subunits in aging. However, in some cases the oxidative modification did not seem to change with age or have any effect to complex function and as seen in quadriceps no effects of increased oxidative modifications on ETC function were observed in aging. Thus, these results do not consistently show whether there is decline in tissue function with age due to increase in oxidative stress. The fact that many of the quadriceps ETC subunits were heavily modified without any age-related differences in ETC activities suggests that these oxidative modifications may play different roles in different tissues and may not always

predict functional consequences in mitochondria with age. In addition, the long-lived Ames DW mutant mice show conflicting results where the oxidative stress markers (IsoPs) are lower in DW mice compared to WT but no significant differences in oxidatively modified proteins or the levels of modifications are seen in all four tissues at any age and except for kidney very few changes in ETC function are seen between WT and DW mice.

Thus, the combined results from this study lead us to conclude that chronic stress due to increased ROS production in aging does specifically target ETC complex subunits and in some cases the increase in oxidative modification correlates the decline in activity of the complex. However, the level of oxidative modification may not always be reliable markers of mitochondrial dysfunction and decline in tissue function as suggested by the free radical theory of aging. Furthermore, the specificity of the site being modified may be more important in elucidating the effects of ROS-mediated damage on complex function rather than general increase in modification. Thus, this study of molecular analysis of mitochondria only partially supports the free radical theory of aging and does not provide any conclusive evidence for or against the role of free radicals in longevity.

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VITA

Kashyap B. Choksi was born on May 30, 1976 in Ahmedabad, India to Bhagawat and Purnima Patel. Kashyap moved to Houston, Texas on November 22, 1992 and completed his high school in Houston. Kashyap then continued his education and received his Bachelor of Science degree in 1998, with a major of Biochemical and Biophysical Sciences from the University of Houston. During his senior year, Kash volunteered to do research in a laboratory under the direction of Dr. Horace Gray and spent 9 months isolating and characterizing a novel BAL31 endonuclease and its potential uses in human genome project. During his time at the University of Houston, he was involved in teaching Calculus III as well as tutoring Biochemistry and Organic Chemistry to a group of students in Scholar Enrichment Program at UH.

He continued his studies in scientific research and received hi Master of Science degree in 2000, with his thesis in the major of Biochemistry from the University of Houston under the guidance of Dr. William R. Widger. The research project involved elucidating the mechanisms of reactive oxygen species production from isolated bovine heart mitochondrial electron transport chain Complex I. He published his thesis titled "The Mechanism of Reactive Oxygen Species Formation by Mammalian Mitochondrial NADH:Ubiquinone Oxidoreductase."

In 2000, Kash entered the School of Medicine at UTMB and was accepted to the M.D.-Ph.D. combined degree program in May, 2001. He did his research rotation in Summer of 2001 under the guidance of Dr. Steve Weinman and focused on determining the effects of Hepatitis C Core protein on mouse liver mitochondrial respiration and oxidative phosphorylation. Upon completion of Year 2 of medical school education Kash did his second research rotation with Dr. John Papaconstantinou and determined the effects of 3-Nitropropionic Acid on mammalian mitochondrial Complex II. He also performed blue-native PAGE with mouse liver and heart mitochondrial electron transport chain complexes. He was very enthusiastic of the research being done in Dr. Papaconstantinou's laboratory that he joined the group and under the mentorship of Dr. Papaconstantinou he pursued his dissertation project focusing on elucidating the mechanisms of aging and longevity using molecular markers of aging. His dissertation is titled "Molecular Analysis of the Role of Mitochondria in Aging and Longevity." Kash also was a Teaching Faculty for School of Medicine undergraduate summer programs at UTMB and taught Embryology to the students in Joint Admissions Medical Program in 2005.

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