Quantitation of cardiolipin molecular species in spontaneously hypertensive heart failure rats using electrospray ionization mass spectrometry

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Abstract **Electrospray ionization mass spectrometry has previously been used to probe qualitative changes in the phospholipid cardiolipin (CL), but it has rarely been used in a quantitative manner. We assessed changes in the amount of individual molecular species of cardiac CL present in a model of congestive heart failure using 1,1′,2,2′-tetramyristoyl cardiolipin as an internal standard. There was a linear relationship between the ratio of the negative molecular ion ($[M\text{-}H]^-$) current from four different CL reference standards and the $[M\text{-}H]^-$ from the internal standard, as a function of the concentration of CL molecular species. Therefore, this internal standard can be used to quantitate many naturally occurring CL molecular species over a wide range of CL concentrations. Using this method, changes to individual molecular species of CL in failing hearts from male spontaneously hypertensive heart failure rats were examined. CL isolated from cardiac mitochondria was compared with left ventricular tissue to demonstrate the feasibility of extracting and quantitating CL from either mitochondrial or tissue samples. The acyl chain composition of individual CL molecular species was identified using tandem mass spectrometry. In animals with heart failure, the major cardiac CL species (tetralinoloyl) decreased significantly, whereas other minor CL species were significantly increased.**—Sparagna, G. C., C. A. Johnson, S. A. McCune, R. L. Moore, and R. C. Murphy. **Quantitation of cardiolipin molecular species in spontaneously hypertensive heart failure rats using electrospray ionization mass spectrometry.** ***J. Lipid Res.*** **2005.** 46: **1196–1204.**

Supplementary key words mitochondria • cardiac • internal standard • tandem mass spectrometry

Cardiolipin [(1′,2′-diacyl-3′-phosphoryl-*sn*-glycerol)2-*sn*-glycerol] (CL) is a unique mitochondrial phospholipid containing four acyl groups and is required for normal mitochondrial function (1, 2). Numerous enzymes involved in electron transport and oxidative phosphorylation have been shown to require CL for normal function (3). In addition, a decrease in CL levels has been implicated in mitochondrial membrane protein activity abnormalities during aging (4–6), ischemia (7), and, most recently, Barth syndrome, a genetic disease characterized by heart abnormalities (8). In addition to the quantitative changes in mitochondrial CL levels that have been implicated in various pathologies of the heart, there is now evidence that alterations in the acyl side chain composition of CL can contribute to aberrant mitochondrial function (9). Recently, alterations in CL have also been strongly implicated in several key events during programmed cell death (apoptosis) in the heart and other tissues (10–17), although the extent to which quantitative versus compositional changes in the CL pool influence these processes is poorly understood.

CL has most often been studied using HPLC, which is able to separate CL from other phospholipids to assess changes in the amount of total CL. To date, determinations of CL acyl side chain composition have been largely qualitative, technically arduous, or limited to only a few of the many CL molecular species known to exist (18). Valianpour et al. (19) reported an important advance in the quantitation of CL molecular species in platelets of Barth syndrome patients, with a quantitation method based on the abundance of the doubly charged species ($[M\text{-}2H]^{2-}$). An alternative approach for the quantitative determination of CL and the resolution of the CL pool on the basis of fatty acyl side chain composition is reported here. A more thorough understanding of CL biosynthesis and how CL bio-

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Abbreviations: CL, cardiolipin; ESI, electrospray ionization; LC-MS, liquid chromatography-mass spectrometry; $[M\text{-}2H]^{2-}$, doubly charged negative molecular ion; $[M\text{-}H]^-$, negative molecular ion; M_4CL, 1,1′,2,2′-tetramyristoyl cardiolipin; MS/MS, tandem collisional mass spectrometry; O_4CL, cardiolipin with four oleates; SHHF, spontaneously hypertensive heart failure.

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synthesis is altered by a variety of pathophysiological and physiological states requires a method for measuring changes in molecular CL species. To this end, we have successfully developed a method using electrospray ionization (ESI) quadrupole mass spectrometry based on the singly charged negative molecular ion ($[M-H]^-$) of CL using a commercially available CL internal standard, 1,1′,2,2′-tetramyristoyl cardiolipin (M_4CL), and have assessed its use in both isolated heart mitochondria and left ventricular cardiac tissue during the progression toward heart failure in spontaneously hypertensive heart failure (SHHF) rats, a well-established model of congestive heart failure (20, 21).

METHODS

Materials

The CL internal standard, M_4CL, and the CL reference standard containing four oleic acid side chains (O_4CL) were obtained from Avanti Polar Lipids (Alabaster, AL) and reported as >99% pure. Beef heart CL was also from Avanti Polar Lipids. Deuterium-labeled [$^2H_4$]linoleic acid (9*Z*,12*Z*-octadecadienoic-9,10,12,13-d_4 acid) was obtained from Cayman Chemicals (Ann Arbor, MI). HPLC solvents and hydrochloric acid were purchased from Fisher Chemicals (Fairlawn, NJ). Remaining chemicals were purchased from Sigma (St. Louis, MO).

Animals

Male SHHF rats were provided out of the breeding colony maintained by S. McCune (University of Colorado at Boulder). All animals were treated according to the guidelines set forth by the University of Colorado Institutional Animal Care and Use Committee.

