Loss of cardiac tetralinoleoyl cardiolipin in human and experimental heart failure

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Abstract The mitochondrial phospholipid cardiolipin is required for optimal mitochondrial respiration. In this study, cardiolipin molecular species and cytochrome oxidase (COx) activity were studied in interfibrillar (IF) and subsarcolemmal (SSL) cardiac mitochondria from Spontaneously Hypertensive Heart Failure (SHHF) and Sprague-Dawley (SD) rats throughout their natural life span. Fisher Brown Norway (FBN) and young aortic-constricted SHHF rats were also studied to investigate cardiolipin alterations in aging versus pathology. Additionally, cardiolipin was analyzed in human hearts explanted from patients with dilated cardiomyopathy. A loss of tetralinoleoyl cardiolipin (L_4CL), the predominant species in the healthy mammalian heart, occurred during the natural or accelerated development of heart failure in SHHF rats and humans. L_4CL decreases correlated with reduced COx activity (no decrease in protein levels) in SHHF cardiac mitochondria, but with no change in citrate synthase (a matrix enzyme) activity. The fraction of cardiac cardiolipin containing L_4CL became much lower with age in SHHF than in SD or FBN mitochondria. In summary, a progressive loss of cardiac L_4CL, possibly attributable to decreased remodeling, occurs in response to chronic cardiac overload, but not aging alone, in both IF and SSL mitochondria. This may contribute to mitochondrial respiratory dysfunction during the pathogenesis of heart failure.—Sparagna, G. C., A. J. Chicco, R. C. Murphy, M. R. Bristow, C. A. Johnson, M. L. Rees, M. L. Maxey, S. A. McCune, and R. L. Moore. **Loss of cardiac tetralinoleoyl cardiolipin in human and experimental heart failure.** *J. Lipid Res.* 2007. **48:** 1559–1570.

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The pathogenesis of heart failure (HF) is a complex, multifactorial process involving progressive cardiovascular alterations at the molecular, cellular, and organ level. Changes in myocardial energy metabolism have been widely observed in human HF and experimental models and may play an important role in the development and/or progression of the disease (see Ref. 1 for review). In humans (2, 3) and animal models of HF (4), reduced activities of mitochondrial respiratory complexes are paralleled by decreases in oxidative phosphorylation potential (5, 6) and a loss of high-energy phosphate content in the heart (7, 8). These types of studies have given rise to the hypothesis that deficiencies in mitochondrial energy metabolism may render the heart unable to meet the energy demands of the hypertrophied and failing heart. However, the specific mechanisms responsible for diminished mitochondrial function in HF have not been clearly identified.

Cardiolipin (CL) is a structurally unique phospholipid found almost exclusively in the inner mitochondrial membrane, where it provides essential structural and functional support for numerous proteins involved in mitochondrial energy metabolism (9). CL is a dimeric phospholipid containing two phosphatidyl moieties with four fatty acyl side chains. The fatty acyl chain pattern of CL is highly specific, being predominantly composed of 18 carbon unsaturated acyl chains, the vast majority of which are linoleic acid (18:2) in most mammalian tissues (10, 11). An 18:2-rich CL profile is particularly evident in the mammalian heart, where 18:2 constitutes 80–90% of CL acyl chains, and tetralinoleoyl cardiolipin (L_4CL) is the most abundant species, making up ~77% and 80% of the ventricular CL in rat and human, respectively (10, 12). The linoleoyl-rich composition of CL is critical for maintaining the enzymatic activity of cytochrome oxidase (COx; complex IV of the mitochondrial respiratory chain) and mitochondrial respiratory capacity, both of which decrease as the quantity of 18:2 in CL fractions is decreased (13, 14). De novo CL

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TABLE 1. Morphological comparison of SHHF and FBN rats

