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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 principle 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

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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 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 carbonylation 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 12h 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 young, middle-aged and old C57BL/6 male mice. Mitochondrial isolation was carried out at 4°C as described [26] with minor modifications [17]. 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 by submitochondrial particles generated by sonication using an oxygen monitoring system (Strathkelvin Instruments Ltd., Glasgow UK) with both NADH and succinate as substrates.

Enzyme activities

Enzyme activities were performed at room temperature using Beckman Coulter DU 530 Spectrophotometer (Beckman Coulter, CA). Citrate synthase activity was measured as described [27]. Complex I (CI), Complex II (CII), Complex III (CIII), Complex IV (CIV), and Complex V (CV) activities were assayed as described [28]. CI-III and CII-III coupled assays were performed as described [28]. All activity results are averages of 4 assays from the pooled sample for each age group. Citrate synthase assay results were used to normalize levels of mitochondrial proteins. Statistical significance was calculated using the Student's 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, MA) 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 mm x 4.6 mm, 5µm 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 nmole) was spiked into 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

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 a 10% separating gel and 8% stacking gel were used for the second dimension SDS-PAGE.

Immunoblotting

Immunoblot analysis was performed as described [17]. Intact mitochondrial ETC complex bands were visualized by antibodies against CI (NDUFA9 subunit), CII (SDHA subunit), CIII (UQCRC1 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 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. HRP-conjugated secondary mouse, rabbit and goat antibodies (Alpha Diagnostic, TX) were used. 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. All oxidative modification detecting immunoblots were stripped using RestoreTM western blot stripping buffer (Pierce Biotechnology, 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 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, 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 CII-III for all three ages (Figures 1 and 2). Rotenone-sensitive CI activity did not change significantly between young and middle age but decreased 15–20% by 20–22 months (Figure 1A). In contrast, there was a 35% increase in CI-III coupled activity in both middle and old age compared to young age (Figure 2A). Malonate-sensitive CII activity decreased by 10% in middle age and by 30% in old age (Figure 1B). The coupled CII-III activity also showed an age-associated decline in activity (Figure 1B), *i.e.*, 15% decline in middle age and 30% decline in old age (Figure 2B). Antimycin A-sensitive CIII activity did not change with age (Figure 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 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 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 have 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 X, ½ X, and ¼ X. The Coomassie-stained first dimensional electrophoretically resolved ETC complexes for these dilutions are shown in Figure 3A. Duplicate gels were run and transferred to PVDF membranes to identify individual complexes by immunoblotting using antibodies specific for ETC complex components (Figure 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 can detect a two- or four-fold difference in complex abundance. Thus, complex-specific antibodies were used to normalize loading in immunoblotting done for specific modifications. We then proceeded to use these techniques to examine potential changes in ETC complex abundance in aged kidney mitochondria. Secondly, having established the validity of Coomassie staining and immunoblotting to quantify complex abundance, 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). No age-related changes in protein levels of complexes in kidney mitochondria were detected by these methods (Figure 4). 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 with aging

To identify the oxidatively modified ETC complex proteins, immunoblotting of second dimension gels was performed to detect individual proteins with MDA (Figure 5A), HNE (Figure 6A) and nitrotyrosine (Figure 7A) adducts. The corresponding change in percent density for protein modification is expressed relative to young protein density and is shown in Figures 5B, 5C, 6B, 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).

Oxidatively modified proteins of Complex I

CI proteins that contained MDA adducts are shown in Figure 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 (#1, #4, #5, #6 – Figure 5B). In addition, NDUFS1 also contained HNE adducts which increased five-fold by

middle age and eight-fold by old age (band 1 – Figure 6A and #1 – Figure 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 5A & 6A), while the CI-associated β chain was modified by MDA and nitration (ATP5B, band 3 – Figure 5A, band 1 – Figure 7A).

