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Decreased Cytochrome *c* Oxidase Subunit VIIa in Aged Rat Heart Mitochondria: Immunocytochemistry

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ABSTRACT

Aging decreases oxidative phosphorylation through cytochrome oxidase (COX) in cardiac interfibrillar mitochondria (IFM) in 24-month old (aged) rats compared to 6-month old adult Fischer 344 rats, whereas subsarcolemmal mitochondria (SSM) located beneath the plasma membrane remain unaffected. Immunoelectron microscopy (IEM) reveals in aged rats a 25% reduction in cardiac COX subunit VIIa in cardiac IFM, but not in SSM. In contrast, the content of subunit IV remains unchanged in both SSM and IFM, irrespective of age. These subunits are localized mainly on cristae membranes. In contrast, semiquantitative immunoblotting, which detects denatured protein, indicates that the content of COX VIIa is similar in IFM and SSM from both aged and adult hearts. IEM provides a sensitive method for precise localizing and quantifying specific mitochondrial proteins. The lack of immunoreaction of COX VIIa subunit by IEM in aged IFM is not explained by a reduction in protein, but rather by a masking phenomenon or by an *in situ* change in protein structure affecting COX activity. Anat Rec, 294:1825–1833, 2011.

Key words: immunoelectron microscopy; cytochrome *c*; mitochondria; oxidative phosphorylation; electron transport chain; aged heart

Abbreviations used: ADP = adenosine diphosphate; ANOVA = analysis of variance; BSA = bovine serum albumin; COX = cytochrome oxidase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IEM = immunoelectron microscopy; IFM = interfibrillar mitochondria; LR white = London resin white; PBS = phosphate buffered saline; PBT = PBS containing 1% w/v bovine serum albumin and 0.01% v/v Tween 20; PCR = polymerase chain reaction; RT-qPCR = real-time quantitative fluorescent polymerase chain reaction; SSM = subsarcolemmal mitochondria; TMPD = *N,N,N*^{1}*,N*^{1} *p*-phenylenediamine.

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INTRODUCTION

Aging leads to cardiac impairment via a loss of myocytes and alters physiologic function (Coleman et al., 1977, Wei, 1992), as does chronic oxidative damage during aging (Sohal et al., 2002). Cardiac mitochondria exist in two functionally distinct populations: subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) (Palmer et al., 1977). SSM are released by tissue homogenization leaving behind skinned myocytes; the liberation of IFM from the skinned myocytes requires a brief exposure to protease (Palmer et al., 1977). Aging-related alterations are limited to IFM, whereas SSM remain unaffected (Fannin et al., 1999). Oxidative phosphorylation was studied in SSM and IFM isolated from 6-month adult and from 24-month aged Fischer 344 rat hearts. Using glutamate as the substrate, the rate of stimulated oxidative phosphorylation is decreased in IFM from elderly groups compared to the 6-month adult controls (Fannin et al., 1999). The coupling of respiration is unaltered by age, as shown by similar rates of ADP-limited state 4 respiration and ADP:O ratios (Fannin et al., 1999).

To further localize the site of aging defects within the electron transport chain, the rate of oxidative phosphorylation was measured using substrates that donate reducing equivalents to specific electron transport complexes (Fannin et al., 1999). IFM exhibited a decrease in the oxidation rate of TMPD-ascorbate, an electron donor to cytochrome oxidase (COX) via the reduction of cytochrome *c*. This observation localizes an aging defect to COX in the electron transport chain of IFM (Fannin et al., 1999). The decrease in respiration through COX in IFM in the aged heart is reflected in a decrease in polarographically-measured COX enzyme activity in freshly-isolated mitochondria. The content of cytochrome aa_3, as measured by the difference of oxidized and reduced spectra, is unaltered (Fannin et al., 1999).

COX is a 13 subunit complex involved in the terminal redox complex in the electron transport chain (Lenka et al., 1998), consisting of three mitochondrially encoded catalytic subunits (*COX I-III*) and 10 nuclear encoded regulatory and structural subunits (*COX IV-VIII*). *COX VIIA (COX7A)* is one of the several nuclear encoded subunits that exist as different isoforms. The designation COX includes the COX7AL isoform in liver and the COX7AH isoform in heart and skeletal muscle (Seelan, et al., 1996). *COX 7 AR* gene is expressed in all tissue types, and Schmidt et al. (2003) showed that in HeLa cells the third isoform is localized to the Golgi apparatus (COX7AR). Recent study indicates that the expression of the mtDNA coded genes is not significantly altered in aged Fisher −344 rat ventricles (Preston et al., 2008). Thus, aging *per se* did not change the content of mitochondrial-encoded catalytic subunits.

An appreciation of the selective effect of aging on IFM is critical to the study of aging-related alterations in mitochondrial physiology. A decrease in the expression of nuclear-encoded subunits in the aging heart has been reported and confirmed by PCR (Preston et al., 2008). IEM of myocardial tissue and of isolated SSM and IFM was used to evaluate the ratio of immunogold labeling of subunit VIIa and IV, and showed a decrease in COX VIIa content in the aged heart. The decreased expression as determined by us matches that found by PCR (Preston et al., 2008).

