Preservation of Cardiolipin Content During Aging in Rat Heart Interfibrillar Mitochondria

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Aging selectively decreases the rate of oxidative phosphorylation in the interfibrillar population of cardiac mitochondria (IFM) located between the myofibers. In contrast, subsarcolemmal mitochondria (SSM), located below the plasma membrane, remain unaffected. IFM from elderly (24-month-old) Fischer 344 rats have a decreased specific activity of complexes III and IV. Complexes III and IV require an inner mitochondrial membrane lipid environment enriched in the oxidatively sensitive phospholipid cardiolipin for maximal activity. We asked if aging decreases the content or alters the composition of cardiolipin as a potential mechanism of the aging defect in IFM. The content and composition of mitochondrial phospholipids were measured in SSM and IFM from adult and aging rat hearts. Aging did not alter the content of mitochondrial phospholipids, including cardiolipin, in either population of mitochondria. The composition of cardiolipin based on characterization of both acyl group and the individual molecular species of cardiolipin was also unaltered by aging. Lipid-mediated oxidative modification of complex III subunits was not detected, making cardiolipin-derived oxidative damage to complex III unlikely. Thus, alterations in cardiolipin are not the mechanism for the aging defect in IFM in Fischer 344 rats.
METHODS

Chemicals
Reagent chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Animal Model of Aging and Isolation of Rat Heart Mitochondria
Male Fischer 344 (F344) rats maintained on NIH-31 Mouse/Rat Sterilizable Diet (7017) were obtained at ages of 6 months (adult) and 24 months (elderly) from the National Institute of Aging colony (Harlan Sprague Dawley, Inc., Indianapolis, IN). The Institutional Animal Care and Use Committee approved the protocol. Rats received heparin (500 U/kg IP) and were anesthetized with pentobarbital (100 mg/kg IP), and their hearts were rapidly excised. SSM and IFM populations of cardiac mitochondria were isolated by a modification of the method of Palmer and colleagues (9). SSM were isolated as described by Palmer and colleagues (9), except that homogenization of the myocardial mince used a polytron tissue processor (Brinkman Instruments, Westbury, NY) theostat setting of 7.0 for 5 seconds. IFM were isolated by incubation of skinned myofibers, obtained following polytron treatment, with 5 mg/g (wt weight) trypsin for 10 minutes at 4°C. Oxygen consumption was measured in freshly isolated mitochondria by using a Clark-type oxygen electrode at 30°C (8). Uncoupled respiration was measured in the presence of 0.3 mM dinitrophe- nol. Mitochondrial protein concentration was determined by the Lowry method, using bovine serum albumin as a standard (22).

Lipid Characterization in Adult and Elderly Isolated Rat Heart Mitochondria
The separation, quantification, and characterization of mitochondrial phospholipids were performed as previously described (23). Phospholipids were extracted from SSM and IFM (1.5 mg of protein) by the method of Folch (24), with 50 μM butylated hydroxytoluene added as an antioxidant. The phospholipid fraction was isolated from other lipid classes by using silica gel chromatography (25). The phospholipid fraction was separated into individual phospholipids by normal-phase high-performance liquid chromatography, using a HP1100 system (Agilent Technologies, Wilmington, DE), Hypersil silica 5 μm (120 Å) 250 mm × 4.6 mm column (Alltech Associates Inc., Deerfield, IL), with a mobile phase of hexane:2-propanol:25 mM potassium acetate (pH 7.0):ethanol:glacial acetic acid (367:490:62:100:0.6, by volume) and a gradient to hexane:2-propanol:25 mM potassium acetate (pH 7.0):acetonytrile:glacial acetic acid (442:490:62:25:0.6, by volume) at 30°C (23).

