Original Contribution

Age-related increases in oxidatively damaged proteins of mouse kidney mitochondrial electron transport chain complexes

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Abstract

Mitochondrial dysfunction generates reactive oxygen species (ROS) which damage essential macromolecules. Oxidative modification of proteins, DNA, and lipids has been implicated as a major causal factor in the age-associated decline in tissue function. Mitochondrial electron transport chain complexes I and III are the primary sites of ROS production, and oxidative modifications to the complex subunits inhibit their in vitro activity. Therefore, we hypothesize that mitochondrial complex subunits may be primary targets for oxidative damage by ROS which may impair normal complex activity by altering their structure/function leading to mitochondrial dysfunction associated with aging. This study of kidney mitochondria from young, middle-aged, and old mice reveals that there are functional decreases in complexes I, II, IV, and V between aged compared to young kidney mitochondria and these functional declines directly correlate with increased oxidative modification to particular complex subunits. We postulate that the electron leakage from complexes causes specific damage to their subunits and increased ROS generation as oxidative damage accumulates, leading to further mitochondrial dysfunction, a cyclical process that underlies the progressive decline in physiologic function seen in aged mouse kidney. In conclusion, increasing mitochondrial dysfunction may play a key role in the age-associated decline in tissue function.

Keywords: Oxidative stress; Mitochondrial dysfunction; Aging; 4-Hydroxynonenal; Malondialdehyde; Nitration

Introduction

The mitochondrial theory of aging proposes that increasing oxidative stress resulting from increasing mitochondrial dysfunction is a basic mechanism of mammalian aging [1,2].

Mitochondria are a major source of ROS production and oxidative stress during the aging process [3–5], and therefore a central factor in the age-associated decline in tissue function. Mitochondrial ROS is produced by in vivo electron leakage from electron transport chain (ETC) complexes during normal respiration. In particular, complex I and complex III are primary sites of ROS production [6–8]. Although free radicals from complex I and complex III have been identified by electron paramagnetic resonance [3,9,10], the short half-life of these radicals makes their accurate measurement difficult. In contrast, the resultant covalent modifications of macromolecules, such as proteins, caused by reactions with ROS are more stable and more easily detectable, and thus can be used as molecular markers of oxidative stress [11]. The relative abundance of modified proteins has, therefore, been used to indicate the level of oxidatively damaged macromolecules that accumulate in

Abbreviations: ACADL, acetyl-CoA dehydrogenase long chain; ACOX1, acyl-CoA oxidase 1; ALDH2, aldehyde dehydrogenase 2; BN-PAGE, blue-native polyacrylamide gel electrophoresis; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V; CoQ, coenzyme Q; DECR1, 2,4-dienoyl CoA reductase 1; DNP, 2,4-dinitrophenylhydrazone; DNPH, 2,4-dinitrophenylhydrazine; ETC, electron transport chain; GGT1, gamma-glutamyltransferase; HNE, 4-hydroxynonenal; MALDI-TOF, matrix-assisted laser disorption–time of flight; MDA, malondialdehyde; MPP, mitochondrial processing peptidase; ROS, reactive oxygen species.

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aged tissues [12]. 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 carboxylation of lysine, arginine, proline, and threonine [13–16]. Oxidatively damaged proteins have been detected by immunoblotting using antibodies specific for these modifications and subsequently identified by mass spectrometry [11,17]. In addition, such oxidative modifications to proteins can result either in reduction of normal function [15,16,18–20] or in the gain of toxic function [21] associated with aging and age-associated diseases. These oxidative modifications are therefore 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.

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 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 [22], and it is prone to relatively high levels of HNE adduct formation in mitochondrial proteins after iron overload [23]. We employed blue-native polyacrylamide gel electrophoresis (BN-PAGE) to further resolve intact ETC complexes [24] 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 [25]. 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.

Materials and methods

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 12-h light/dark cycle and fed ad libitum on a standard chow diet before sacrifice.