Our study presents a novel approach to investigate the *in situ* changes in protein expression originally suggested by studies of transcriptional responses. The aging-induced decreases in COX VIIa (25% reduction in IFM) were observed using IEM. Changes in COX VIIa occur only in IFM from hearts of aged rat, but COX IV remains unaltered with aging. At the same time, these subunits in SSM are unchanged, irrespective of age. This study confirms the reduction of oxidative phosphorylation in IFM in aged rat, and provides a mechanism whereby this reduction takes place.

MATERIALS AND METHODS

Chemicals

All reagents and chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Anti-complex IV subunit IV [COX IV (MS407)] and –complex IV subunit VIIa [COX VIIaHL (MS415) monoclonal antibodies, which recognizes both the heart and liver isoforms, was purchased from Mitosciences (Eugene OR). Gold-conjugated secondary antibodies were purchased from Amersham Life Sciences (Arlington, IL).

Animal Model of Aging and Isolation of Rat Heart Mitochondria

Animal studies were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University School of Medicine and by the Louis Stokes Cleveland Department of Veterans Affairs Medical Center. Male Fischer 344 (F344 202 barrier and 217 barrier) rats at 6 months (adult) and 24 months (aged) were obtained from the National Institute of Aging colony (Harlan Sprague Dawley, Indianapolis, IN). SSM and IFM populations of cardiac mitochondria were isolated as previously described by Palmer et al. (1977) and by Fannin et al. (1999), except that trypsin was used as the protease (Moghaddas et al., 2002) to release IFM. Oxygen consumption was measured in freshly isolated mitochondria using a Clark-type oxygen electrode at 30°C as described by Palmer et al. (1997). Mitochondrial protein concentration was determined by the Lowry method, using bovine serum albumin (BSA) as a standard (Lowry, 1951).

IEM

In general, membranes, including those of mitochondria, are not optimally preserved for IEM by means of conventional fixatives. In the absence of osmium postfixation, the double membrane structure of mitochondria is largely effaced (Watkins et al., 1987, Vincent et al., 1994, Lobo et al., 2000, Bowes, et al., 2007, Brezová et al., 2007). To circumvent this problem, we designed a method that improved the retention of antigenicity, combined with improved structure. We substituted 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, which appears to be innocuous in terms of effects on mitochondrial function, but which yields mitochondria of conventional structure. We added magnesium ion to the fixative (Palay et al., 1962) to help stabilize the membrane protein. We pretreated specimens with a mixture of paraformaldehyde and a monofunctional imidate,

ethyl acetimidate, which preserved antigen integrity before glutaraldehyde fixation (Tokuyasu, et al., 1983).

Left apical ventricular tissue and cardiac SSM and IFM isolated from the remainder of the selfsame heart were used for immunocytochemical analysis. Cardiac tissues and isolated mitochondria from adult and aged hearts were lightly fixed for 5 min at 4°C with 3% formaldehyde and 20-mM ethylacetimidate in HEPES-buffered saline (30-mM HEPES, 151-mM NaCl, 4.7-mM KCl, 1.2-mM $MgCl_2$, 7.8-mM glucose, pH 7.3) at 4°C. These samples were washed, further fixed for 45 min in 3% w/v formaldehyde containing 0.25% w/v glutaraldehyde in HEPES buffered saline at 4°C, then dehydrated in ethanol and embedded in LR White resin (Polysciences, Warrington, PA). Thin sections were blocked with phosphate buffered saline (PBS) containing 1% w/v bovine serum albumin and 0.01% v/v Tween 20 (PBT). Grids were then incubated with either anti-COX IV or COX VIIaHL monoclonal antibody (Mitosciences, OR) at 1:25 and 1:200 dilution, respectively, in PBT for 12 h at 4°C. Negative controls included normal mouse serum and PBT replaced as the primary antibody. After washing, grids were incubated for 1 h in 5-nm gold-conjugated goat anti-mouse IgG (Amersham Life Sciences, Arlington, IL) diluted 1:30 in PBT, rinsed with PBS, and fixed with glutaraldehyde to stabilize the gold particles. Samples were stained with uranyl acetate and lead citrate, then examined in either a Zeiss CEM902 electron microscope (Oberkochen, Germany) or a JEOL 1200EX electron microscope (Tokyo, Japan). Primary antibody dilution titer was determined by the lowest feasible antibody concentration and minimum background signals.

Quantitative IEM

(1) Concentration of antibodies: The amount of antibodies was determined by a dilution experiment. Antibody titer used in these experiments was optimized for obtaining the highest gold labeling and lowest nonspecific labeling.

(2) COX IV and COX VIIa epitopes were evenly distributed in the mitochondria: We examined several different portions of pellets of isolated mitochondria at different levels and counted the gold particles, which indicate endogenously expressed COX IV and COX VIIa. The density of the gold particles/μm^2 in different mitochondria was evaluated in different areas of the section.

(3) Single and double-side labeling of the sections: The relative density of COX IV and COX VIIa was calculated after labeling either a single side or both sides of the sections. As expected, the average number of gold particles with double-side labeling is about two-fold that of single side labeling.

(4) Gold labeling of serial sections: Serial thin sections were used for detecting the presence of COX IV or COX VIIa protein. Alternate grids were stained for either COX IV or COX VIIa protein. This was done not only to verify the homogeneity of antigens inside a given mitochondrion, but also to determine the sensitivity of two different (COX IV and COX VIIa) antigens using immuno-EM.

(5) Gold labeling occurs only on exposed epitopes at the surface of the 70-nm thick sections. It is possible to calculate the densities of an exposed antigen in the mitochondria; this allows a reliable quantification of the number of gold particles in both isolated and in situ mitochondria (Bergersen et al., 2008), especially in isolated organelles, which are spherical (round in section).