CL reference standard preparation and assessment

The CL reference standards with four linoleate side chains (L_4CL), three linoleates with one oleate (L_3OCL), and two linoleates and two oleates (L_2O_2CL) were obtained from beef heart CL and purified by preparative reverse-phase HPLC. Briefly, a Chromolith Performance RP-18e (4.6 × 100 mm) column (Merck KGaA, Darmstadt, Germany) was used to separate the beef heart CL molecular species (1 mg repeated for four runs) using a mobile phase of isopropanol-20 mM ammonium acetate in water (2:1, v/v) for solvent A and hexane-isopropanol-20 mM ammonium acetate in water, pH 5.5 (30:40:7, v/v/v), for solvent B. Flow rate was 1 ml/min. The gradient was isocratic (3.5% B) for 15 min and increased to 20% B by 30 min, to 95% B by 35 min, and was isocratic at 95% B until 45 min, after which the solvents returned to their initial conditions, for a 55 min total run time. Fractions were collected at 30 s intervals with 1% directed into an ESI triple quadrupole mass spectrometer (API 2000; Applied Biosystems, Foster City, CA; operating conditions are detailed below). Each fraction was transferred to a separate sample vial and rechecked for purity (>95%) using direct injection into the API 2000 mass spectrometer, and the CL species of interest were separately pooled. These CL molecular species along with commercially available O_4CL (listed in **Table 1**) served as the reference standard solutions to establish the standard curve. The concentration of the CL species in each reference standard solution was established by GC-MS as described below.

Gas chromatography-mass spectrometry

The fatty acyl side chains of the four reference standards and the internal standard were first removed from the CL backbone by base hydrolysis. A fatty acid internal standard solution of [$^2H_4$] linoleic acid (0.35 nmol) was added to each sample. Saponification was carried out by the addition of 1.0 N NaOH (0.5 ml; previously extracted with isooctane to remove any fatty acid impurities). After a 30 min incubation, the reaction was stopped by the addition of 1.0 N HCl (0.5 ml; previously extracted with isooctane). The fatty acids were then extracted two times in isooctane (1 ml), and extracts were dried in a vacuum centrifuge. Pentafluorobenzyl esters were made as described previously (22). Briefly, pentafluorobenzyl bromide (1% in acetonitrile, 25 μl) and diisopropylethylamine (1% in acetonitrile, 25 μl) were added to each sample. Samples were mixed and incubated at room temperature for 30 min. After evaporation to dryness under a stream of N_2, the derivatives were resuspended in decane (50 μl) for GC-MS analysis. A standard curve containing [$^2H_4$]linoleic acid (0.35 nmol) and known quantities of linoleic and oleic acids was also derivatized and analyzed by GC-MS. The system was a Thermo Finnigan Trace DSQ GC-MS quadrupole mass spectrometer (San Jose, CA), which used a splitless injector and a 15 m × 0.25 mm inner diameter × 0.25 μm film thickness ZB-1 column (Phenomenex, Torrance, CA) and helium as the carrier gas. The column was temperature programmed from 150°C to 300°C at 15°C/min. The mass spectrometer was operated in the negative ion chemical ionization mode with methane used as the reagent gas. Data were acquired in the single ion monitoring mode. Ions were monitored for elution of myristic, palmitic, linoleic, oleic, and steric acids from the capillary GC column at charge-to-mass ratios (*m/z*) of 227, 255, 279, 281, and 283, respectively. The stable isotope-labeled [$^2H_4$]linoleic acid was also monitored at *m/z* 283 but eluted at the retention time of linoleic acid. The peak area for fatty acids was divided by the [$^2H_4$]linoleic acid peak area as the quantitative measure of each fatty acid in the sample. A standard curve using known quantities of linoleic and oleic acids was used to calculate the concentrations of fatty acids over the range of 0.003–3.6 nmol.

After quantitation of CL content in each reference standard solution, varying quantities of CL reference standard (0.0043–1.1 nmol) were mixed with a fixed amount (0.081 nmol) of M_4CL internal standard, and the samples were resuspended in hexane-isopropanol (30:40, v/v) before analysis on the API 2000 mass spectrometer. For each sample, the area under the curve for the extracted singly charged species, $[M-H]^-$, was determined for both reference and internal standards. With the API 2000 ion source, mobile phase, and ESI operating conditions used (see below), the singly charged molecule was dominant in these spectra, with

TABLE 1. CL molecular species used as reference standards for the quantitative assay

	Number of Each Fatty Acyl Group on the CL Backbone			
m/z	14:0	18:1	18:2	Abbreviation in Text[a]
1240[b]	4			M_4CL[c]
1448			4	L_4CL[d]
1450		1	3	L_3OCL[d]
1452		2	2	L_2O_2CL[d]
1456		4		O_4CL[c]

[a] CL, cardiolipin; L, linoleate (18:2); M, myristate (14:0); O, oleate (18:1).

[b] The exact mass of each molecular species ranged from *m/z* 1239.84 to 1456.03 as a result of the large number of hydrogen atoms in these molecules, but they are indicated as the closest integer values in this table and throughout the text.

[c] Commercially available.

[d] Isolated from bovine heart as described in Methods.