Group	Age	n	BW	Heart Weight	Heart/BW	LV Weight	LV/BW
	months		g	mg	mg/g	mg	mg/g
SHHF	2	4	269 ± 4	971 ± 26	3.6 ± 0.1	415 ± 25	1.6 ± 0.1
FBN	2	4	244 ± 6	700 ± 31	2.9 ± 0.1	346 ± 4	1.4 ± 0.0
SHHF	5	4	407 ± 24	1,534 ± 65	3.8 ± 0.3	719 ± 27	1.8 ± 0.1
FBN	5	4	384 ± 10	882 ± 40	2.3 ± 0.1	459 ± 7	1.2 ± 0.0
SHHF	15	5	415 ± 10	1,592 ± 42	3.8 ± 0.1	787 ± 48	1.9 ± 0.1
FBN	15	5	510 ± 13	902 ± 189	1.8 ± 0.2	408 ± 20	0.8 ± 0.0
SHHF, early HF	21–24	5	395 ± 14	1,833 ± 86	4.6 ± 0.2	957 ± 22	2.4 ± 0.1
SHHF, late HF	21–24	6	394 ± 8	2,609 ± 39	6.6 ± 0.2	1,079 ± 50	2.7 ± 0.1
	21–24	5	568 ± 14	1,092 ± 25	1.9 ± 0.1	538 ± 35	1.0 ± 0.1
FBN	30						

BW, body weight; FBN, Fisher Brown Norway; HF, heart failure; LV, left ventricle; SHHF, Spontaneously Hypertensive Heart Failure. Data are presented as means ± SEM. SHHF rats exhibited substantially greater relative (mg/g BW) and absolute (mg) heart and LV weights compared with FBN rats throughout life ($P < 0.01$ at all time points).

biosynthesis initially generates nascent CL with a random acyl composition. Linoleoyl enrichment of CL is accomplished by an acyl chain remodeling process that is thought to involve the phospholipid acyltransferase tafazzin (15, 16). Tafazzin gene deletion models and the X-linked cardioskeletal myopathy known as Barth syndrome (associated with a nonfunctional tafazzin mutation) result in severe L_4CL deficiency (17), destabilization of the mitochondrial respiratory chain supercomplex (18), and mitochondrial dysfunction (19, 20).

Abundant evidence indicates that any condition that results in a decrease in the content or linoleoyl-rich composition of CL in the heart is associated with impaired mitochondrial respiratory function and cardiac pathology (see Ref. 21 for review). Cardiac CL deficiencies have been reported in various rodent models of aging (22), myocardial ischemia reperfusion (23), and hypothyroidism (24), in which they have been associated with mitochondrial protein dysfunction. The most convincing evidence for a pathological role of CL alterations in human heart disease is Barth syndrome, in which a loss of L_4CL has been implicated as a primary mechanism responsible for the associated mitochondrial dysfunction and infantile or childhood development of dilated cardiomyopathy (25, 26).

Recently, our laboratories examined the CL molecular species profile of failing hearts obtained from aged Spontaneously Hypertensive Heart Failure (SHHF) rats using electrospray ionization mass spectrometry (27). We discovered that between 5 months old and HF, there was a specific loss of L_4CL in subsarcolemmal (SSL) mitochondria, whereas the content of minor CL species containing oleic acid (18:1) and arachidonic acid (20:4) increases (27). Several studies, often done in rat strains that are prone to cardiac pathology, have reported CL alterations in cardiac mitochondria during aging (22, 28–30). Therefore, the extent to which changes in the aged SHHF rat are attributable to chronic cardiac pressure overload, as opposed to aging per se, requires further clarification. This study was conducted to *1*) determine, using a wide range of SHHF rat ages, the time course and onset of cardiac CL alterations during the pathogenesis of HF in the SHHF rat model in SSL as well as interfibrillar (IF) mitochondria; *2*) distinguish between the effect of chronic cardiac overload and the normal aging process on the cardiac CL profile; *3*) determine whether a loss of L_4CL content in the SHHF rat heart is associated with reduced COx activity; and *4*) examine the extent to which CL is altered in the failing human heart.

MATERIALS AND METHODS

Animals

Male SHHF rats were obtained from the breeding colony maintained by S.A.M. at the University of Colorado at Boulder. Fisher Brown Norway (FBN) rats were purchased from the colony maintained by the National Institute on Aging (Bethesda, MD). Sprague-Dawley (SD) rats were purchased from Harlan (Indianapolis, IN). All animals were treated according to the guidelines