(6) Gold particle size: Maximal detecting sensitivity is in inverse proportion to the gold particle size. The detecting sensitivity of 5-nm gold particles is approximately four times higher than that of 10-nm particles (Gu and D'Andrea, 1989). Therefore, we used 5-nm gold particles, which are relatively easy to visualize.

(7) Number of gold particles per unit area: Quantification of the number of gold particles in isolated mitochondria allowed calculation of the densities of exposed antigens on individual organelles. The number of gold particles per unit area in isolated mitochondria was calculated as follows: we measured the long (a) and short axis (b) of each isolated mitochondrion and determined in square μm the area of each mitochondrion.

(8) Nonspecific alkaline phosphatase treatment of sections: Thin sections, all from the same block (24-month IFM), were incubated overnight at 37°C on a drop containing 1.2 U alkaline phosphatase (SIGMA P6774) in 0.1 M Tris, pH 8.0, rinsed, and processed for IEM. Two controls were performed; (1) buffer without alkaline phosphatase and (2) no treatment at all. Gold particles, representing COX VIIaHL, were counted in 20 isolated IFMs from each treatment. The number of gold particles per unit area/single side in isolated mitochondria was calculated using the same method as described above.

(9) Statistical analysis: Gold particles, which indicate the localization of COX IV and VIIa, were counted in 50 SSMs and 50 IFMs in each experiment. Myocardial tissue samples from three adult and three aged rat hearts were evaluated by IEM, and the remaining tissues used to prepare isolated SSM and IFM from the individual heart also to be examined by IEM. The ratio of immunogold labeling of subunit VIIa vs. subunit IV is expressed as group means ± SD. A decrease in the expression of subunit VIIa in IFM in aged rat heart was determined using parametric one-way ANOVA test and Student-Newman-Keuls as *post-hoc* test; differences at $P < 0.05$ were considered significant.

Immunoblotting Studies Using COX IV and COX VIIa Antibodies

SSM and IFM (Palmer et al., 1977, Fannin et al., 1999, Moghaddas et al., 2002) and rat liver mitochondria (Hoppel et al., 1979) were subjected to SDS-PAGE using a 12% v/v separating gel and run with 50 μg of mitochondrial protein per lane using both cardiac mitochondria and also liver mitochondria populations. The separated proteins (polypeptides) were transferred to a 0.2-μm polyvinylidene difluoride membrane. Membranes were immunodetected with either anti-COX IV or -COX VIIaHL antibody (Mitosciences, OR) at 1:250 and 1:500 dilution, respectively, and were visualized by colorimetric detection using alkaline phosphatase-conjugated

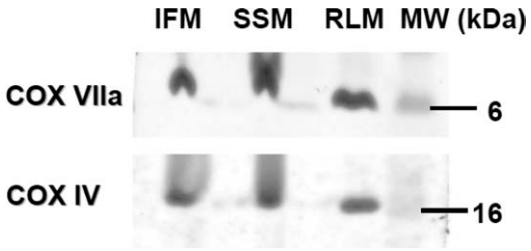

Fig. 1. Immunoblot analysis of COX VIIaHL monoclonal antibody. The immunoblot experiment was performed as described in Materials and Methods, except each well was loaded with 43 μg mitochondrial protein and 10% Tricine gel. As a quality control, the membrane also was incubated with COX IV. The two rows were from the same immunoblot, but are based on different exposure times.

second antibodies (BioRad, Hercules, CA). Immunodetected COX IV was used as a loading control. COX VIIa immunodetected protein bands were quantified by densitometry relative to COX IV (unchanged by aging) using NIH ImageJ 1.32 software (http://rsb.info.nih.gov/ij/).

RESULTS

Functional Characterization of SSM and IFM From Adult and Aged Hearts

The maximal rate of ADP-stimulated respiration was significantly decreased by aging in IFM using glutamate as substrate (6 months: 438 ± 35 nAO/min/mg, n = 3; 24 months: 245 ± 85 nAO/min/mg, n = 3; $P < 0.05$). Respiration in SSM was unaltered by aging (6 months: 189 ± 48 nAO/min/mg, n = 3; 24 months: 141 ± 33 nAO/min/mg, n = 3; p = NS). The protein yield of IFM was decreased in the aged heart. Respiration was well coupled in both populations of mitochondria and was unaltered by age. TMPD-ascorbate stimulated respiration also was decreased in IFM from the aged heart (6 months: 1087 ± 179 nAO/min/mg, n = 3; 24 months: 711 ± 45 nAO/min/mg, n = 3; $P < 0.05$). These results are similar to those from our laboratory (Fannin et al., 1999; Lesnefsky et al., 2001a, Lesnefsky et al., 2001b, Hoppel et al., 2002).

IEM

Before performing actual IEM, it was necessary to characterize the antibody in a precise fashion. Individual antibodies against the two isoforms of COX VIIa, liver and heart isoforms, are not commercially available. The currently available monoclonal antibody against COX VIIa used in this study is marketed as detecting both liver and heart isoforms. Liver contains only the liver isoform whereas heart contains both isoforms. However, the company's Web site and direct communication with them did not provide evidence that the COX VIIaHL antibody recognizes the liver isoform. So we performed a Western blot on rat liver and heart SSM and IFM using the commercial antibody (Fig. 1). The antibody recognized COX VIIaHL in liver mitochondria at a similar intensity as in the heart mitochondria. As a quality control, the antibody directed at subunit COX IV was used with similar results. These analyses strongly support the validity of our antibody for IEM.