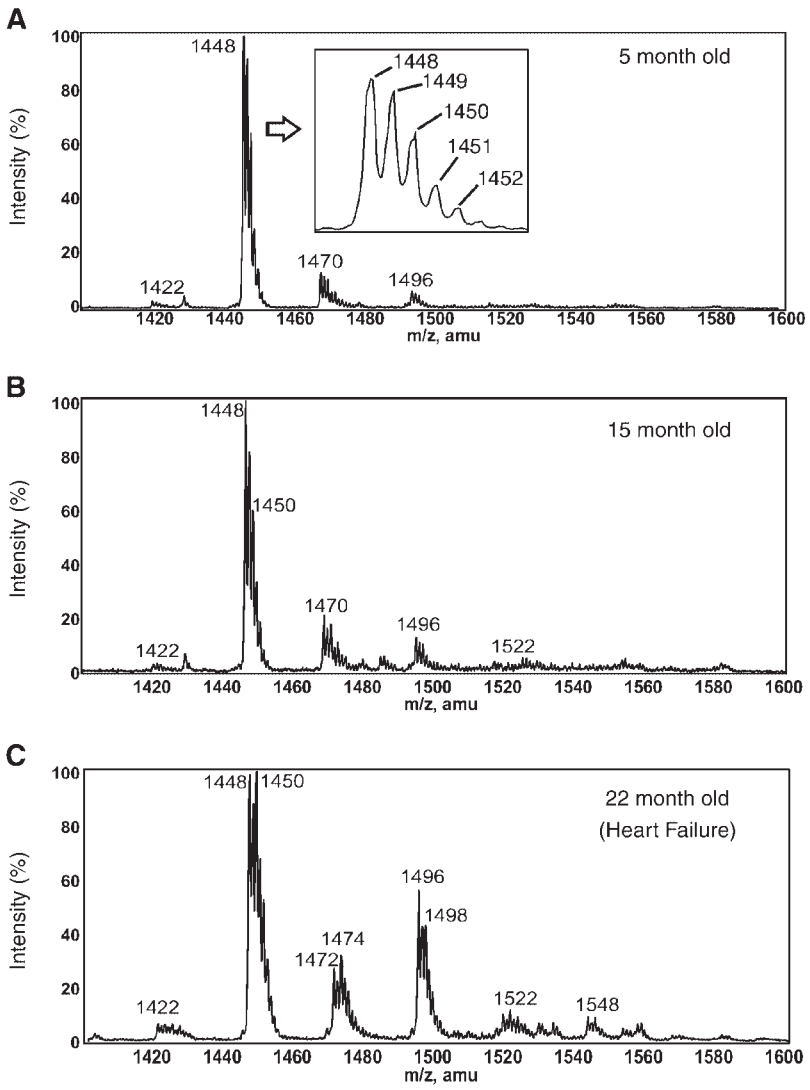

Fig. 1. Representative mass-to-charge spectra of spontaneously hypertensive heart failure (SHHF) rat heart mitochondria. Samples were taken from rats of three different ages, 5 months (A), 15 months (B), and ~22 months, when they went into symptomatic congestive heart failure (C). Numbers next to peaks denote integral *m/z* values. Note the relative increase in cardiolipin (CL) minor molecular species during heart failure compared with *m/z* 1448 (L_4CL). The inset in A shows isotope peaks of L_4CL.

~95% singly charged $[M\text{-}H]^-$ and 5% doubly charged $[M\text{-}2H]^{2-}$ L_4CL ions. Under the same solvent conditions, analysis of CL using the QSTAR quadrupole time-of-flight instrument (Applied Biosystems) gave a higher percentage of the doubly charged $[M\text{-}2H]^{2-}$ species (~25%). Using a third type of mass spectrometer, the API 3000 (Applied Biosystems), the percentage of doubly charged $[M\text{-}2H]^{2-}$ M_4CL was even higher, ~40%. This suggests that the instrument design has a significant influence on the efficiency of singly charged CL $[M\text{-}H]^-$.

Isolation of phospholipids from SHHF rat heart tissue and mitochondria

Male SHHF rats aged 5, 15, and ~22 months (in heart failure) were deeply anesthetized using 35 mg/kg ip injected sodium pentobarbital. After diminution of animal reflexes, hearts were excised, placed in ice-cold saline, and dissected into left and right ventricles and septum. Fractions of these three sections (~0.5 g total) were then immediately used for subsarcolemmal mitochondrial isolation, and the rest of the tissue was frozen in liquid nitrogen for later isolations of CL from whole tissue. Mitochondria were isolated by first washing the excised heart with buffer containing 100 mM KCl, 50 mM MOPS, 5 mM $MgCl_2$, 1 mM ATP, and 1 mM EGTA, pH 7.4, and roughly chopped dry with scissors. The chopped tissue was then washed further and dissociated using a Polytron (Brinkman Instruments, Westbury, NY) for 10 s at medium speed. The homogenate was centrifuged at 1,500 *g* for 4 min, the supernatant was put aside, and the pellet was resuspended and centrifuged again. The supernatants were pooled and centrifuged at 5,000 *g* for 10 min, and the mitochondrial pellet was resuspended in a solution containing 200 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 0.01 mM $MgCl_2$, 5 mM succinate, and 2 mM HEPES, pH 7.2. The mitochondrial samples were immediately frozen at −80°C until experiments were performed.