Mitochondrial isolation

Mice were sacrificed by decapitation and their kidneys were harvested immediately, rinsed in ice-cold PBS, and prepared for subcellular fractionations. Mitochondria were prepared from the pooled kidneys of 9 young, 10 middle-aged, and 8 old C57BL/6 male mice. Mitochondrial isolation was carried out at 4°C as described [26] with minor modifications [17]. Briefly, kidneys were blended using a Brinkman Polytron PT 3000 with large blade for 10–15 s 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 a Teflon pestle glass homogenizer and the homogeneous solution was centrifuged at 800g for 20 min. 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 800g for 20 min. The supernatant was combined with the one from the previous step and centrifuged two additional times at 800g for 20 min. Each time the supernatant was transferred to a new tube and pellet was discarded. The final supernatant was centrifuged at 8000g for 20 min to pellet the mitochondria. The mitochondrial pellets were washed twice with half volumes and centrifuged at 8000g and the supernatants were discarded. The final mitochondrial pellets were resuspended in a minimal volume of isolation buffer and stored in aliquots at –80°C. For each analysis, fresh aliquots were used. To verify respiratory activity, oxygen consumption was measured using an oxygen monitoring system (Strathkelvin Instruments Ltd., Glasgow UK) with both NADH and succinate as substrates in sonicated mitochondria generated with a Branson Model 250 Digital Sonifier (2 cycles of 1.8 s each, 6 s total time for each cycle, 1 min between each cycle, amplitude 30% pulse time on for 0.3 s, pulse time off for 0.7 s).

Enzyme activities

Enzyme activities were performed at room temperature using a Beckman Coulter DU 530 spectrophotometer (Beckman Coulter, CA). Citrate synthase activity was measured as described [27]. Briefly, in each 1 mL assay reaction mixture containing reaction buffer (50 mM potassium phosphate, pH 7.4, 5 mM MgCl2, 2 mM EDTA, 0.1% Triton X-100, and 1 mg/mL BSA) 24–26 μg of sonicated mitochondria (as described above) was added followed by the 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 [20,28]. 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), 24–26 μM of sonicated mitochondria, 2 mM KCN, 3.7 μM antimycin A, and 100 μM Q1. The reaction was initiated by the addition of 140 μM NADH and after 3 min 20 μM rotenone was added to inhibit the enzyme activity. The final rate was measured by subtracting rotenone-insensitive rate from 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°C for 20 min containing reaction buffer (50 mM potassium phosphate, pH 7.4, 5 mM MgCl2, and 1 mg/mL BSA), 24–26 μg of sonicated mitochondria, 20 μM rotenone, 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 Q1. After 3 min 10 mM malonate was added to inhibit the enzyme activity. The final rate was measured by subtracting maitonate-insensitive rate from initial rate. CIII activity was measured at 550 nm (ε = 22 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), 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 decylbenzoquinol with or without 7.4 μM antimycin A. The final rate was measured by subtracting antimycin A-insensitive rate from rate without the addition of the inhibitor. CV 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 MgCl2, and 1 mg/mL BSA), 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 rate without the 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 MgCl2, and 250 mM sucrose), 24–26 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 min 15 μM oligomycin was added to inhibit the enzyme activity. The final rate was measured by subtracting oligomycin-insensitive rate from initial rate.

Cl–III- and ClII–III-coupled assays were performed as described [20,28]. Briefly, Cl–ClIII activity was measured at 550 nm (ε = 19 mM⁻¹ cm⁻¹) in 1 mL reaction mixture initially incubated at 30°C for 10 min containing reaction buffer (50 mM potassium phosphate, pH 7.4, 5 mM MgCl2, and 1 mg/mL BSA), 24–26 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 min, 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 initial rate. CII–ClIII activity was measured at 550 nm (ε = 19 mM⁻¹ cm⁻¹) in 1 mL reaction mixture initially incubated at 30°C for 20 min containing reaction buffer (50 mM potassium phosphate, pH 7.4, 5 mM MgCl2, 0.5 mM EDTA, and 1 mg/mL BSA), 24–26 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 min 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 results are averages of four assays from the pooled sample for each age group. Citrate synthase assay results were used to calculate ratios of young to middle-age and young to old mitochondrial protein levels and these ratios were multiplied to normalize each enzyme activity for specific age group. Statistical significance was calculated using the Student t test with P < 0.05 and P < 0.001 considered significant and highly significant, respectively.