To determine the density of endogenously expressed mitochondrial COX IV and COX VIIa, we used immunogold labeling of sections of chemically-fixed specimens embedded in water-soluble LR White resin. The various steps of the procedure (Experimental Procedures) were optimized for obtaining low-background noise. For quantitative analysis, we counted gold particles on mitochondria, a total of 50 per experiment; these organelles were scattered through a number of different sections. IEM revealed specific labeling of COX IV and COX VIIa, predominantly on the cristae membrane of the isolated SSM and IFM from adult and aged hearts (Fig. 2). In three independent experiments using anti-COX IV and COX VIIa antibodies on isolated IFM and SSM from three adult and three aged hearts, COX IV content (number of gold particles per unit area) remained unchanged in both SSM and IFM (Fig. 3) (6-month SSM = 48.2 ± 6.7 gold particles/μm^2, 24-month SSM = 51.0 ± 12.2 gold particles/μm^2, 6-month IFM = 50.9 ± 8.9 gold particles/μm^2, 24-month IFM = 50.2 ± 6.2 gold particles/μm^2).

A statistical decrease in the expression of subunit COX VIIa in IFM in the aged rat heart ($P < 0.05$) was determined as having occurred using parametric one-way ANOVA test (6-month SSM = 61.0 ± 2.2 gold particles/μm^2, 24-month SSM = 60.7 ± 1.9 gold particles/μm^2, 6-month IFM = 59.6 ± 3.9 gold particles/μm^2, 24-month IFM = 38.0 ± 10.4 gold particles/μm^2). The density of the COX VIIa protein content decreased in isolated IFM from aged hearts (Fig. 3). Because isolated mitochondria are globular, serial thin sections of a given mitochondrion revealed a series of progressively enlarging organelle profiles, followed by a steady decline in profile diameter. Counts of gold particles in such series remained steady in terms of particles/μm^2, showing that the plane of section through any mitochondrion does not in any way influence the count, and that these subunits in question are uniformly distributed within each mitochondrion (Fig. 4), guaranteeing that our counts are unbiased by label sequestration. These observations of label uniformity apply equally to the 6- and 24-month rat mitochondria.

In like manner, the data from isolated mitochondria were confirmed in *in situ* SSM and IFM from both adult and aged rat heart (Figs. 5 and 6). IEM of cardiomyocytes revealed specific labeling of COX IV and COX VIIa exclusively in mitochondria (Fig. 5), which are arranged in longitudinal rows, paralleling myofibrils. The labeled mitochondria showed a preferential localization of gold particles on inner membranes (Fig. 5). For quantification of the number of gold particles in *in situ* mitochondria, the organelles selected for examination were of approximately equal size. A decrease in the expression of subunit COX VIIa in IFM in the aged rat heart ($P < 0.05$) was determined using parametric one-way ANOVA test (COX IV: 6-month SSM = 44.2 ± 3.0 gold particles/mitochondrion, 24-month SSM = 44.8 ± 4.1 gold particles/mitochondrion, 6-month IFM = 38.0 ± 7.4 gold particles/mitochondrion, 24-month IFM = 36.4 ± 6.3 gold particles/mitochondrion, COX VIIa: 6-month SSM = 57.6 ± 4.0 gold particles/mitochondrion, 24-month SSM = 55.1 ± 4.0 gold particles/mitochondrion, 6-month IFM = 48.8 ± 3.7 gold particles/mitochondrion, 24-month IFM = 35.2 ± 2.3 gold particles/mitochondrion) (Fig. 6). Thus, IEM uncovered a decrease in COX VIIa protein content in IFM only in aged rat.