Phospholipids were isolated using a modified method of Bligh and Dyer (23). For mitochondria, a protein determination was performed (bicinchoninic acid; Pierce, Rockford, IL). For each sample, 120 μg of mitochondrial protein and 0.081 nmol of M_4CL

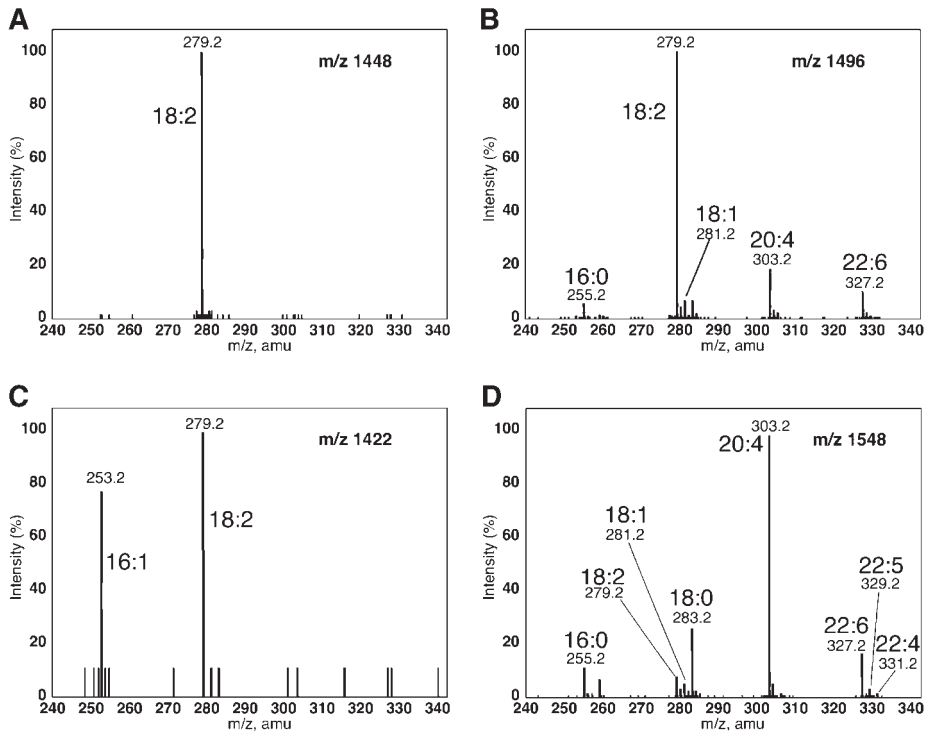

Fig. 2. Tandem collisional mass spectrometry (MS/MS) analysis of CLs isolated from failing rat heart mitochondria shown in Fig. 1C. Four representative MS/MS spectra product ions were obtained by collisional activation of the indicated precursor ion in a tandem quadrupole/time-of-flight mass spectrometer. The major carboxylate anions are identified with the acyl group and observed mass. CLs with observed negative molecular ions at *m/z* 1447.96 (A), 1495.97 (B), 1421.95 (C), and 1547.996 (D) were chosen for illustration.

was added, and extraction proceeded as with the tissue samples described below. For previously frozen heart tissue, 10–20 mg of previously frozen (in liquid N_2) left ventricular free wall tissue was homogenized using a glass-on-glass homogenizer in methanol (500 μl), and its protein concentration was determined. For each sample, 40 μg of tissue protein and 0.081 nmol of M_4CL were added. For extraction of phospholipids, the following reagents were added to either tissue or mitochondria: methanol (795 μl), chloroform (790 μl), and HCl (715 μl of 0.1 N containing 11 mM ammonium acetate). Samples were vortexed for 1 min and centrifuged at 3,000 *g*, and the lower organic layer was transferred to a clean glass tube and dried under a stream of nitrogen. Dried phospholipid samples from heart tissue or mitochondria were resuspended in 100 μl of hexane-isopropanol (30:40, v/v).

ESI mass spectrometry of CL

Samples for CL quantitation were injected (25 μl) into a normal-phase HPLC column (Prodigy 5 μm silica 100 Å, 1.0 × 150 mm column; Phenomenex) coupled to a API 2000 mass spectrometer running in negative ion mode. Negative ion ESI was carried out at −4,000 V, with declustering potential of −100 V, focusing potential of −350 V, and entrance potential of −10 V. Unit resolution was used with a step size of 0.1 amu in the range of 600–1,700 amu over a period of 4 s. The mobile phase flow rate was 50 μl/min. The solvent system consisted of hexane-isopropanol-20 mM ammonium acetate in water, pH 5.5, in a ratio of 30:40:7 (v/v/v) for solvent A and hexane-isopropanol (30:40, v/v) for solvent B. Initial conditions were 50% B for 6 min, linearly increasing from 50% to 95% B from 6 to 15 min, and remaining at 95% B until 30 min, after which it returned to the initial condition of 50% B. CL molecular species eluted off the column between 4 and 10 min.

Quantitation of CL molecular species

The nanomoles of individual molecular species of CL were calculated by determining the ratio of the peak area of the CL molecular species of interest to the internal standard. This ratio, along with the slope and intercept of the standard curve, was used to calculate nanomoles of CL using the formula for a line ($y = mx + b$, where y is ratio, b is intercept, m is slope, and x is nanomoles of CL). By dividing nanomoles of CL by milligrams of protein per sample, the value in nanomoles per milligram of protein (mitochondrial or tissue) was determined.

Collisional activation with tandem mass spectrometry

To determine the fatty acyl composition of CL species of interest, mitochondrial samples from rats in heart failure were made as described above starting with 3 mg of mitochondrial protein and omitting the internal standard. Analysis of the samples was performed by nanospray injection into a QSTAR quadrupole time-of-flight mass spectrometer operated in the negative ion mode with a collision energy of −60 V (laboratory frame of reference) and N_2 as the collision gas. Analysis was also performed using the API 2000 triple quadrupole liquid chromatography-tandem mass spectrometry (LC-MS/MS) system operated in the negative ion full-scan mode with a collision energy of −80 V and N_2 as the collision gas. Using either system, CL species of interest were collisionally activated, resulting in the production of ions caused by the loss of acyl side chains from the CL backbone. Individual fatty acids were detected as the carboxylate anion permitting molecular species identification (24).