TABLE 2. Patient characteristics

Patient No.	Age	Sex	BP	EF
	years		mmHg	%
HF1	46	F	88/59	20
HF2	60	F	98/55	16
HF3	60	F	84/52	8
HF4	57	F	106/71	14
HF5	53	F	82/62	14
HF6	39	F	123/85	30
HF7	65	M	102/62	24
HF8	48	M	84/64	27
HF9	57	M	114/66	18
HF10	63	M	106/74	18
HF11	39	M	95/60	11
NF1	50	F	106/56	NA
NF2	59	F	119/81	55
NF3	39	F	163/74	80
NF4	61	F	115/63	NA
NF5	69	F	115/51	NA
NF6	41	F	110/67	55
NF7	68	M	110/52	NA
NF8	19	M	101/62	44
NF9	37	M	94/54	50
NF10	20	M	90/58	44

BP, blood pressure; EF, left ventricular rejection fraction; F, female; HF, heart failure; M, male; NA, data not available; NF, nonfailing. Data are presented as means ± SEM.

established by the University of Colorado Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

SHHF rat models of HF

The SHHF rat represents a well-characterized animal model of idiopathic dilated cardiomyopathy (IDC) and was selected because of its proven similarity to the hallmark biochemical and pathophysiological features of IDC in humans (31). The lean male SHHF rats used in this study typically develop hypertension by 2 months of age, with severe hypertension becoming evident in all animals by 5 months of age. In our laboratory, SHHF rats develop classic signs of HF [left ventricular (LV) contractile dysfunction, cardiac hypertrophy, piloerection, congested lungs, peritoneal fluid, etc.] by 21–24 months of age. Lean male SHHF rats were euthanized at 2 months (n = 4), 5 months (n = 4), and 15 months (n = 5) of age and after the development of overt symptoms of HF (21–24 months; n = 11). Based on the extent of

cardiac hypertrophy, animals in HF were further divided into "early" HF (heart weight < 2 g) and "late" HF (heart weight > 2 g) at the time of euthanization (**Table 1**).

A separate group of young male SHHF rats were induced to rapid HF by thoracic aortic banding (TAB) surgery at 8 weeks of age. One week after surgery, animals were either euthanized (TAB; n = 4) or administered a high-sodium diet (8% NaCl, w/w) for 3 weeks until the development of classic HF symptoms by 12 weeks of age (TAB+HS; n = 4). Surgeries were performed under an induction dose of 5% isoflurane in a holding cage. A polyethylene tracheal tube was inserted into the animal and mechanically ventilated with a 2% maintenance dose of isoflurane. A 1.5–2.0 cm incision was made between the fourth and fifth ribs, and a small retractor was positioned to spread the ribs to permit visualization of the lungs, heart, and thoracic aorta. The aorta was isolated, and a loop of 4-0 surgical silk suture was placed around the aorta. A 22 gauge template was placed in the suture loop, and the loop was rapidly closed and secured to completely occlude the aorta. The template was then removed, leaving a 22 gauge suture constriction around the thoracic aorta. Sham
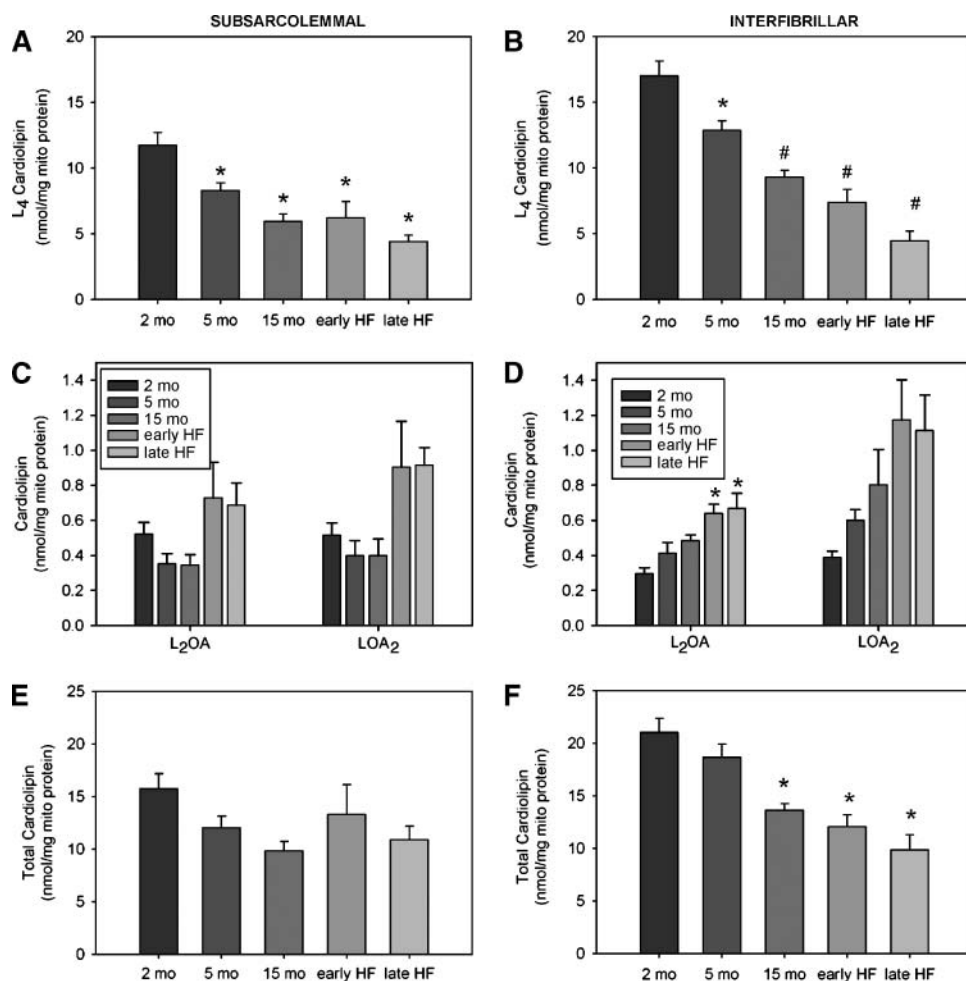