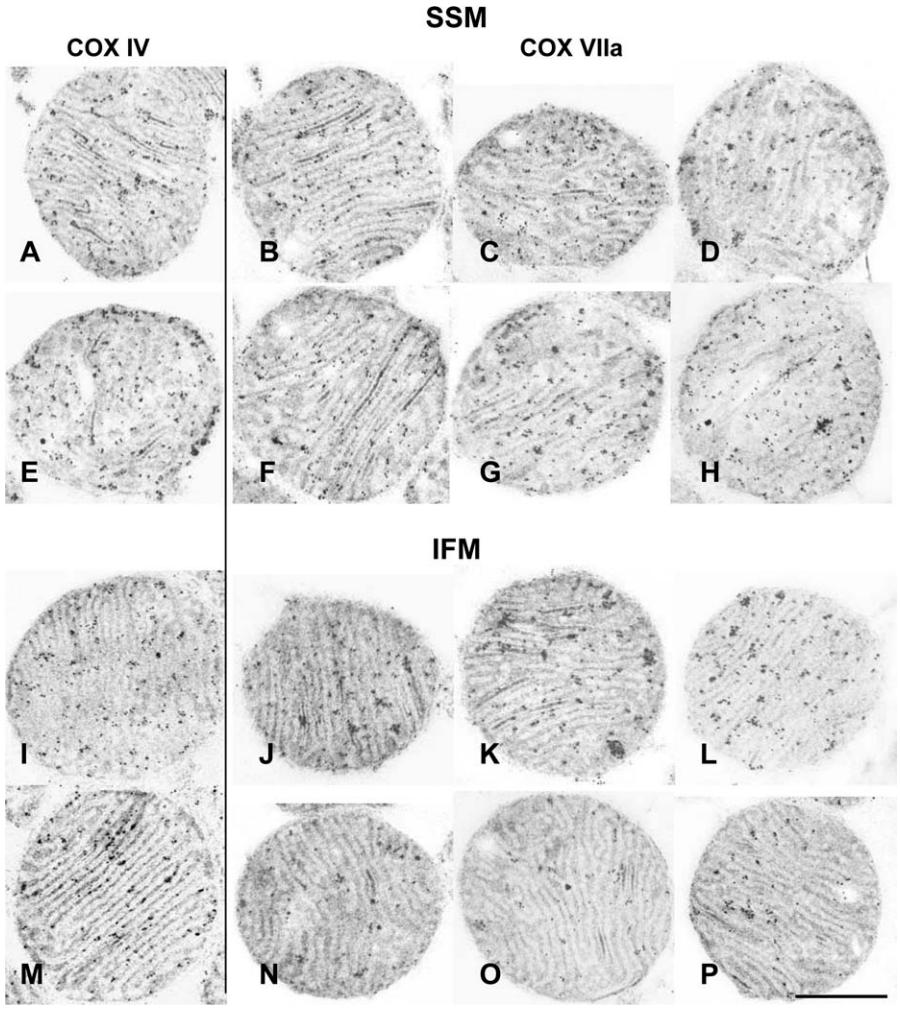

Fig. 2. Immunoelectron microscopic analysis of COX IV (**A**, **E**, **I**, **M**) and COX VIIa (**BD**, **FH**, **JL**, and **NP**) in isolated cardiac SSM and IFM from 6 months. (AD, and IL) and 24 months. (EH, and MP) Fisher 344 rats. Gold particles (5 nm) indicate the COX IV or COX VIIa contents of the mitochondria. Vertical line separates these mitochondria labeled for COX IV and COX VIIa. Scale bar = 1μm; all micrographs on this plate are at the same magnification.

The COX VIIaHL content (number of gold particles per unit area) remained unchanged in these three groups (Fig. 7, [alkaline phosphatase treated 24-month IFM = 34.8 ± 2.4 gold particles/μm^2; buffer without alkaline phosphatase treated 24-month IFM = 34.7 ± 2.9 gold particles/μm^2; no treatment of 24-month IFM = 34.3 ± 2.7 gold particles/μm^2]).

Assessment of COX VIIa Content by Immunoblotting

The content of COX VIIa also was assessed by semi-quantitative immunoblotting using antibody to COX VIIa with immunoblotting of COX IV as a loading control. We found that the COX IV expression was not altered by aging. The densitometry response to COX VIIa was nearly linear from 10- to 50-μg mitochondrial protein (with $R^2 = 0.98$). Since the goal was to observe a potential decrease of 20–30% in COX VIIa content, 50 μg of protein was chosen. Six and eight paired analyses were performed between adult and aged hearts, respectively, for each population of mitochondria. The relative density was normalized for COX VIIa band in the 6-month heart and was set as 1.0. As shown in Figure 8, the content of COX VIIa was not different with age in either isolated SSM or IFM.

DISCUSSION

Immunogold electron microscopy is a reliable technique to quantify and to compare the molecular contents of intracellular compartments of the same and different cell types (Bergersen et al., 2008). Based on IEM, we found a 25% reduction in cardiac COX subunit VIIa in IFM from aged rat. We established a new procedure for quantitative immuno-EM that can detect the epitopes of COX VII and IV in complex IV in both adult and aged mitochondrial subpopulations (SSM and IFM). Commercially available anti-COX VIIaHL and COX IV antibodies recognize antigenic determinants in both SSM and IFM from adult and aged rat. Using the IEM methodology, the morphology of *in situ* and isolated SSM and IFM

was seen to be intact and unchanged from those prepared by conventional techniques. For example, there was no obvious change in number or configuration of cristae and no obvious swelling of either the isolated SSM or IFM. The number of particles/unit mitochondrial area of COX IV and COX VIIaHL was determined after labeling either a single side or both sides of the sections. As expected, the average number of gold particles with double side-labeling is about twice that of single side-labeling, showing that the antigen is present along the full breadth of the cristae membranes within a section thickness. We examined several different portions of pellets of isolated mitochondria at different planes of section including serial thin sections. Inspection of the distribution of the gold particles verified that within any given organelle there is no clustering or sequestration of particles, showing the homogeneity of the antigens.

The 25% reduction in the content of COX VIIa in IFM mirrors the aging-related 25% reduction of oxidative phosphorylation through COX of the intact IFM found by biochemical means (Fannin et al., 1999). The permeabilized (hypotonic phosphate-expressed) or solubilized (DOC-expressed) freshly isolated IFM from aged rat in our study also showed a 25% reduction of COX activity compared to IFM from adult rats. Because Fannin et al. (1999) demonstrated that despite the aforementioned decrease in COX activity, this activity can be restored by addition of phospholipid liposomes or by freezing-thawing of mitochondria, indicating that the active protein remained in cytochrome *c* oxidase. To further explore