Statistics

All statistics were determined using SPSS (SPSS Software, Chicago, IL). Details of statistical analyses are shown, if applicable, in individual figure legends.

RESULTS

Characterization of CL in failing mitochondria

Relatively little is known about the changes that occur in individual molecular species of cardiac CL during pathological situations. To investigate changes to CL during heart failure, negative ion ESI mass spectrometry was used to study CL in mitochondria from failing rat hearts. The resulting mass spectra (**Fig. 1**) from 5, 15, and ~22 month old (in heart failure) SHHF rat heart mitochondrial samples revealed numerous molecular species of CL. Obvious changes in CL species were evident in the failing heart, most notably an increase in the fraction of the CL pool composed of CL molecular species other than L_4CL (*m/z* 1447.96, labeled nominally as *m/z* 1448 in Fig. 1). The molecular species distribution in Fig. 1 was consistent with a decrease in L_4CL, an increase in minor species, or a combination of both. To clarify the nature of the changes to CL during heart failure, a method was developed to quantify CL using the addition of a synthetic, commercially available internal standard, M_4CL.

The identities of some of the CL molecular species present in the failing hearts were further analyzed using collisional activation and tandem mass spectrometry (MS/MS) to assess their fatty acyl substituents. A subset of these MS/MS spectra are shown in **Fig. 2**. Collisional activation of the most abundant CL species (*m/z* 1448; Fig. 2A) yielded a product ion spectrum with a single carboxylate anion at *m/z* 279.2 corresponding to linoleate. The majority of the ion abundance at *m/z* 1448 was most likely L_4CL, as expected. At higher mass-to-charge ratios, the possible combinations of acyl groups making up each CL molecular species were quite complex. For example, collisional activation of *m/z* 1496 (Fig. 2B) revealed five major carboxylate anions corresponding to esterified palmitate, oleate, linoleate, arachidonate, and docosahexaenoate (abbreviated P, O, L, A and D, respectively). In fact, if all common fatty acyl groups from 12:0 to 22:6 were used to calculate all possible molecular species of CLs, there would be more than 1,000 possible species having an $[M\text{-}H]^-$ at *m/z* 1496 with fatty acyl groups that added up to 76:12 (acyl carbons:double bonds). When the actual fatty carboxylate anions observed by MS/MS are used to constrain the possible 76:12 species, five species became the most likely candidates that made up this mixture. These were CLs with acyl side chains composed of PPDD, PLAD, PAAA, LLLD, and LLAA. Interestingly, the lower molecular weight CL species, *m/z* 1422 (Fig. 2C), had an even larger number of possible combinations (more than 1,200) but was limited to a single species by the presence of only two major carboxylate anions at *m/z* 253.2 (palmitoleate, abbreviated Po) and 279.2 (linoleate) for this 70:7 species. Thus, the majority of this species was likely a CL with the acyl side chains PoLLL. At this level of analysis, however, it was not possible to distinguish regioisomer content. The ions corresponding to the observed CL species of very high molecular weights were quite complex in terms of the actual number of different molecular species present. Collisional activation of *m/z* 1548 (Fig. 2D) revealed at least eight distinct carboxylate anions. This $[M\text{-}H]^-$ ion would correspond to those species having total acyl carbons and double bonds of 80:14. Considering only the most abundant carboxylate anions, the likely species present would contain the side chains (where S is stearate, 18:0) LSDD, OODD, and SAAD, but clearly, many more minor species were present at this mass. Polyunsaturated fatty acyl-containing CLs were clearly dominant in these larger CL species. A summary of the major CL molecular species observed in the failing rat heart are presented in **Table 2**, along with their observed molecular ions as $[M\text{-}H]^-$. A subset of these peaks in the 5 month old rat were also studied using MS/MS and found to contain the same relative composition of CL molecular species in the rats in heart failure.

To convert ESI mass spectrometry ion abundances to a quantitative measure of CL, it was necessary to establish a valid internal standard method. Although most quantitative mass spectrometric methods have used stable isotopic

TABLE 2. Fatty acid side chain composition of various CL molecular species in failing rat heart mitochondria as determined by MS/MS

			Number of Each Fatty Acyl Substituent on Individual CL Molecular Species						
m/z	Acyl Carbons: Double Bonds	Approximate Abundance[a]	16:0	16:1	18:0	18:1	18:2	20:4	22:6
1422[b]	70:7			1			3		
1424	70:6		1				3		
1448	72:8						4		
1450	72:7					1	3		
1470	72:8						4[c]		
1472	74:10						3	1	
1474	74:9					1	2	1	
1496	76:12	Major					3		1
		Intermediate					2	2	
		Minor	1				1	1	1
		Minor	2						2
		Minor	1					3	
1498	76:11	Major				1	2		1
		Major				1	1	2	
1520	78:14	Major					2	1	1
		Major					1	3	
		Minor	1				1		2
1522	78:13					1	1	1	1
1548	80:14	Major			1			2	1
		Intermediate			1		1		2
		Minor				2			2

Each CL molecular ion was collisionally activated in either a tandem quadrupole or quadrupole time-of-flight mass spectrometer, and assignment of fatty acyl groups in each molecular species was determined by the occurrence of fatty acyl carboxylate anions.

[a] Estimated relative composition of the molecular ion based on tandem collisional mass spectrometry spectra like those shown in Fig. 2.

