Original Contribution

Age-related alterations in oxidatively damaged proteins of mouse heart mitochondrial electron transport chain complexes

Kashyap B. Choksi, John Papaconstantinou *

Department of Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555-0643, USA

Received 23 October 2007; revised 10 December 2007; accepted 8 January 2008
Available online 13 February 2008

Abstract

Mitochondrially generated ROS increase with age and are a major factor that damages proteins by oxidative modification. Accumulation of oxidatively damaged proteins has been implicated as a causal factor in the age-associated decline in tissue function. Mitochondrial electron transport chain (ETC) complexes I and III are the principle sites of ROS production, and oxidative modifications to their complex subunits inhibit their in vitro activity. We hypothesize that mitochondrial complex subunits may be primary targets for modification by ROS, which may impair normal complex activity. This study of heart mitochondria from young, middle-aged, and old mice reveals that there is an age-related decline in complex I and V activity that correlates with increased oxidative modification to their subunits. The data also show a specificity for modifications of the ETC complex subunits, i.e., several proteins have more than one type of adduct. We postulate that the electron leakage from ETC 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 of the aged mouse heart.

© 2008 Elsevier Inc. All rights reserved.

Keywords: Aging; Carbonylation; 4-Hydroxynonenal; Mitochondrial dysfunction; Nitration; Oxidative stress; Free radicals

Increasing ROS production, an aging cardiomyocyte phenotype, is a major consequence of mitochondrial dysfunction [1,2] that contributes to age-associated cardiomyocyte injury in normal aging as well as during myocardial ischemia [3,4]. However, although the site of ROS production in myocardial dysfunction has been localized to specific electron transport chain (ETC) complexes, the consequences of this oxidative stress, i.e., the oxidative damage to proteins of the ETC complexes, are not understood. In this study we identify oxidatively modified proteins in aging cardiomyocytes as an important step in understanding their potential role in myocardiacyte aging and injury.

Complex I (CI) and complex III (CIII) are the major sites for ROS production in aging and ischemia–reperfusion injury of the heart [5–7]. The NADH dehydrogenase site of CI, which is also the site of electron leakage, is located in the matrix side of the inner mitochondrial membrane [8]. Thus, oxidant production from CI is directed into the mitochondrial matrix where oxidative damage to mitochondrial proteins may occur. Our studies have shown that oxidative modification of proteins of CI–CV accumulate in aging mouse kidney, suggesting that such modifications may play a role in age-associated mitochondrial dysfunction [9].

CIII, also a key site of ROS generation [6,10–12], has been shown to release superoxide to both sides of the inner mitochondrial membrane [10,13–15]. The sites of release of ROS suggest that the protein components most proximal to these sites may also be at risk for oxidative damage. Thus, the identification

Abbreviations:
- ATP5A1, complex V α chain; ATP5B, complex V β chain; BN–PAGE, blue native polyacrylamide gel electrophoresis; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V; COX4, cytochrome c oxidase subunit 4; DNP, 2,4-dinitrophenylhydrazine; DNPH, 2,4-dinitrophenylhydrazine; ETC, electron transport chain; HNE, 4-hydroxynonenal; MALDI-TOF–TOF, matrix-assisted laser desorption/ionization time of flight–time of flight; MDA, malondialdehyde; MPP, mitochondrial processing peptidase; NDUF81, NADH dehydrogenase Fe–S subunit 1; NDUFV1, NADH dehydrogenase flavoprotein subunit 1; ROS, reactive oxygen species; SDHA, succinate dehydrogenase subunit 1; UQCRC1, Core 1 subunit; UQCRC2, Core 2 subunit.
* Corresponding author. Fax: +1 409 772 9216.
E-mail address: japacon@utmb.edu (J. Papaconstantinou).

0891-5849/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2008.01.032
of oxidatively modified proteins of the ETC complexes may contribute to further understanding of the molecular mechanisms of mitochondrial dysfunction in aging and age-associated cardiomyocyte injury. In this study we have focused upon the identification of oxidatively modified proteins of the ETC complexes of the aged mouse heart.