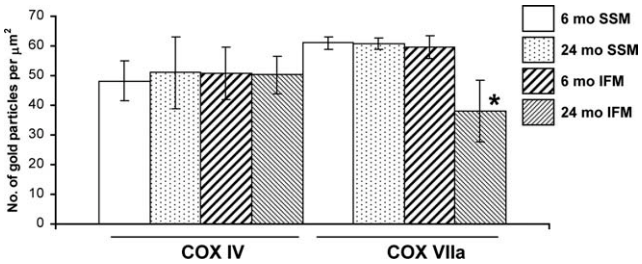

Fig. 3. Histogram showing the average number of gold particles per μm^2 mitochondrion (*Y*-axis; mean ± SD) *in vitro*. Electron micrographs of 50 mitochondria were obtained from each experiment and the total number of gold particles counted for each mitochondrion. Three independent experiments were performed using anti-COX IV and COX VIIaHL antibodies on isolated SSM and IFM from three adults and three aged rats (total = 150 organelles). Each bar indicates the mean number of gold particles from 150 mitochondria±SD * A decrease in the expression of subunit COX VIIa in IFM in the 24-month rat compared with the expression of subunit COX VIIa in the 6-month rat heart was determined using parametric one-way ANOVA test ($P < 0.05$).

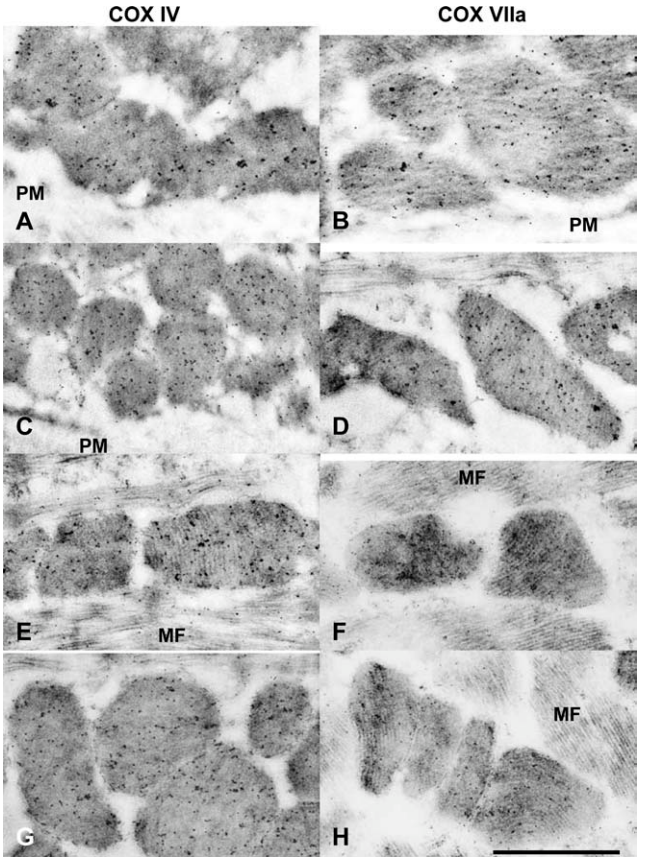

Fig. 5. Representative immunoelectron microscopic analysis of COX IV (**A**, **C**, **E**, and **G**) and COX VIIa [**B**, **D**, **F**, and **H**] in *in situ* mitochondria from 6 months. SSM (A and B), 6 months. IFM (E and F), 24 months. SSM (C and D), and 24 months. IFM (G and H). Gold particles (5nm) indicate the COX IV or COX VIIa contents. Myofibrils (MF). Plasma membrane (PM). Because of aldehyde fixation without osmium postfixation, limiting membranes of these mitochondria are variously discernible. Scale bar = 1μm; all micrographs are at the same magnification.

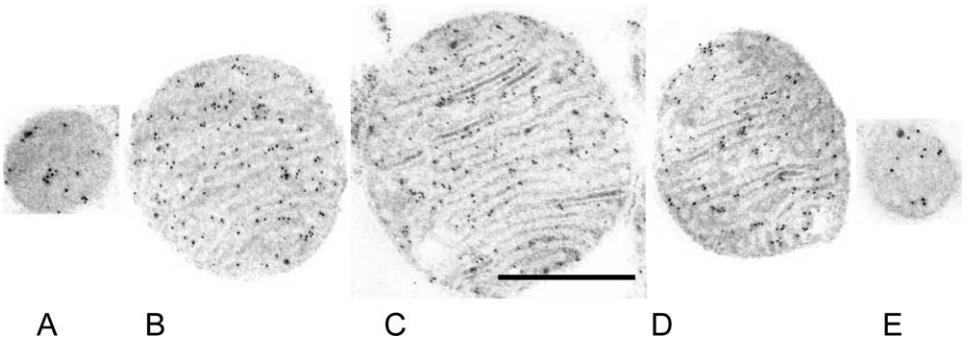

Fig. 4. IEM of COX VIIa in serial but not consecutive sections of an isolated cardiac IFM (**A**–**E**) from 6 months. Fischer 344 rat. Gold particles (5nm) indicate COX VIIa in the mitochondrion. Scale bar = 1 μm; all micrographs are at the same magnification.

this peculiarity in behavior of COX, we subjected subunit IV and VIIa H/L in mitochondria to semiquantitative immunoblots. Using this methodology, we found that the content of both subunit IV and VIIa is consistent in the SSM (6 and 24 months) and IFM (6 and 24 months). We also found that the migration in gels of COX VIIa from IFM in aged heart is the same as that from IFM in adult heart. In other words, the protein content *per se* has remained constant with no change in molecular weight.