Chromatographic peaks were identified and collected by using ultraviolet (UV) detection at 206 nm. Individual phospholipid peaks were identified by comparison to the retention time of standards. Phospholipids were quantified by organic phosphate measurements (23) and also by absorbance at 206 nm. Organic phosphate measurements were performed by the method of Bartlett (26), except that measurements were performed at 815 nm to increase sensitivity (23). Measurements were performed on serial 2-ml fractions from the normal-phase HPLC by using a Spectra- max 96-well plate reader to generate a “phosphorous chromatogram.” The assay was linear from 2.5 to 75 nmol organic phosphate with a limit of detection of 2.5 nmol. The acyl-group composition of cardiolipin was determined by collection of cardiolipin from normal-phase HPLC followed by alkaline hydrolysis, derivitization to methylesters, and subsequent gas chromatography with mass selective detection (25).

Molecular species of cardiolipin were separated by using reversed-phase HPLC followed by electrospray ionization–mass spectrometry, performed as previously described with minor modifications (23). The normal-phase fraction containing cardiolipin was evaporated under N2 and brought up in 70 μl of acetonitrile:methanol:15 mM ammonium acetate pH 7.4 60:30:10 (v:v:v). The resulting sample (20 μl) was then injected onto a Hypersil MOS 3 μm, 150 × 2.1 mm (ThermoQuest, Hypersil Division, UK) column (23). Electrospray ionization mass spectrometry was performed on a Finnegan LCQ-Deca (San Jose, CA) in the negative ion mode with nitrogen as sheath and auxiliary gas. The respective parameters and data collection were performed as previously reported (23), except that only the first 2 minutes of the effluent flow were diverted to the waste. This data-dependent MS3 fragmentation analysis from reverse-phase HPLC separation was utilized to identify and characterize individual cardiolipin molecular species in SSM and IFM obtained from hearts of 6-month-old (n = 3) and 24-month-old (n = 3) Fischer rats.

Immunoreactivity Studies Using HNE and DODA Antibodies
Aging may have resulted in lipid-mediated oxidative modification of complex III as a mechanism of the defect in IFM. For lipid-mediated oxidative modification to be examined, immunoblots of complex III subunit peptides were performed with antibodies to stable HNE adducts and HNE-pyrrole end product adducts (27). Antiserum to HNE adducts was purchased from Alexis Chemicals (San Diego, CA). The 9,12-dioxodecenoic acid (DODA)-keyhole limpet hemocyanin (KLH) antibody was used to detect HNE-pyrrole epitopes derived from linoleic acid (27). Complex III was isolated under native conditions as described by Schägger (28), the complex III band was excised and subjected to second-dimension electrophoresis to separate subunits (13,29), and subunits were transferred to a polyvinylidene difluoride (PVDF) 0.2-μm membrane (27). Immunoreactivity was increased by treating electroblotted membranes with 0.2 N of potassium hydroxide (KOH) prior to immunodecorating with DODA-KLH antibody (27). Separ- ate gels and membranes were used for each antibody. Membranes were immunodecorated with either antibody at 1:250 or 1:500 dilution and were visualized by colormetric detection using alkaline phosphatase conjugate substrate (BioRad, Hercules, CA).

Statistical Analysis
Data are expressed as mean ± standard error of the mean (SEM). Differences between adult and aging rat hearts are
compared by a one-way analysis of variance (30). A value of $p < .05$ was considered significant.

**RESULTS**

**Characterization of SSM and IFM in Adult and Elderly Rat Hearts**

The protein yield of SSM was similar in adult and aging rat hearts (6-month-old adult = 11.3 ± 0.6 mg/g wet weight, $n = 9$; 24-month-old elderly = 10.6 ± 0.7 mg/g wet weight, $n = 10$, $p = NS$). Aging decreased the protein yield of IFM (6-month-old 12.7 ± 0.5 mg/g wet weight, $n = 9$; 24-month-old 10.6 ± 0.7, $n = 10$, $p < .05$) as previously observed (12,13). The maximal rate of adenosine diphosphate (ADP)-stimulated oxidative phosphorylation was unchanged by aging in SSM, consistent with previous results (data not shown).

The rate of ADP-stimulated respiration was significantly decreased by aging in IFM as previously described (dihydroquinoxine as substrate: 6-month-old 695 ± 61 nanomolars of atomic oxygen [nAO]/min/mg, $n = 9$; 24-month-old 503 ± 34 nAO/min/mg, $n = 10$, $p < .05$). The rate of state 4 respiration, respiratory control ratio, and ADP:O ratio were unaltered by aging in both populations, which is in agreement with previous results (data not shown).