Increasing oxidative stress resulting from progressive mitochondrial dysfunction, as proposed by the free radical theory of aging, is a basic mechanism of mammalian aging [16,17]. Mitochondrial ROS production plays a central role in the age-associated decline in tissue function [18–20]. Mitochondrially generated ROS, produced by in vivo electron leakage from ETC CI and CIII, play a key role in the modification of mitochondrial proteins [18,21–25]. These modifications have served as molecular markers of oxidative stress [26,27]. In these experiments we identify the oxidatively modified ETC CI–CV proteins in aged mouse heart and whether these proteins accumulate with age and affect ETC complex function.

The relative abundance of modified proteins is indicative of the level of accumulation of oxidatively damaged macromolecules in aged tissues [9,27]. Protein modifications caused by ROS include the formation of lipid peroxidation adducts (4-hydroxynonenal, or HNE, and malondialdehyde, or MDA); carboxylation of lysine, arginine, proline, and threonine; and nitration of tyrosine [28–31]. Oxidatively damaged proteins have been detected and identified by mass spectrometry [9,26,32]. We propose to determine whether such oxidative modifications cause mitochondrial dysfunction associated with aging and age-associated diseases [9,30–33]. The accumulation of these oxidatively modified proteins occurs in various tissues [9] and their accumulation in cardiovascular tissue may, therefore, be an important molecular marker of age-associated decline in cardiovascular function. We propose that oxidative modification may play an important role in the molecular mechanisms of aging and development of age-associated diseases, including cardiovascular disease.

In this study we analyzed the activities of ETC CI–CV to identify potential age-associated functional changes and whether the modifications of specific proteins correlate with changes in enzyme activities. We chose hearts to test the hypothesis that oxidative modification of proteins may lead to a decline in cardiovascular function and whether specific proteins of CI–CV proximal to the sites of ROS production are susceptible to oxidative damage. Our studies provide further support for the mitochondrial theory of aging as indicated by the loss of function of oxidatively modified ETC proteins leading to a decline in tissue function.

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, USA). 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 hearts were harvested immediately, rinsed in ice-cold PBS to remove blood, and prepared for subcellular fractionations. Mitochondria were prepared from the pooled hearts of 9 young, 10 middle-aged, and 8 old C57BL/6 male mice. Mitochondrial isolation was carried out at 4°C as described with minor modifications [9,34,35].

Enzyme activities

Enzyme activities were performed at room temperature using a Beckman Coulter DU 530 spectrophotometer (Beckman Coulter, CA, USA). Citrate synthase activity was measured as described [19,36]. 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) 7–8 μg of sonicated mitochondria (as described above) were added, followed by addition of 0.1 mM acetyl-CoA and 2 mM DTNB. The reaction was initiated with the addition of 40 μM oxaloacetate and the enzyme activity was recorded at 412 nm (ε=13.6 mM⁻¹ cm⁻¹). Rotenone-sensitive CI activity, malonate-sensitive complex II (CII) activity, antimycin A-sensitive CIII activity, KCN-sensitive complex IV (CIV) activity, and oligomycin-sensitive CV activity were assayed as described [9,26,33]. Briefly, CI activity was measured at 340 nm (ε=6.81 mM⁻¹ cm⁻¹) in 1 ml reaction mixture containing reaction buffer (50 mM potassium phosphate, pH 7.4, 5 mM MgCl₂, and 1 mg/ml BSA), 7–8 μg 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 the rotenone-insensitive rate from the initial rate. CII activity was measured at 600 nm (ε=19.1 mM⁻¹ cm⁻¹) in 1 ml reaction mixture initially incubated at 30°C for 20 min containing reaction buffer (50 mM potassium phosphate, pH 7.4, 5 mM MgCl₂, and 1 mg/ml BSA), 7–8 μg of sonicated mitochondria, 20 mM succinate, and 0.2 mM ATP. The reaction was initiated by the addition of 20 μM rotenone, 2 mM KCN, 3.7 μM antimycin A, 50 μM DCPIP, and 100 μM Q₁. After 3 min 10 mM malonate was added to inhibit the enzyme activity. The final rate was measured by subtracting the malonate-insensitive rate from the initial rate. CIII activity was measured at 550 nm (ε=19 mM⁻¹ cm⁻¹) in 1 ml reaction mixture containing reaction buffer (50 mM potassium phosphate, pH 7.4, 5 mM MgCl₂, and 1 mg/ml BSA), 1 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 dicyclobenzoxquinol with or without 7.4 μM antimycin A. The final rate was measured by subtracting the antimycin A-insensitive rate from the rate without the addition of the inhibitor. CIV activity was measured at 550 nm (ε=19 mM⁻¹ cm⁻¹) in 1 ml reaction mixture containing reaction buffer (10 mM Tris–HCl, pH 7.4, 20 mM KCl, and 1 mg/ml BSA), 0.5–1 μg of sonicated mitochondria, and 1 mM dodecyl-β-D-maltoside. The
reaction was initiated by the addition of 11 μM ferrocyanochrome c with or without 2 mM KCN. The final rate was measured by subtracting the KCN-insensitive rate from the 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), 7–8 μg of sonicated mitochondria, 25 units of pyruvate kinase, 25 units of lactate dehydrogenase, 20 μM rotenone, 2 mM KCN, 5 mM phosphoenol pyruvate, 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 the oligomycin-insensitive rate from the initial rate.