A likely explanation of the differences obtained with IEM and western blot might be based on the dependency of function on structure. In the case of the western blot, what is being measured is the total content of COX VIIa, whereas with IEM it is only the VIIa H/L in functional cytochrome *c* oxidase that is being measured. As long as one is dealing with *in situ* mitochondria the functional cytochrome *c* oxidase can be measured. In contrast, once isolated, mitochondria are divested of their membrane by freeze-thawing or detergent treatment, all of the cytochrome *c* oxidase – including that which was originally nonfunctional—now can be measured. In other words, the total cytochrome *c* oxidase content of the mitochondria has become available for testing. Because the IEM was carried out on intact mitochondria, whether isolated or in situ, only the functional cytochrome *c* oxidase was measured. The number of gold particles on the mitochondria is totally independent of

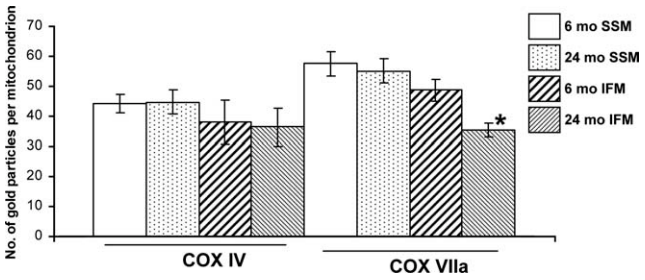

Fig. 6. Histogram showing the average number of gold particles per mitochondrion (*Y*-axis; mean ± SD) *in vivo*. Electron micrographs of 50 mitochondria were obtained from each experiment and the total number of gold particles counted for each mitochondrion. Three independent experiments using anti-COX IV and COX VIIaHL antibodies on isolated SSM and IFM from three adults and three aged rats were performed (total =150 organelles). Each bar indicates the mean number of gold particles from 150 mitochondria±SD. * A decrease in the expression of subunit COX VIIa in IFM in the 24-month rat compared with the expression of subunit COX VIIa in the 6-month rat heart was determined using parametric one-way ANOVA test ($P < 0.05$).

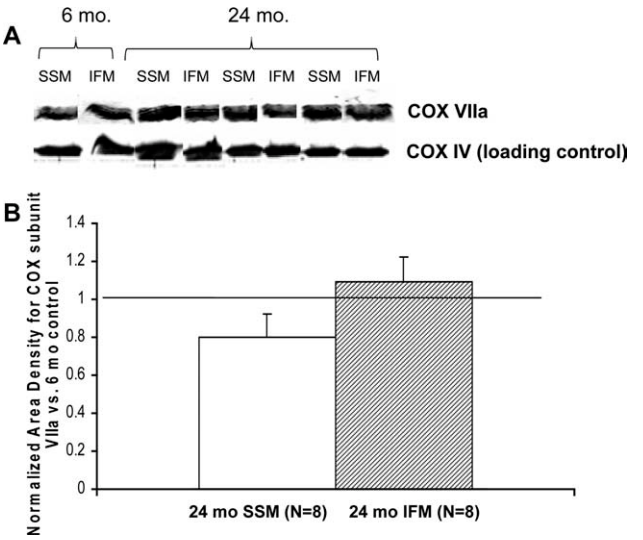

Fig. 8. Quantification of densitometry measurements of COX VIIa bands of western blots. **A**: Representative Western blots of COX IV and COX VIIa bands from SSM and IFM of three 24-month F344 rats are compared to those from SSM and IFM of one typical 6-month F344 rat. **B**: Protein bands of COX VIIa and IV were quantified by densitometry using NIH Image J 1.32 software (http://rsb.info.nih.gov/ij/). In the histogram, the plotted value is the ratio of the density of COX VIIa in the 24-month mitochondria to the density of COX VIIa in the 6 months; comparison is based on SSM and IFM in the two different age groups (6 months, N = 8; 24 months, N = 8). The horizontal line at 1 refers to the 6-month mitochondria, and aids in comparing the 24-month mitochondria with their younger counterparts. This figure contains the data from the rats used in the IEM study plus those used in the experiments originally performed for immunoblotting only.