Oxidatively modified proteins of Complex II

Complex II proteins containing MDA and HNE adducts include subunit 1 (SDHA, band 7 – Figure 5A) and subunit 2 (SDHB, band 5 – Figure 6A), respectively. Compared to young, the modification of SDHA by MDA increased by 23% at middle age and by 82% in old age (#7 – Figure 5B). In contrast, the extent of modification of SDHB subunit decreased with aging (#5 – Figure 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. 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 5A and #8 – Figure 5B; band 3 – Figure 6A and #3 – Figure 6B; and band 2 – Figure 7A and #2 – Figure 7B, respectively); whereas IDH2 showed an increase in MDA modification (band 9 – Figure 5A and #9 – Figure 5B) at old age compared to young and DECR1 showed a decrease in HNE modification (band 4 – Figure 6A and #4 – Figure 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 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 5C), ISP shows a three-fold increase in middle age and a five-fold increase by old age compared to young kidney (#13 – Figure 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 b_L 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 co-migrate with CIII and was immunoreactive to anti-MDA showing an increase in modification at old age (band 10 – Figure 5A and #10 – Figure 5C) and to anti-HNE showing a decrease at old age (band 6 – Figure 6A and #6 – Figure 6B).

Oxidatively modified proteins of Complex IV

Subunit 2 (COX2) was the only CIV protein found to be modified by MDA (band 16 – Figure 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 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 5A and #14 & #15 – Figure 5C) and showed an increase in anti-HNE immunoreactivity at old age (bands 8 & 9 – Figure 6A and #8 & #9 – Figure 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 6A and #7 – Figure 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 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 (#17 & #18 – Figure 5C). The α and β chains were also modified by HNE (ATP5A1 & ATP5B, bands 10 & 11 – Figure 6A). In contrast to MDA modification, HNE modification levels increased by 50% in middle age compared to young and either remained unchanged (α chain, #10 – Figure 6C) or returned to the young level (β chain, #11 – Figure 6C) by old age. In addition, the γ polypeptide of CV (ATP5C1, band 12 – Figure 6A) was also modified by HNE and the level of modification decreased with age (#12 – Figure 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 [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. On the other hand, the

coupled enzyme activities of CI-CIII, which increase with age, are not consistent with the CI enzyme activity, which decreases with age, whereas 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 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 [7,9,33], 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-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, 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 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 [34,35]. 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 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 [36–41], 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* [7,33], 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* [10,42]. 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 [42]. 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 [8,42]. 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 [43]. 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 [44]. 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 [45–49].

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

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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
DECRI	2,4-dienoyl CoA reductase 1
DNP	2,4-dinitrophenylhydrazine
DNPH	2,4-dinitrophenylhydrazine
ETC	electron transport chain
GGT1	gamma-glutamyltransferase

HNE

4-hydroxynonenal

MALDI-TOF

matrix-assisted laser desorption – time of flight

MDA

malondialdehyde

MPP

mitochondrial processing peptidase

ROS

reactive oxygen species

Figure 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 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.

Figure 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 μ g (1X), 80 μ g (1/2X), and 40 μ g (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 μ g (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.

Figure 4.

Protein abundance of ETC complexes in young middle-aged and old kidney mitochondria. Young, middle-aged and old kidney mitochondria (160 μ g) 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-aged kidney mitochondria and O = old kidney mitochondria.

Figure 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 2.

Figure 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 2.

Figure 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 2.

Figure 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.

Table 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 ± 1.3 ^{**}
Old	105.5 ± 0.6	33.8 ± 1.5	139.2 ± 1.6 ^{**} , [†]

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 CoQ₉ + CoQ₁₀ 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.

Table 2

MDA, HNE, and Nitrotyrosine-Modified Protein Subunits of Mouse Kidney Mitochondrial Electron Transport Chain Complexes.

Band #	Gene Name	ProFound Protein ID (MW)	Z score ^a	Localization
MDA-Modified (Figure 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	UQCRCFS1	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 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 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.