CI–CIII and CII–CIII coupled assays were performed as described [9,37]. Briefly, CI–CIII 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), 7–8 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 the inhibitor-insensitive rate from the initial rate. CII–CIII activity was measured at 550 nm (ε = 19 mM⁻¹ cm⁻¹) in 1 ml reaction mixture initially incubated at 30°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), 7–8 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 the malonate-insensitive rate from the 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 each 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.

Polyacrylamide gel electrophoresis

BN–PAGE and SDS–PAGE were carried out by established methods with minor modifications [9,26,38]. 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, USA) were used for the second-dimension SDS–PAGE.

Immunoblotting

Immunoblot analysis was performed as described [9,34]. 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, USA). Immunoreactive bands were detected by chemiluminescence using the Immobilon Western HRP substrate (Millipore, MA, USA), and images were recorded using Kodak X-Omat AR films. Films were analyzed using an Alpha Innotech FluorChem IS-8900 imager (Alpha Innotech Corp., CA, USA) 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, USA). The CIV-specific antibody is against the 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, USA), anti-MDA goat polyclonal antibody (Academy Bio-Medical, TX, USA), and anti-HNE fluorophore rabbit polyclonal antibody (EMD Biosciences, CA, USA). Carboxylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH) to generate a stable 2,4-dinitrophenylhydrazone (DNP) adduct at the carbonyl group [39,41]. Anti-DNP rabbit polyclonal antibody (Molecular Probes) was then used to detect DNP-derivatized proteins. All oxidative modification-detecting immunoblots were stripped using Restore Western blot stripping buffer (Pierce Biotechnology, IL, USA) per the manufacturer’s recommendations and reprobed 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 the density of young protein bands as 100%. Data represented in the figures are from the same samples for each age group, for which tissues were pooled from 9 animals in the young group, 10 animals in the middle-aged group, and 8 animals in the old age group. Pooling 8–10 tissues maximizes the recovery of mitochondria and minimizes outliers and thus, the observed differences in oxidative modification levels are taken as true biological variations.

Maldi-Tof–Tof

Individual ROS-modified protein bands were excised from the second-dimension SDS–PAGE run simultaneous 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, USA); the tryptic peptides were then analyzed by MALDI-TOF–TOF [26]. 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 aging on heart mitochondrial ETC complexes, we measured the enzymatic activities of all five complexes as well as the coupled activities of CI–III and CII–III for all three ages (Figs. 1 and 2). As evident from the data, only CI and CV from the aged heart showed a decrease in enzyme activities. In contrast, CII as well as CI–CIII and CII–CIII coupled activities showed an age-related increase in enzyme function (Figs. 1 and 2). Rotenone-sensitive CI activity decreased by ~9% at middle age and by ~13% at old age compared to young (Fig. 1A). In contrast, there was an ~20% increase in CI–III coupled activity in both middle and old age compared to young age (Fig. 2A). Malonate-sensitive CII activity also showed an increase with age, i.e., CII activity increased by ~12 and ~19% at middle and old age, respectively (Fig. 1B). Similarly, the CII–CIII coupled activity increased with age, i.e., there was an ~18% increase at middle age and an ~13% increase by old age (Fig. 2B). Antimycin A-sensitive CIII activity also increased by ~14% at old age (Fig. 1C). KCN-sensitive CIV activity did not show any change with aging (Fig. 1D). Oligomycin-sensitive CV activity showed