**Coenzyme Q levels**

Total mitochondrial coenzyme Q was quantified using an HPLC method [29,30]. Briefly, coenzyme Q was extracted from mitochondrial preparations at 4°C with ethanol–n-hexane (2:5, v/v). Samples were dried to completion 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, MA) system equipped with a UV-Visible (Model 528, ESA Biosciences) detector. Isocratic separations were performed by pumping mobile phase consisting of 50 mM NaClO4 dissolved in ethanol–methanol–70% HClO4 (700:300:1, v/v) through a C18 column (150 × 4.6 mm, 5 μm Alltech Associates, IL) while continually monitoring absorbance at 275 nm. Elution positions of reduced and oxidized coenzyme Q9 (CoQ9) and coenzyme Q10 (CoQ10) 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 CoQ9 and CoQ10 with appropriate calibration curves. To account for variations in extraction efficiency, decylubiquinone (~20 nmol) was spiked into mitochondrial preparations and served as an internal standard. Statistical significance was calculated using the Student t test with P < 0.05 and P < 0.001 considered significant and highly significant, respectively.

**Polyacrylamide gel electrophoresis**

BN-PAGE and SDS-PAGE were carried out by established methods [24] with minor modifications [11]. 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 [17]. Briefly, all immunoblots were generated after overnight transfer and were blocked with 5% nonfat blocking grade milk (Bio-Rad) in TBS-T (Tris base saline, pH 7.4, and 0.05% Tween 20) and incubated with appropriate antibody dilutions in blocking
solution for 1 h or overnight. The blots were washed three times for 5 min each with TBS-T and probed with appropriate secondary antibodies conjugated with HRP (Alpha Diagnostic, 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, CA) and density values were calculated according to the manufacturer's instructions.

Intact mitochondrial ETC complex bands were visualized by antibodies against CI (NDUFA9 subunit), CII (SDHA subunit), CIII (UQCRFS1 subunit), CIV (COX1), and CV (ATP5A1 subunit) (Molecular Probes, OR). CIV-specific antibody is against 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 (Upstate Biotechnology, NY), anti-MDA goat polyclonal antibody (Academy Bio-Medical, TX), and anti-HNE Fluorophore rabbit polyclonal antibody (EMD Biosciences, CA). Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH) to generate a stable 2,4-dinitrophenylhydrazone (DNP) adduct at the carbonyl group [31]. Anti-DNP rabbit polyclonal antibody (Molecular Probes, OR) was then used to detect DNP-derivatized proteins. All oxidative modification detecting immunoblots were stripped using Restore Western blot stripping buffer (Pierce Biotechnology, IL) per the manufacturer's recommendations and reprobed with complex-specific antibodies as noted 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 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 immuno blotted and analyzed by the Proteomics Core Facility at UTMB. The proteins were eluted from the gel and digested with trypsin (Promega, WI); the tryptic peptides were then analyzed by MALDI-TOF-TOF [11]. Mass spectral peak data were submitted to the ProFound (Rockefeller University) online search engine for protein identification using the NCBI database.

**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 CI–II–III for all three ages (Figs. 1 and 2). Rotenone-sensitive CI activity did not change significantly between young and middle age but decreased 15–20% by 20–22 months (Fig. 1A). In contrast, there was a 35% increase in CI–III-coupled activity in both middle and old age compared to young age (Fig. 2A). Malonate-sensitive CII activity decreased by 10% in middle age and by 30% in old age (Fig. 1B). The coupled CII–III activity also showed an age-associated decline in activity (Fig. 1B), i.e., 15% decline in middle age and 30% decline in old age (Fig. 2B). Antimycin A-sensitive CIII activity did not change with age (Fig. 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 (Fig. 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 (Fig. 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.

**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 1). Although the CoQ9 and CoQ10 homologues make up about 95% of pool present in mitochondria, CoQ9 is the predominant form in mouse tissues [29,30]. Thus, we measured both CoQ9 and CoQ10 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 young, there is ~33% increase in CoQ levels at middle age and ~19% increase at old age, which indicates that aging does not affect the CoQ substrate availability for ETC complexes. In fact the data suggest that there is more CoQ available for these complexes.