**Content of Phospholipids in SSM and IFM From Adult and Elderly Rat Hearts**

Figure 1 shows the normal-phase HPLC separation of phospholipids from adult and elderly rat IFM. The chromatographic separation patterns of phospholipids are similar in adult and elderly rat hearts (Figure 1). Mitochondria contain phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and cardiolipin (CL). Phospholipids were quantified by measurement of organic phosphate in serial 2-ml fractions (23). The content of the cardiolipin was unaltered by aging in SSM and IFM (Figure 2A). The content of the remaining phospholipids also was unchanged with age in SSM and IFM (Figure 2B). Thus, aging did not alter phospholipid content in IFM that contain aging-induced defects in mitochondrial oxidative physiology.

Total phospholipid phosphorous was independently measured in phospholipid fractions from silica gel chromatography and compared with the sum of the four individual phospholipids (Table 1). The recovery of total phospholipid phosphorous as the sum of individual phospholipids was excellent in both adult and aging hearts (Table 1). Furthermore, measurement of organic phosphate in serial 2-ml fractions from the normal-phase HPLC did not provide evidence for the presence of lysophospholipids (23) or other unanticipated phospholipid species in the aging heart. The excellent recovery of phospholipids demonstrated by balance studies shows that the presence of any additional lipid phosphorous containing compounds is unlikely. The ratio of CL content to total lipid phosphate content was unchanged by aging in either population of mitochondria (Table 1).

Other investigators have quantified CL solely by using absorption at 206 nm (18). We also utilized a similar approach to quantify CL based on 206-nm absorbance from phospholipids (Table 1). A: Quantification of CL content by phosphate measurement per mg mitochondrial protein (nmol P/mg pr) in SSM and IFM from 6-month-old (n = 9) and 24-month-old (n = 10) hearts (± SEM; $p = NS$ 24-month-old elderly vs 6-month-old adult). B: Quantification of remaining phospholipids by phosphate measurement per mg mitochondrial protein (nmol P/mg pr) in adult (n = 9) and elderly (n = 10) SSM and IFM (± SEM; $p = NS$ elderly vs adult; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol).

**Table 1. Recovery of Phospholipid Phosphate in Mitochondria from Adult and Elderly Rat Hearts**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SSM 6 mo (n = 9)</th>
<th>SSM 24 mo (n = 10)</th>
<th>IFM 6 mo (n = 9)</th>
<th>IFM 24 mo (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total P</td>
<td>331 ± 15</td>
<td>320 ± 20</td>
<td>350 ± 20</td>
<td>324 ± 16</td>
</tr>
<tr>
<td>Summed P</td>
<td>297 ± 13</td>
<td>317 ± 26</td>
<td>308 ± 14</td>
<td>324 ± 21</td>
</tr>
<tr>
<td>% recovery</td>
<td>102 ± 7</td>
<td>102 ± 9</td>
<td>91 ± 7</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>% CL/Total P</td>
<td>14.6 ± 0.8</td>
<td>13.6 ± 0.9</td>
<td>13.5 ± 0.9</td>
<td>12.7 ± 0.7</td>
</tr>
</tbody>
</table>

Notes: numbers are ± SEM; $p = NS$ elderly vs corresponding adult; CL = cardiolipin; summed P = sum of individually measured phospholipids; total P = total phospholipid phosphate; % recovery = summed P divided by total P expressed as a percent; SSM = subsarcolemmal mitochondria; IFM = interfibrillar mitochondria.
the normal-phase HPLC chromatograms used for organic phosphate measurement. Bovine CL was used as a standard to generate a standard curve from 0 nmol to 64.5 nmol CL phosphate content. The standard curve of the area of CL absorbance at 206 nm versus measured CL phosphate was linear ($r^2 = .999$). The CL content (nmol/mg protein) was calculated based on the observed optical density (OD) 206 nm of the CL peak in the injected sample using the standard curve. The integrated areas of the 206-nm absorbance peaks were similar in adult and aging IFM (6-month-old adult = 11,096 ± 1203 peak area at OD 206, n = 5; 24-month-old elderly = 12,282 ± 1260 peak area at OD 206, n = 5, ± SEM, p = NS). The CL content estimated by peak area of 206-nm absorbance using the standard curve was also similar in IFM from adult and aging rat hearts (data not shown).