Fig. 1. Measurement of ETC complex activities from 3- to 5-, 12- to 14-, and 20- to 22-month-old mouse heart 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±SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (3–5 months), middle-aged (12–14 months), and old (20–22 months) heart ETC CI–CV are plotted as follows: (A) CI activity with aging. Coefficients of variance were 2 (young), 5.4 (middle age), and 4.1% (old). (B) CII activity with aging. Coefficients of variance were 3.2 (young), 4.6 (middle age), and 3% (old). (C) CIII activity with aging. Coefficients of variance were 5.7 (young), 7.8 (middle age), and 6.3% (old). (D) CIV activity with aging. Coefficients of variance were 5.4 (young), 4.6 (middle age), and 2.9% (old). (E) CV activity with aging. Coefficients of variance were 6.5 (young), 9.1 (middle age), and 6% (old). *p<0.05 compared to young, **p<0.001 compared to young, and †p<0.05 compared to middle-aged.
the most dramatic decline with aging, especially at middle age, i.e., ~25% decrease in middle age and no further decline in old age (Fig. 1E).

Overall, the mitochondrial ETC enzyme activities of mouse heart show a differential pattern such that CI and CV enzyme activities decline, CII and CIII as well as CI–CIII and CII–CIII coupled activities increase, and CIV activity does not change with age.

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

To determine if the changes in enzyme function with aging are due to changes in enzyme levels, the CI–CV complexes were resolved by BN–PAGE and the levels of ETC complexes were measured by using immunoblotting with complex-specific antibodies. In previous studies we established the use of immunoblotting of BN–PAGE-resolved complexes with complex-specific antibodies as a method to determine complex abundance [9]. This procedure has also been used in these studies to determine whether there are age-related quantitative differences in levels of individual complexes [9,40]. Our results show very few age-related changes in complex levels in heart mitochondria (Fig. 3). Though the data support a 15–20% increase in CIV and CV levels in middle age (Fig. 3B), the changes are not statistically significant and the enzyme activities of both individual complexes were not affected. However, because the ETC complexes are multiprotein complexes, our procedure does not detect any minor changes in components of these complexes.

Oxidative modification of ETC complex subunits with aging

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

Fig. 2. Measurement of coupled mitochondrial ETC complex activities from 3- to 5-, 12- to 14-, and 20- to 22-month-old mouse heart mitochondria. CI–III and CII–III coupled enzyme activities were measured spectrophotometrically as described under Materials and methods. All activity results are averages of four assays from the pooled sample±SEM for each age group. Citrate synthase assay results were used to normalize mitochondrial proteins. Activities for young (3–5 months), middle-aged (12–14 months), and old (20–22 months) heart ETC CI–III and CII–III are plotted as follows: (A) CI–CIII coupled activity with aging. Coefficients of variance were 4.9 (young), 3.8 (middle age), and 1.9% (old). (B) CII–CIII coupled activity with aging. Coefficients of variance were 6.8 (young), 1.5 (middle age), and 4.8% (old). *p<0.05 compared to young and **p<0.001 compared to young.

Fig. 3. Protein abundance of ETC complexes in young, middle-aged and old heart mitochondria. Young, middle-aged, and old heart mitochondria (160 μg) were solubilized and the ETC complexes were separated by BN–PAGE as described under Materials and methods. (A) Representative immunoblots of heart BN–PAGE using complex-specific antibodies. The complexes are shown according to their mass and position in the BN gels. Lanes Y, M, and O represent young, middle-aged, and old heart mitochondrial ETC complexes, respectively. (B) Density values of each ETC complex band are plotted as a percentage of young complexes. All results are averages of three immunoblot analyses from the pooled sample±SEM for each age group. Coefficients of variance for CI were 2.2 (young), 4.6 (middle age), and 6.5% (old). Coefficients of variance for CII were 13 (young), 1.1 (middle age), and 13% (old). Coefficients of variance for CIII were 6 (young), 7.4 (middle age), and 7% (old). Coefficients of variance for CIV were 6.3 (young), 5.1 (middle age), and 5.1% (old). Coefficients of variance for CV were 22.7 (young), 14 (middle age), and 3.6% (old).
(Fig. 4A), as well as HNE (Fig. 5A), nitrotyrosine (Fig. 6A), and MDA (none detected) adducts. The corresponding change in percentage density for protein modification is expressed relative to young protein density and is shown in Figs. 4B, 5B, and 6B. Duplicate second-dimension gels were run simultaneously for each immunoblot and used for identification of modified proteins by MALDI-TOF–TOF and are summarized in Table 1. All experiments were performed twice to check for accuracy of results and showed almost identical results. This along with the results from Fig. 3 confirmed that the results we obtained from immunoblots to detect oxidative modifications were valid and led us to make further conclusions as discussed below.