**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 [25]. 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×, 1/2×, and 1/4×. The Coomassie-stained first-dimensional electrophoretically resolved ETC complexes for these dilutions are shown in Fig. 3A. Duplicate gels were run and transferred to PVDF membranes to identify individual complexes by immu-
Fig. 1. Measurement of ETC complex activities from 3- to 5-, 12- to 14-, and 20- to 22-month-old mouse kidney mitochondria. Individual complex enzyme activities were measured spectrophotometrically as described under Materials and methods. All activity results are averages of four 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 nmol/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 nmol/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 nmol/min/mg and coefficients of variance were 8.3% (young), 6.6% (middle-age), and 6.6% (old), respectively. (D) Kidney CIV activity with aging. Young CIV activity was 2731.2 nmol/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 nmol/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.
n blotting using antibodies specific for ETC complex components (Fig. 3B). The densities of the individual complex bands were measured and compared to the $1 \times$ sample set as 100%. The results clearly show that both Coomassie staining and immunoblotting procedures can detect a 2- or 4-fold difference in complex abundance. Furthermore, the coefficients of variance of both methodologies for each complex at $1 \times$ sample amount (which was 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 (Fig. 4). No age-related changes in protein levels of complexes in kidney mitochondria were detected by these methods (Fig. 4). However, since all ETC complexes are multiprotein 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, immunoblotting of second-dimension gels was performed to detect individual proteins with MDA (Fig. 5A), HNE (Fig. 6A), and nitrotyrosine (Fig. 7A) adducts. The corresponding change in percentage density for protein modification is expressed relative to young protein density and is shown in Figs. 5B, 6C, and 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 2. Anti-DNP immunoblots used to detect carbonylated proteins indicated that there were no age-associated differences in modification levels (data not shown). All experiments were performed twice to check for accuracy of results and showed almost identical results. This along with results from Figs. 3 and 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 Fig. 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 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 (Nos. 1, 4, 5, and 6 in Fig. 5B). In addition, NDUFS1 also contained HNE adducts which increased 5-fold by middle age and 8-fold by old age (band 1 in Fig. 6A and No. 1 in Fig.

<table>
<thead>
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<th>Age</th>
<th>CoQ9 total (nmol/mg)</th>
<th>CoQ10 total (nmol/mg)</th>
<th>CoQ9±CoQ10 (nmol/mg)</th>
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<tbody>
<tr>
<td>Young</td>
<td>86.9±1.3</td>
<td>30.2±0.3</td>
<td>117.1±1.3</td>
</tr>
<tr>
<td>Middle-age</td>
<td>118.2±0.5</td>
<td>37.3±1.1</td>
<td>155.5±1.3**</td>
</tr>
<tr>
<td>Old</td>
<td>105.5±0.6</td>
<td>33.8±1.5</td>
<td>139.2±1.6**</td>
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Pooled kidney mitochondrial samples were used for each age group. Coenzyme Q was extracted and quantified as described under Materials and methods. Data are an average of three experiments±SE. Coefficients of variance for CoQ9±CoQ10 are 7.1% (young), 3.9% (middle-age), and 12.6% (old), respectively. **$P<0.001$ compared to young, $\dagger P<0.05$ compared to middle-aged.
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 comigrated with CI. The CI-associated α chain contained both lipid peroxidation adducts, MDA and HNE (ATP5A1, band 2 in Figs. 5A and 6A), while the CI-associated β chain was modified by MDA and nitration (ATP5B, band 3 in Fig. 5A, band 1 in Fig. 7A).

Oxidatively modified proteins of complex II

Complex II proteins containing MDA and HNE adducts include subunit 1 (SDHA, band 7 in Fig. 5A) and subunit 2 (SDHB, band 5 in Fig. 6A), respectively. Compared to young, the modification of SDHA by MDA increased by 23% at middle age and by 82% in old age (No. 7 in Fig. 5B). In contrast, the extent of modification of SDHB subunit decreased with aging (No. 5 in Fig. 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 cofactor and the active site for substrate binding. However, SDHB is mostly embedded in the inner mitochondrial membrane. 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 comigrate 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 in Fig. 5A and No. 8 in Fig. 5B; band 3 in Fig. 6A and No. 3 in Fig. 6B; and band 2 in Fig. 7A and No. 2 in Fig. 7B, respectively); whereas IDH2 showed an increase in MDA modification (band 9 in Fig. 5A and No. 9 in Fig. 5B) at old age compared to young and DECR1 showed a decrease in HNE modification (band 4 in Fig. 6A and No. 4 in Fig. 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 Fig. 5A and include Core 1 (UQCR1, band 11), Core 2 (UQRC2, 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, Nos. 11 and 12 in Fig. 5C), ISP shows a 3-fold increase in middle age and a 5-fold increase by old age compared to young kidney (No. 13 in Fig. 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. ISP is completely embedded in the inner mitochondrial membrane and near the heme bL of cytochrome b subunit where the second CoQ is thought to associate for single electron transfer [8,10]. 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 comigrate with CIII and was immunoreactive to anti-MDA, showing an increase in modification at old age (band 10 in Fig. 5A and No. 10 in Fig. 5C) and to anti-HNE showing a decrease at old age (band 6 in Fig. 6A and No. 6 in Fig. 6B).