Composition and Characterization of Cardiolipin in SSM and IFM From Adult and Aging Hearts

We next asked if aging altered the composition of CL as a potential mechanism of the defect in IFM. The acyl-group composition of CL was measured following alkaline hydrolysis, derivitization to form fatty-acid methyl esters, and separation and quantitation by gas chromatography (25). Cardiolipin was composed predominantly of linoleic acid (C18:2) with minor amounts of oleic acid (C18:1) and stearic acid (C18:0). The fraction of total measured acyl groups represented by linoleic acid was similar in SSM and IFM from both adult and aging rat hearts (SSM 6 month, n = 5: 93 ± 1%; 24 month, n = 5: 89 ± 4%; IFM 6 month, 93 ± 2%; 24 month, 89 ± 2%; all p = NS).

Reverse Phase HPLC/Electrospray Ionization Mass Spectrometry Characterization of CL Molecular Species in Adult and Aging SSM and IFM

The composition of CL molecular species was also examined by using reverse-phase HPLC followed by electrospray ionization mass spectrometry. Figure 3A shows the reconstructed ion chromatogram of the major and minor molecular species of CL obtained from IFM from 6-month-old adult and 24-month-old elderly rat hearts. The composition of CL was similar in IFM from adult and aging hearts.

The composition of the major (Figure 3B) and minor molecular species of CL was determined by using data-dependent MS$^n$. The predominant precursor ion (m/z 1448) of CL, when subjected to collision-induced fragmentation, generated glyceroldiphosphatic acid with two C18:2 (m/z 831); phosphatic acid with two C18:2 acyl groups (m/z 695); and lysophosphatic acid with one C18:2 (m/z 415). The most intense peak (m/z 695) was further fragmented by collision-induced dissociation to yield lysophosphatic acid with one C18:2 (m/z = 415) and linoleic acid (C18:2) (m/z = 279). This fragmentation pattern corresponds to cardiolipin containing four C18:2 acyl residues, as shown in Figure 3B, (i)–(iii). The representative chemical structure with the discussed fragmentation, is shown in Figure 3B, (iv).

The minor molecular species of CL were characterized in a similar fashion. Based on fragmentation patterns using collision-induced dissociation, the molecular species with m/z 1450 consists of CL containing one C18:1 and three C18:2 acyl groups. The other minor molecular species (m/z 1452) correspond to CL containing one C18:0 and three C18:2 acyl residues. There were no aging-related alterations in the composition of the CL in either mitochondrial population, based on the study of CL obtained from SSM and IFM.

Immunodetection of Protein Adducts Resulting From Oxidative Lipid Modification in SSM and IFM From Adult and Aging Rat Hearts

Cardiolipin contains predominately oxidatively sensitive linoleic acyl residues (C18:2). Complex III contains six tightly bound CL residues (16). Despite the absence of aging-related alterations in the content or composition of CL in IFM, we considered the possibility that aging-induced oxidative damage to CL, perhaps the tightly bound residues, could result in lipid-mediated oxidative protein modification in complex III subunit peptides as a mechanism of the aging defect in complex III. Oxidative reactions with lipid acyl groups generate HNE or lipid peroxides (6). HNE is an α,β-unsaturated alkenal that adds to nucleophilic groups including peptide thiol and amines to form stable Michael protein adducts (31). The DODA-KLH antibody detects stable pyrrole epitopes resulting from oxidative reactions of C18:2-generated lipid peroxides with proteins (27). Complex III was isolated from SSM and IFM from adult and elderly rat hearts by blue native PAGE electrophoresis, and the 11 subunits of complex III were separated by second-dimension tricine sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13,28,29). Immunoblotting of subunits was performed by using a polyclonal antibody to HNE-modified Michael adducts and a polyclonal antibody that recognizes stable pyrrole end products of protein modification from C18:2-derived lipid peroxides (DODA-KLH; 27). Epitopes corresponding to lipid-mediated oxidative modification of the subunits of complex III were not detected in SSM or IFM from adult or aging rat hearts (data not shown). The antibodies recognized HNE-modified bovine serum albumin (BSA) and DODA-BSA used as positive controls. Thus, modification of subunits of complex III by reactive lipid products did not occur in IFM from the aging heart.