Oxidatively modified proteins of complex I

The carbonylated and HNE-modified CI protein, shown in Figs. 4A and 5A was identified as the Fe–S subunit 1 (NDUFS1, band 1). With respect to age, this protein shows a mild decrease in carbonylation (No. 1—Fig. 4B) and an increase in HNE (No. 1—Fig. 5B). Oxidative modification of NDUFS1 shows very little change with age, and because this subunit is part of the iron–sulfur protein region, it bears significance that it is specifically modified at all ages. In contrast, NDUFV1, part of the flavoprotein (FP) region, was heavily modified by nitration in an age-dependent manner (Fig. 6A). The nitration of NDUFV1 increased to more than 7-fold by middle age and ~14.5-fold at old age. Again, because this protein is a component of the FP region, the dramatic modification of this subunit shows that it may be specifically targeted by ROS-mediated damage. It is also of particular interest to note that the α chain of CV also comigrated with CI and was modified by carbonylation (band 2—Fig. 4A) and contained HNE (band 2—Fig. 5A) adducts. The CI-associated α chain showed an age-associated decrease in carbonylation levels (No. 2—Fig. 4B) and in HNE levels (No. 2—Fig. 5B).

Oxidatively modified proteins of complex II

Subunit 1 (SDHA, band 3—Fig. 5A) is the only protein of complex II that was oxidatively modified by HNE adducts. Though the modification of SDHA was seen in all ages, there was a decrease in levels of HNE modification at middle age.

Fig. 4. Identification of carbonylated proteins of young, middle-aged, and old heart mitochondrial ETC complex subunits. Heart mitochondrial ETC complexes were resolved into individual subunits and DNP-derivatized after transfer to PVDF membrane as described under Materials and methods followed by immunoblotting. (A) Immunoblot of young, middle-aged, and old heart mitochondrial ETC complex subunits using anti-DNP antibody. Modified proteins were numbered according to their complex localization followed by the highest to the lowest molecular weight of the proteins. Protein loading was normalized using complex-specific antibodies as described under Materials and methods. Normalized density values of each individual carbonylated protein are plotted as a percentage of the young heart protein density for all five ETC complex subunits. (B) Densitometry for modified proteins found in CI (1 and 2), CII (3), CIV (4) and CIII (5). Identification of each numbered band is summarized in Table 1.
and no change in old age (No. 3—Fig. 5B) compared to young mice. Because 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, its differential modification with lipid peroxidation suggests that it is specifically targeted by ROS-mediated damage.

Oxidatively modified proteins of complex III

The oxidatively modified CIII proteins are shown in Figs. 4A, 5A, and 6A and include Core 1 (UQCRCl, band 4—Fig. 5A and band 3—Fig. 6A) and Core 2 (UQCRCl2, band 3—Fig. 4A). Whereas Core 1 is modified by nitration and HNE, Core 2 is carbonylated. The modification of Core 2 shows an age-related decrease in carbonylation and by old age it decreases by ~46% compared to young mice (No. 3—Fig. 4B). Core 1, on the other hand, shows a differential profile of decreased HNE modification and very high increase in nitration with age. The HNE levels in Core 1 decrease by ~24% in middle age and ~14% at old age compared to young age (No. 4—Fig. 5B). In contrast, Core 1 is heavily nitrated in an age-dependent manner and the nitration increases by more than 7.5-fold in middle age and ~10-fold at old age (Fig. 6). Both Core 1 and Core 2 proteins are anchored to the inner mitochondrial membrane with most of the protein exposed to the matrix side. Because CIII is one of the ROS-generating sites in mitochondria, the topographical arrangement of these CIII proteins and the proximity to electron transfer sites may explain the differential modifications for these proteins.