[b] The exact mass of each molecular species ranged from *m/z* 1421.95 to 1547.996 as a result of the large number of hydrogen atoms in these molecules, but they are indicated as the closest integer values in this table and throughout the text.

[c] The majority of this peak is composed of the sodium adduct of the *m/z* 1448 peak.

internal standards, the number of possible molecular species and the lack of labeled CL molecules focused attention on an analog with a molecular weight far removed from the naturally occurring molecular species (*m/z* 1350–1650). This internal standard method was tested with regard to whether the ratio between the analyte peak area and the internal standard peak area would remain linear with respect to the concentration of analyte through the range of concentrations expected in the cardiac extracts and independent of CL molecular species. The internal standard, M_4CL (0.081 nmol), was combined separately with four different CL molecular species (see Table 1 for side chain and mass spectral parameters) over a wide range of concentrations (**Fig. 3**). The CL profiles of these combined samples were then tested using LC-MS, and the ratio was taken from the peak area for the reference standard to that of the internal standard. In the range tested, the relationship of the ratio of the peak areas of CL reference standard and internal standard versus nanomoles of reference standard was linear ($r^2 = 0.98$). Because the points on the curve in Fig. 3 fell on the line independent of their side chain composition, the feasibility of using this curve to quantitate all CL molecular species regardless of acyl chain identity was accepted. A slope of 10.09/nmol and a y-intercept of 0.43 were determined for the linear fit in Fig. 3.

CL in rat heart mitochondria

The standard curve in Fig. 3 was then applied to heart mitochondrial samples with added M_4CL internal standard to assess changes in CL molecular species during heart failure. Mitochondrial samples from SHHF rat hearts of three different ages approaching heart failure were used. **Figure 4** shows the peak area profiles of *m/z* 1447.96 (L_4CL) and 1239.84 (M_4CL, internal standard) as these individual molecular species eluted from the HPLC system in the

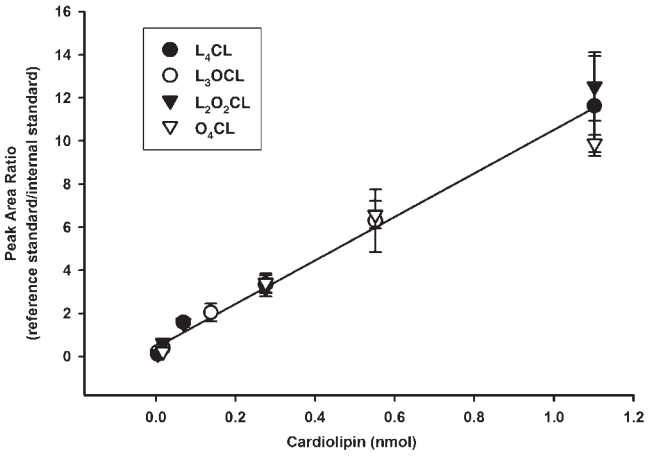

Fig. 3. Standard curve using the ratio of CL reference standards to the internal standard. Internal standard (0.081 nmol) and reference standards (0.0004–1.1 ng) were combined, and the peak area under the curve from the mass spectrum was used. The *m/z* values of the four reference standards are listed in Table 1 along with their fatty acyl groups. Error bars indicate SD; n = 3.

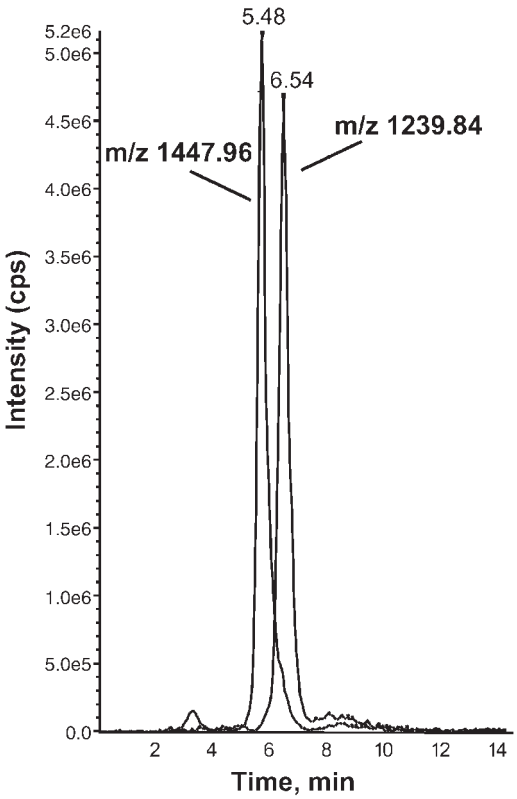

Fig. 4. Spectra of cardiac CL isolated from an SHHF rat. Typical spectrum of ions extracted from a total ion count spectrum of liquid chromatography-mass spectrometry electrospray ionization showing temporal separation between the internal standard, 1,1′,2,2′-tetramyristoyl cardiolipin (*m/z* 1239.8), and the CL reference standard with four linoleate side chains (L_4CL; *m/z* 1448.0). cps, counts per second.

LC-MS assay. The peaks were shifted temporally, as a result of the higher polarity of the internal standard. Using the slope and intercept from Fig. 3, the ratio of peak areas of CL molecular species to the internal standard yielded the nanomoles of CL, which was then corrected for the amount of mitochondria (or heart tissue) protein used in the sample. When heart mitochondrial samples were analyzed in this manner, changes in CL molecular species with heart failure were apparent. There was a significant loss in L_4CL with increasing severity of heart failure (**Fig. 5A**). There were also many CL minor species that increased during heart failure (Fig. 5B). The acyl chain compositions of these CLs are listed in Table 2.