Oxidatively modified proteins of complex IV

Subunit 2 (COX2) was the only CIV protein found to be modified by MDA (band 16 in Fig. 5A). Modification of this subunit compared to young increased 50% at 12–14 months and further increased nearly 2-fold by 20–22 months (No. 16 in Fig. 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 comigrate 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 and 15 in Fig. 5A and Nos. 14 and 15 in Fig. 5C) and a decrease at old age (bands 8 and 9 in Fig. 6A and Nos. 8 and 9 in Fig. 6C), while ALDH2 only contained HNE adducts and showed a very high increase at middle age.

Fig. 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 under Materials and methods. (A) The gel was stained with Coomassie G-250 stain. Lanes 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.
that lowered at old age but was still higher than young age (band 7 in Fig. 6A and No. 7 in Fig. 6C).

**Oxidatively modified proteins of complex V**

CV proteins that contained MDA adducts include the α and β chain (ATP5A1 and ATP5B, bands 17 and 18 in Fig. 5A). Both proteins show a slight decrease in MDA modification in middle age compared to young age but show similar levels of modification by old age (Nos. 17 and 18 in Fig. 5C). The α and β chains were also modified by HNE (ATP5A1 and ATP5B, bands 10 and 11 in Fig. 6A). In contrast to MDA modification, HNE modification levels increased by 50% in middle age compared to young and either remained unchanged (α chain, No. 10 in Fig. 6C) or returned to the young level (β chain, No. 11 in Fig. 6C) by old age. In addition, the γ polypeptide of CV
(ATP5C1, band 12 in Fig. 6A) was also modified by HNE and the level of modification decreased with age (No. 12 in Fig. 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...

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 under Materials and methods 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 under Materials and methods. 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 and 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 2.

Fig. 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 under Materials and methods 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 under Materials and methods. 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 and 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 2.
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 [12,32]. 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 [28]. However, these investigators measured their enzyme activities at 30°C compared to our enzyme activity assay conditions which 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

**Fig. 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 under Materials and methods 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 under Materials and methods. 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 2.
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 [28] 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 [33,34].

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 the 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 consideration. 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 [7,9,35], 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 lead 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, first-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-mediated 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, there-

### Table 2

<table>
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<td>2.38</td>
<td>Complex I</td>
</tr>
<tr>
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<td>α Chain (59.7 kDa)</td>
<td>2.39</td>
<td>Complex V</td>
</tr>
<tr>
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<td>β Chain (56.3 kDa)</td>
<td>2.41</td>
<td>Complex V</td>
</tr>
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</tr>
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<tr>
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<tr>
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<td>Gamma-glutamyltransferase 1 (61.5 kDa)</td>
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<td>Peroxisome</td>
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</table>

| **Nitrotyrosine-modified (Fig. 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.
fore, suggest that accessibility may play a key role in the ROS-mediated modification of these subunits. The continuous decline of inhibitor-sensitive activity of CI as well as the coupled CI–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 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 [36,37]. 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, even though there is a 5-fold increase in HNE modification of NDUF51 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 either that a threshold in the number of sites modified has been exceeded or structural that 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 [38–43], and this correlates with the amount of damaging adduct 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 region that are located in the mitochondrial matrix. Interestingly, since the CI iron–sulfur clusters donate electrons to molecular oxygen in vitro [7,35], 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 NDUF51 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 [10,44]. 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_{56} of the cytochrome b subunit [44]. 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 [8,44]. 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 [45]. 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 (Cu_{A}) that mediates electron transfer between reduced cytochrome c and heme a in COX1 [46]. 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 [47–51].

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 middle age, the enzyme activity was not affected. Paradoxically, in aged kidney, 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 conigrated 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 $\alpha$ and $\beta$ 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 a 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 CI, CII, and CIV show a direct correlation between increased oxidative damage and decreased enzyme function. Although CV shows an 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 an 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 (Fig. 8). Our study provides important insight into physiological effects of oxidative modifications on mitochondrial function and their role in aging.

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References