Oxidatively modified proteins of complex IV

Subunit 4 (COX4) is the only CIV protein that is both carbonylated (band 4—Fig. 4A) and HNE modified (band 5—Fig. 5A). COX4 showed a similar pattern of modification, i.e., an ~10% (carbonylation) and ~30% (HNE modification) increase in middle age and a decrease back to basal levels by old age (No. 4—Fig. 4B and No. 5—Fig. 5B, respectively). COX4 mainly plays a role in stability of this enzyme in the mitochondrial inner membrane and is not involved in the physiological activity of CIV [42].

Oxidatively modified proteins of complex V

Both the α and the β chains of CV are differentially oxidatively modified with aging. The α chain was carbonylated (ATP5A1, band 5—Fig. 4A), whereas the β chain was both HNE modified (band 6—Fig. 5A) and nitrated (band 2—
Fig. 6A). The α chain shows an age-related decrease in carbonylation level by ~10% in middle age and ~18% by old age (No. 5—Fig. 4B). Interestingly, carbonylated α chain was also found to comigrate with CI and showed a similar profile of modification (band 2—Fig. 4A and No. 2—Fig. 4B). Although, the α chain is HNE modified, the oxidatively damaged protein is not detected in the intact complex but is, instead, associated with CI (band 2—Fig. 5A). The β chain, on the other hand, shows an age-associated increase in HNE modification (band 6—Fig. 5A) that increases to ~56% in middle age and decreases to ~17% in old age, compared to young mice (No. 6—Fig. 5B). In addition, the β chain is heavily nitrated (band 2—Fig. 6A) and shows a more than 7.5-fold increase in modification in middle age and increases further to more than 11-fold in old mice. Both α and β chains are involved in ATP biosynthesis that is coupled with proton translocation and are not directly proximal to electron transfer sites of ETC. Thus, the differential, dramatic, and highly reproducible modification of these subunits with aging suggests that they are specific targets of ROS-mediated damage.

Discussion

Our studies identified oxidatively modified mouse heart mitochondrial ETC proteins whose levels of modification in some cases correlated with the decrease in complex activity as is predicted by the free radical theory of aging [9,16,17,27,33], whereas in others, modifications had no effect on functions, thus suggesting the interaction of other factors. The decrease in CI and CV enzyme function is consistent with our hypothesis that mitochondrial dysfunction in the aging heart may be due to the accumulation of oxidatively modified proteins. On the other hand, the continuous decrease in CI enzyme activity and increase in coupled activity of CI–CIII activity with age are not consistent with the decline in CI function. Neither are the increases in CII enzyme activity and CII–CIII coupled activities consistent with the concept of age-associated progressive increase in mitochondrial dysfunction. We propose that this increase in CII activity may be due to a tighter coupling between CII and CIII in response to the loss of CI enzyme activity. Although the mechanism of this unique response is not understood, we propose that it may enable the cell to balance the decline in CI activity by shunting electrons through CII and increasing the efficiency of electron transfer between CII and CIII. Thus, although this may be a less favorable pathway, the increased efficiency may lessen the adverse effect of loss of CI activity.

The fact that CII function increases with age was a surprising observation that led us to consider that the loss of enzyme...
Thus, any number below the threshold was considered significant.

Whether this affects the overall mitochondrial and tissue function is yet to be determined. Our studies show that there is an age-related decline in mouse heart CI and CV function.

Although oxidative damage to the ETC complexes in vitro leads to a decline in enzyme function [51–56], and this correlates with the amount of damaging adducts, the in vivo modification of several complex proteins did not severely affect their activity. Thus, the decrease in nitration of NDUFV1 and increased carbonylation of COX4, with age, did not severely affect either CI or CV function, suggesting that if the modification caused a structural change(s) this did not affect their function.