An additional calculation was performed to separate out molecular species concentrations that differed by a single double bond, for example, the CL at *m/z* 1472 (three linoleate and one arachidonate) and the species at *m/z* 1474 (two linoleate, one oleate, and one arachidonate). The percentage of the *m/z* 1474 abundance that was attributable to the *m/z* 1472 [M+2] abundance was calculated using a previously published formula (25) with the newly updated mole fraction values for ^{2}H, ^{13}C, ^{17}O, and ^{18}O of 0.000115, 0.0107, 0.0038, and 0.00205, respectively (26). The component of the peak area of *m/z* 1474 that was attributable to the presence of isotopes adding

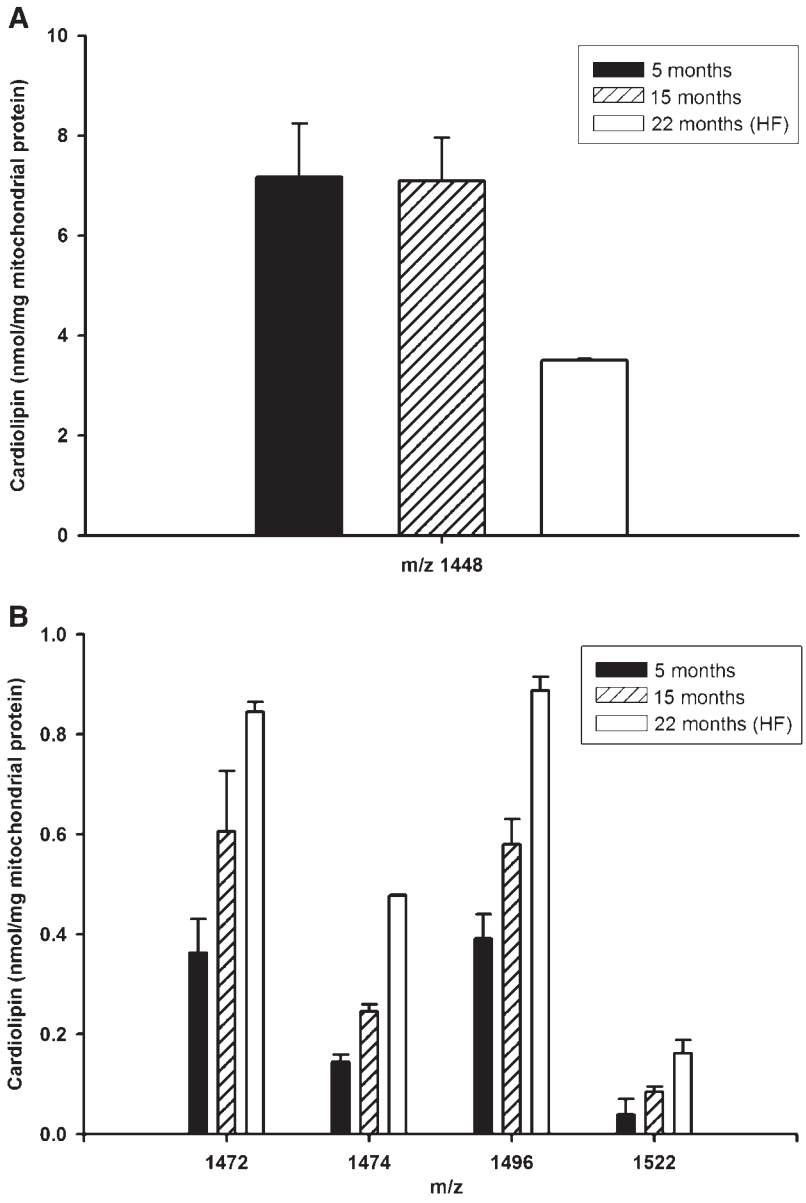

Fig. 5. Quantification of CL molecular species in SHHF rat heart mitochondria. CL was quantified using the standard curve in Fig. 3. A: Decrease in L_4CL with heart failure (HF). $P = 0.02$ by one-way ANOVA. B: Molecular species of CL that increase during heart failure include *m/z* 1472, 1474, 1496, and 1522, which are identified in Table 2. *P* values for these are 0.053, 0.004, 0.007, and 0.004, respectively, by one-way ANOVA. Error bars represent SEM; n = 4 animals.

2 amu to the *m/z* 1472 species was 45.4% of the *m/z* 1474 peak area.

CL in tissue samples

In the rat heart, CL is usually studied using isolated mitochondria. Although this provides a more concentrated CL sample, mitochondrial samples are not always available, as in banks of tissue biopsies from human patients, where the tissue is frozen and mitochondria (which require fresh tissue for their isolation) are unable to be isolated. Because CL is confined almost exclusively to mitochondrial membranes and, in the heart, the percentage of mitochondria is larger than in other tissues, measurement of CL in cardiac tissue samples was very plausible. To determine the feasibility of using previously frozen heart tissue samples, lipids were isolated from left ventricular free wall samples of the same rats from which the mitochondrial samples (Fig. 5A, B) were isolated. Because of the unique size of the singly ionized CL molecule, approximately twice the observed mass-to-charge ratio of other phospholipids, and the ability of CL to be separated from other phospholipid classes using liquid chromatography, there was little if any interference by other cellular phospholipids. As shown in **Fig. 6**, the quantitative profile of L_4CL in left ventricular heart tissue was very similar to the quantitative L_4CL profile taken from isolated mitochondria (Fig. 5A).