Discussion

We found that aging does not alter the content or composition of cardiolipin in IFM. Cardiolipin content is preserved in IFM that contain aging-related decreases in the rate of oxidative phosphorylation and the activities of complex III (12,13) and complex IV (12). Thus, alterations in the content or composition of CL do not contribute to aging-induced defects in IFM in this model. Furthermore, CL does not participate in lipid-mediated oxidative protein modification to complex III. Hence, oxidative damage to CL is not the mechanism for the aging-induced decrement in complex III activity in IFM in this model.

Complexes III and IV both require a lipid environment enriched in CL for optimal activity (15–17). Each complex contains tightly bound CL moieties that are released only upon dissociation of the complex (15–17). The aging defect in complex IV was relieved by freezing–thawing, an intervention that alters the membrane environment (12). Complex IV enzyme activity was increased by the addition of...
Figure 3. Identification and characterization of cardiolipin molecular species from interfibrillar mitochondria (IFM) from 24-month-old aging rat hearts and 6-month-old adult controls using data-dependent electrospray ionization mass spectrometry (MS) in the negative ion mode. A: Reverse-phase high-performance liquid chromatogram, showing the separation of molecular species of cardiolipin. The major molecular species (m/z 1448) eluted at a retention time of 9.9 min and the minor species at 12 (m/z 1450) and 13 (m/z 1452) min. (1A): the reconstructed ion chromatogram for cardiolipin isolated from IFM from a heart from a 6-month-old rat (normalized sensitivity: 2.22 × 10^7). (2A): the reconstructed ion chromatogram for cardiolipin isolated from IFM from a heart from a 24-month-old rat (normalized sensitivity: 3.87 × 10^7). The molecular species were similar in IFM from adult and aging hearts. B: Mass spectrum of the predominant cardiolipin molecular species and the chemical structure determined by data-dependent MS^n fragmentation. Collision-induced fragmentation patterns for the most intense peak is depicted in subpanels (i)–(iii), and subpanel (iv) represents the illustrated chemical structure. The dotted line represents the chemical structures for each cardiolipin fragment identified by collision-induced fragmentation.
phospholipid-containing liposomes (18). The aging defect in complex III was not associated with a decrease in the content of peptide subunits (13). Although a decrease in the content of CL seemed to be a plausible unifying mechanism for the defect, as reported herein this does not occur.

The content of total phospholipids was similar in IFM from adult and aging rat hearts. The sum of individual mitochondrial phospholipids accounts for the measured total phospholipids (Table 1), excluding any appreciable loss of phospholipids as well as the presence of other unexpected phosphorous-containing lipids.

Alternatively, a modification in the composition of CL can alter the activity of electron transport chain complexes (14–17). Hence, we examined the composition of CL in aging compared with adult IFM by using two complimentary approaches, acyl-group analysis by gas chromatography with mass spectrometry and CL molecular species measured with reverse-phase HPLC with electrospray ionization mass spectrometry. Aging did not alter the composition of CL in SSM or IFM assessed by either technique. Rat heart CL consists mostly of a molecular species containing four C18:2 acyl groups.