CIII is also a major site of ROS production in vitro [25,57]. Because Core 1 and Core 2 span the entire inner mitochondrial membrane and are in proximity to the predicted site of ROS generation at CIII [57], it is not surprising that they are both targets of oxidative modifications. However, the differential Core 2 carbonylation and Core 1 HNE modification and nitration raise the question of the physiological environment that supports adduct-specific multiple types of modification of specific subunits. Thus, dramatically increased Core 1 nitration and decreased Core 2 carbonylation with age suggest highly localized and specific environmental conditions for such modifications. Despite the heavy Core 1 nitration in both middle and old age, the CIII enzyme function did not change and in fact it increased slightly at old age, suggesting that the Core 1 nitration does not affect CIII activity. Interestingly, Core 1 and Core 2 proteins have a dual function in CIII, i.e., they play a key role in providing structural stability to the complex as well as being members of the mitochondrial processing peptidase (MPP) family that are involved in processing and proper folding of proteins imported into the matrix [58]. If increased Core 1 nitration is due to oxidative damage, a decrease in MPP activity could result in impairment of protein processing and increased age-associated mitochondrial dysfunction that is not related to ETC function.

Although F1F0-ATP synthase of CV is not part of the ETC processes, its location within the matrix, as well as its parameters, our studies show that there is an age-related decline in mouse heart CI and CV function.
abundance, makes it a prime candidate for oxidative modification. Thus, any change in CV activity is important because of its role in ATP homeostasis. Although the α chain is oxidatively damaged, the fact that the HNE-modified α chain was found to comigrate only with CI (and not in the intact CV) suggests that the modification causes its dissociation from CV. Thus, removal of the oxidatively damaged α chain and its replacement with unmodified protein may be a protective mechanism. This implies, however, that the modification and dissociation would trigger de novo α-chain synthesis, which remains to be tested. Alternatively, failure to replace the dissociated α chain may account for the decreased ATP production, which is a hallmark of increasing mitochondrial dysfunction with aging. Previously, it has been shown that MDA-modified β chain is associated with decreased heart CV function with age [33]. The fact that we did not find MDA-modified β chain in BN–PAGE-separated CV suggests that it may have dissociated from the intact complex. Thus, we propose that the modifications of the α and β chains may alter their binding affinity for CV and that dissociation of both modified proteins from CV may be a protective function.

The specific and dramatic 10-fold increase in β-chain nitration correlates directly with the decline in CV function with aging. Interestingly, the major decrease in enzyme function which occurs at middle age does not correlate with the continued modification of the β chain with age, suggesting that the specificity of the modifications rather than abundance may be a determinant of the consequences of modification to ETC and mitochondrial function.

In conclusion, many of the ETC complex subunits are specific targets of ROS-mediated oxidative modifications, some of which cause a decline in function. In this study we have reported two novel consequences of oxidative modification in aged heart mitochondria. First, the dissociation of modified CV subunits raises the question of whether structural changes due to oxidatively damaged α chain and its replacement with unmodified protein may be a protective mechanism. This implies, however, that the modification and dissociation would trigger de novo α-chain synthesis, which remains to be tested. Alternatively, failure to replace the dissociated α chain may account for the decreased ATP production, which is a hallmark of increasing mitochondrial dysfunction with aging. Previously, it has been shown that MDA-modified β chain is associated with decreased heart CV function with age [33]. The fact that we did not find MDA-modified β chain in BN–PAGE-separated CV suggests that it may have dissociated from the intact complex. Thus, we propose that the modifications of the α and β chains may alter their binding affinity for CV and that dissociation of both modified proteins from CV may be a protective function.

The specific and dramatic 10-fold increase in β-chain nitration correlates directly with the decline in CV function with aging. Interestingly, the major decrease in enzyme function which occurs at middle age does not correlate with the continued modification of the β chain with age, suggesting that the specificity of the modifications rather than abundance may be a determinant of the consequences of modification to ETC and mitochondrial function.

In conclusion, many of the ETC complex subunits are specific targets of ROS-mediated oxidative modifications, some of which cause a decline in function. In this study we have reported two novel consequences of oxidative modification in aged heart mitochondria. First, the dissociation of modified CV subunits raises the question of whether structural changes due to modification affect their assembly with CV. Second, modifications on mitochondrial function and their role in aging.

Acknowledgments

This publication was supported by USPHS Grant 1P01 AG021830-04 awarded by the National Institute on Aging, USPHS Grant 1 P30 AG024832-03, the Claude D. Pepper Older Americans Independence Center grant awarded by the National Institute on Aging, and the Sealy Center on Aging.

References