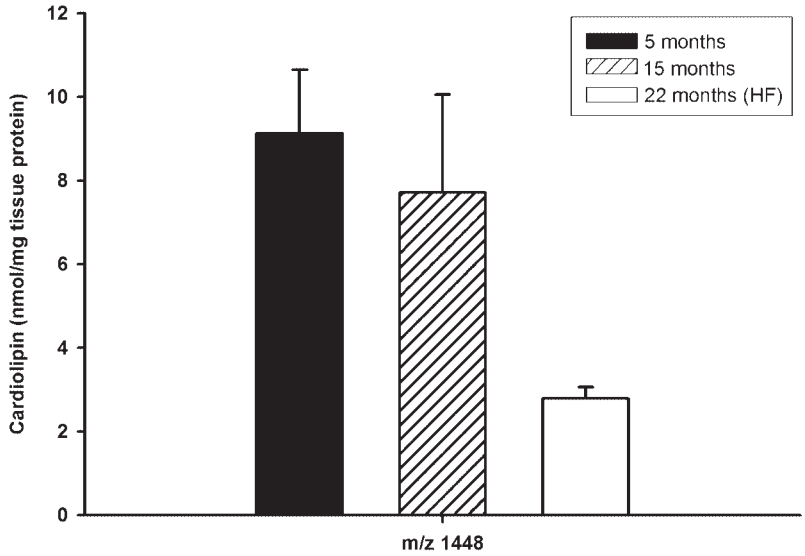

Fig. 6. Analysis of CL from SHHF rat heart tissue samples. CL species quantification of L_4CL (*m/z* 1448) in previously frozen left ventricular free wall samples from SHHF rats approaching heart failure (HF). Error bars represent SEM; n = 4 animals.

DISCUSSION

CL is increasingly being recognized as an essential component in the regulation of mitochondrial energy production and as a molecule that may, when altered under pathological conditions, be the cause of mitochondrial dysfunction. An overwhelming majority of studies on CL are carried out with HPLC alone, a technique that isolates the total pool of CL in a sample so it can be measured, but this technique lacks the ability to separate out different CL molecular species from each other. It is clear, however, that the composition of CL, in addition to its quantity, plays a large role in its function. Valianpour et al. (19) have used ESI mass spectrometry to study CL in platelets of patients with Barth syndrome. Even though they also used M_4CL as an internal standard, there are several differences between our studies. They measured the doubly charged CL species, making it much more difficult to resolve the naturally occurring stable isotope peaks (which are separated by 0.5 amu) and accurately measure abundance. Analysis of the singly charged ion permitted the resolution of these isotopic ions and a more accurate correction for molecular species abundance when CL species are separated by only 2 or 4 amu. We have also experimentally determined that the standard curve was virtually identical regardless of changes in CL acyl side chain composition over the range of investigation.

In the data presented here, the identities and absolute quantities of several CL molecular species present in mitochondrial or tissue samples were determined. The rat heart CL from SHHF rats approaching heart failure were found to have a significant decrease in the amount of the major CL molecular species (L_4CL), whereas the amounts of several minor species increased with the onset of heart failure. In the heart, enrichment of L_4CL is accomplished in two separate steps. First, CL is synthesized, via CL synthase, from phosphatidylglycerol containing random fatty acyl constituents. The newly synthesized CL contains random fatty acyl groups and must undergo a further remodeling step via either monolysocardiolipin acyltransferase (27) and phospholipase A_2 or transacylase (28) to replace these fatty acyl groups with linoleic acid. During the progression to heart failure, L_4CL decreases and CL containing other fatty acyl constituents increases. This could indicate either *1*) a further remodeling of the L_4CL that is present, or *2*) a defect in the remodeling step so that less L_4CL is made. Further experiments are needed to determine which of these scenarios occurs during heart failure.

In a comparison of mitochondrial and tissue CL molecular species, a similar amount and increase in L_4CL was observed (Figs. 5A, 6). These amounts are normalized to mitochondrial or tissue protein, respectively, but because mitochondrial protein makes up 40% of total heart tissue protein (29), the quantity of L_4CL found in tissue is higher than expected. This discrepancy is most likely attributable to differences between the tissue and mitochondrial samples. These samples, although they came from the same rats, were likely different for two reasons: *1*) mitochondria were isolated from the entire heart, whereas the tissue samples in Fig. 6 were from the left ventricle only, and *2*) isolated mitochondria were only from the subsarcolemmal population, which did not contain interfibrillar mitochondria, whereas tissue contained both. We have found that left ventricular tissue, which makes up ~50% of the heart by weight, has twice the CL content of right ventricular or septal tissue (data not shown). It has been reported previously that interfibrillar mitochondria (30) show much less of a change in total CL than the subsarcolemmal mitochondria with age (31) and with ischemia (7). The regional and mitochondria-type differences in L_4CL quantity help to explain the higher than expected value of CL in heart tissue.

In summary, this newly described technique should prove valuable for the evaluation of changes that occur in CL during various pathologies and treatments. Specifically, it should facilitate the expansion of CL analyses to include both the quantitative and compositional alterations that occur in a variety of pathophysiological settings. This approach will undoubtedly be critical in elucidating the processes that underlie alterations in CL biosynthesis and remodeling and the consequences of these alterations to normal mitochondrial function.

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