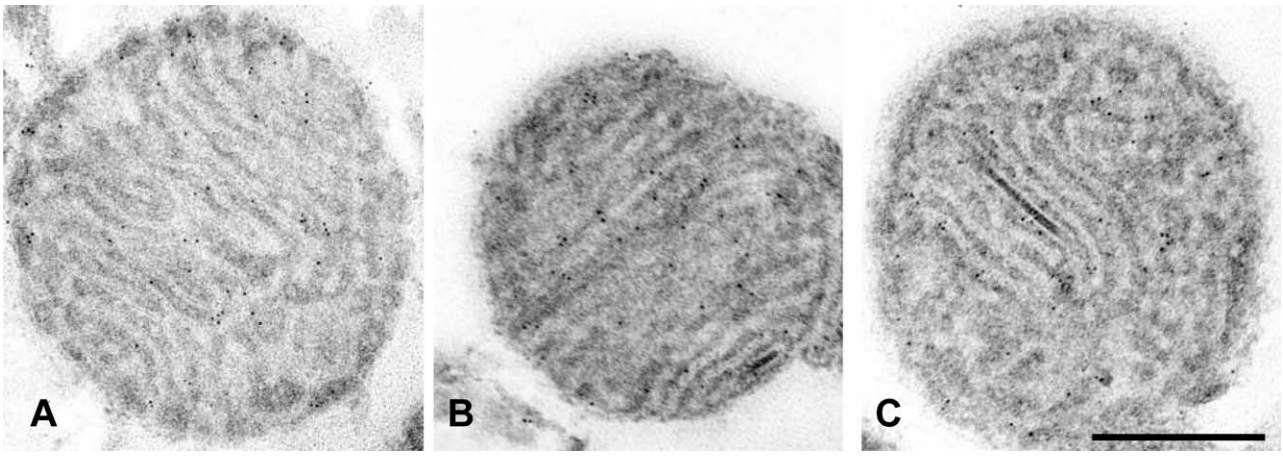

Fig. 7. Immunoelectron microscopic analysis of COX VIIa in isolated cardiac IFM from 24 months. Sections were dephosphorylated with non-specific alkaline phosphatase. **A**: Alkaline phosphatase treatment; **B**: Buffer without alkaline phosphatase; **C**: No treatment. The results are identical in all three cases. ***Bar = 0.5 μm.

the state of phosphorylation as revealed by treatment with non-specific alkaline phosphatase.

Although not directly concerned with the structure-function relationships, some previous studies provide evidence that may aid in understanding this issue in cardiac mitochondria in aging. The net release of H_2O_2 is increased in IFM isolated from aged Fischer 344 rat hearts compared with adult-aged control, but there is no increased ROS production in isolated SSM (Suh et al., 2003). Increased ROS production within IFM indicates increased oxidative stress in hearts during aging (Judge et al., 2005). The effect of H_2O_2 on cytochrome *c* oxidase of bovine heart is a 50–60% decrease in content of COX VIa and VIIa (Musatov et al., 2004). The partial dissociation of the COX VIIa results in rearrangement of its tertiary structure with formation of non-native forms of the polypeptide. Therefore, IEM analysis might reflect a decrease in the reaction intensity of COX VIIa. The 10 nuclear encoded subunits of complex IV most likely play a structural and/or regulatory role (Kadenbach and Merle, 1981). For example, modification of subunit VIIc and dissociation of subunit VIIa are coupled events that are responsible for the inactivation of cytochrome c oxidase (Musatov et al., 2004). Such structure-functional alterations probably are the basis for the loss of cytochrome *c* oxidase activity in intact mitochondria. In like fashion, these changes might be responsible for the diminution in COX VIIaHL recognition as measured by IEM in our study.

As a supplement to our IEM studies, we applied gene chip array analysis with follow-up RT-qPCR. These latter studies revealed a decrease in COX VIIa liver isoform transcripts in hearts of aged rats as reported by Preston et al. (2008), but Western blot analysis showed no change concomitant with aging in this factor in either SSM or IFM. In contrast, IEM showed that although COX VIIaHL remained unchanged in SSM, this factor decreased in the IFM from aged rat. The discrepancy between our RT-qPCR, array and IEM versus Western blot analysis might have several possible explanations. We used the antibodies in the same fashion as predecessors (Koves et al., 2005, Wall et al., 2006, Murray et al., 2007). Conceivably, the existence of two COX VIIa isoforms could lead to misinterpretation; it has been reported that these isoforms are present in adult rodent heart in the ratio of 2:1 for heart and liver isoforms. This ratio was measured on mRNA transcripts (Jaradat et al., 1998, Schmidt et al., 1999), and the protein level of isoforms in fetal and adult rat heart was reported (Kuhn-Nentwing and Kadenbach, 1985). The ratio of transcripts does not necessarily match that of proteins (Preiss and Lightowlers, 1993). Isoforms of subunits VIa, VIIa, and VIII, designated heart (H) and liver (L), can be transcribed in mammalian hearts. The subunit VIII is transcribed universally, but the liver isoform is undetectable in bovine heart and skeletal muscle (Preiss and Lightowlers, 1993). In contrast, protein sequence analysis of subunit VIa in mouse hearts reveals about 80% heart isoform and 20% liver isoform (Radford et al., 2002). However, little is known about the translational regulation of subunit VIIa isoforms. We are left without precise knowledge of the ratio of the H/L isoforms in the F344 adult and aged rat hearts. Our antibody, anti-COX VIIaHL, reacts with both isoforms, so what we measured by IEM and Western blot includes both. What is clear from our study is that COX VIIa, regardless of the ratio of isoforms, is reduced in IFM from 24-month old rats, whereas it remains unaltered in SSM.

Differentially expressed genes identified using microarray analysis (Affymetrix GeneChip® Rat Genome 230 2.0 array) of cDNA prepared from cardiomyocytes from left ventricles of adult and aged hearts has been reported (Preston, et al., 2008). Expression of nuclear-encoded *COXVIIa L* was down-regulated 1.3-fold in the aged heart. Our preliminary data using array analysis (data not shown) agreed with the data presented by Preston et al. (2008), so we discontinued this part of the study.

In conclusion, we have used IEM in a rarely employed fashion to determine quantitative changes in specific mitochondrial populations as a result of aging. This methodology has a clear advantage over Western blot analysis in that it determines active COX VIIa. We have applied this technique to both *in situ* and isolated mitochondria, and our results are in perfect agreement for both types of preparations, showing that the isolation procedure is not responsible for the reduction in activity of IFM. IEM may become an important tool in the diagnosis of mitochondrial changes in various types of pathology.

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