Mitochondria have been proposed as a source of oxidants that lead to the chronic oxidative damage observed during the aging process (32). CL is enriched in oxidatively sensitive C18:2 residues. Despite the lack of an observed decrease in the content of CL, oxidative reactions involving the tightly bound CL of complex III could direct lipid-mediated oxidative damage to the subunits of complex III, modifying peptide subunits and contributing to the aging defect in complex III in IFM. In order to address this question, complex III was isolated intact by blue native PAGE (28), separated into subunits by second-dimension electrophoresis (13,28,29), and subunits probed for lipid-mediated oxidative damage resulting from C18:2. C18:2 generates HNE by chain scission (31). HNE reacts with nucleophilic functional groups on proteins to form Michael adducts (27,31). Alternatively, abstraction of an allylic hydrogen from C18:2 generates conjugated diene acyl radicals that add molecular oxygen to form lipid peroxides (6). Lipid peroxides react with proteins, forming stable pyrrole intermediates (27). Lipid-mediated oxidative modification of subunits of complex III was not detected in the aging heart by using antibodies both to epitopes of HNE-derived Michael adducts or lipid peroxide-derived pyrrole adducts. Thus, oxidative modification derived from reactive lipid species does not appear to contribute to the aging defect in complex III in IFM.

The content of CL was previously reported to decrease in the aging heart (18–20). Paradies and colleagues utilized an experimental approach that studied a mixed population of mitochondria of uncertain composition (18). These investigators estimated CL content relative to the other phospholipids (18). The content of CL, other phospholipids, and total mitochondrial phospholipids were not reported (18). Information regarding recovery of CL also was not supplied (18). In the absence of quantification, a comparison of the results of the present study with those of the previous investigation is difficult to perform. However, in the present study, OD 206 was also used to estimate cardiolipin content, as performed by Paradies and colleagues (18). Whether based on integrated peak area of OD 206 absorbance, a standard curve based on OD 206, or even on the fractional composition of total phospholipids represented by CL (Table 1), a decrease in CL content was not observed in IFM or SSM from the aging heart.

McMillin and colleagues (19) found a decrease in CL content in a mixed population of mitochondria prepared from hearts of 30-month-old Fischer 344 rats by digestion with nagarse, a bacterial protease. CL content, quantified by comparison to standards, decreased from 39 ± 2 nmol/mg mitochondrial protein (± SEM) in adults to 30 ± 2 in 30-month-old rats (19). The rate of oxidation of glutamate and succinate was similar in these mitochondria (19). In contrast, the oxidative defect in IFM is observed at 24 and 28 months of age (12). The mortality rate increases in the Fischer 344 rat between 28 and 30 months (29-month median survival) (33). In the current study, we elected to study rats younger than the median survival age.

Pepe and colleagues (20) reported a decrease in the mole percent composition of CL measured relative to other phospholipids, utilizing a mixed population of mitochondria also prepared by nagarse digestion of hearts from elderly Wistar rats. In these mitochondria, succinate oxidation was unaltered by aging. As discussed above, when the quantitative CL data obtained in the current study are expressed as mole percent, CL content remains unchanged by aging. The relative decrease in CL observed by Pepe and colleagues may have occurred secondary to strain differences or as a result of dietary intervention (20). Both McMillin and colleagues (19) and Pepe and colleagues (20) found decreases in CL with aging by using a mixed population of cardiac mitochondria with preserved rates of oxidative phosphorylation. The failure to observe oxidative defects with aging by using mixed populations of mitochondria likely results from the inclusion of SSM that do not contain the oxidative defect (12), but this does not explain the decreases in CL reported in these two studies.

Conclusions

In conclusion, the current study used a systematic analytical approach to quantify cardiolipin supported by a balance study of phospholipid recovery. The analytical approach used in the current study provides the ability to (i) identify lysophospholipids if present, (ii) observe and quantify novel organic phosphorous-containing compounds by measurement of serial fractions from normal-phase HPLC, and (iii) assess phospholipid recovery by means of balance studies (23). No other study of cardiolipin in aging used a balance study approach to assess phospholipid recovery (18–21). Aging-related defects in mitochondrial oxidative physiology occur selectively in IFM (12). Aging did not alter the content of mitochondrial phospholipids, including cardiolipin, in IFM. The relative composition of cardiolipin also was unchanged by aging in IFM. Thus, cardiolipin, either by a decrease in content, alteration in composition, or participation in oxidative reactions, does not contribute to the genesis of the aging-related defect in complex III in IFM.

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