Fig. 1. Time course of cardiolipin (CL) changes in cardiac mitochondria obtained from male Spontaneously Hypertensive Heart Failure (SHHF) rats. Tetralinoleoyl cardiolipin (L_4CL) content declined as the animals progressed to late heart failure (HF) in subsarcolemmal (SSL) (A) and interfibrillar (IF) (B) mitochondria. There was a general trend for an increased content of CL species containing oleic (O) and arachidonic (A) acid side chains when animals developed HF (C, D). No significant change in total CL levels was observed in SSL mitochondria (E), whereas a significant decrease was observed by 15 months in IF mitochondria (F). * $P < 0.05$ versus 2 months; # $P < 0.01$ versus 2 and 5 months. Values shown are means ± SEM; n = 4–6 animals per group.

animals (n = 4) were treated identically to banded animals except that no suture was placed around the aorta. After surgery, animals were housed in individual holding cages that were maintained via heating lamps at temperatures between 25°C and 30°C for 30–60 min. Animals were observed intermittently over a 4 h period before being returned to their usual housing facilities. Thereafter, animals were monitored twice daily over a 3–5 day period to deal with any issues regarding suture failure or signs of wound infection and to monitor daily food intake.

HF patients

Patient characteristics are presented in **Table 2**. LV tissues were obtained from explanted hearts of patients diagnosed with IDC (52 ± 3 years; n = 4 male, 6 female; mean ejection fraction, 17 ± 2%). Nonfailing (NF) LV samples were obtained from patients who had no history of cardiac or pulmonary disease (46 ± 5 years; n = 4 male, 6 female; mean ejection fraction, 48 ± 2%). All hearts were dissected and flash-frozen in a timely manner to ensure consistency between samples. There were no significant differences in mean age between the IDC and NF control groups. NF and failing hearts were obtained via an Institutional Review Board-approved protocol maintained by the University of Colorado Health Sciences Center Cardiac Tissue Bank. Hearts donated for research purposes were obtained under written consent from family members of organ donors whose hearts could not be used for transplantation or by direct written consent from end-stage HF patients undergoing cardiac transplantation.

Isolation of IF and SSL mitochondria

Cardiac myocytes contain two functionally distinct populations of mitochondria: SSL located beneath the plasma membrane, and IF residing between the myofibrils (32). Given evidence that these populations may be affected differently by cardiomyopathy (6) and aging (33), both mitochondrial fractions were isolated from rat heart ventricles using differential centrifugation and nargarse digestion of myofibrils, as described previously by Palmer, Tandler, and Hoppel (32). In FBN heart samples, only SSL mitochondria were isolated.
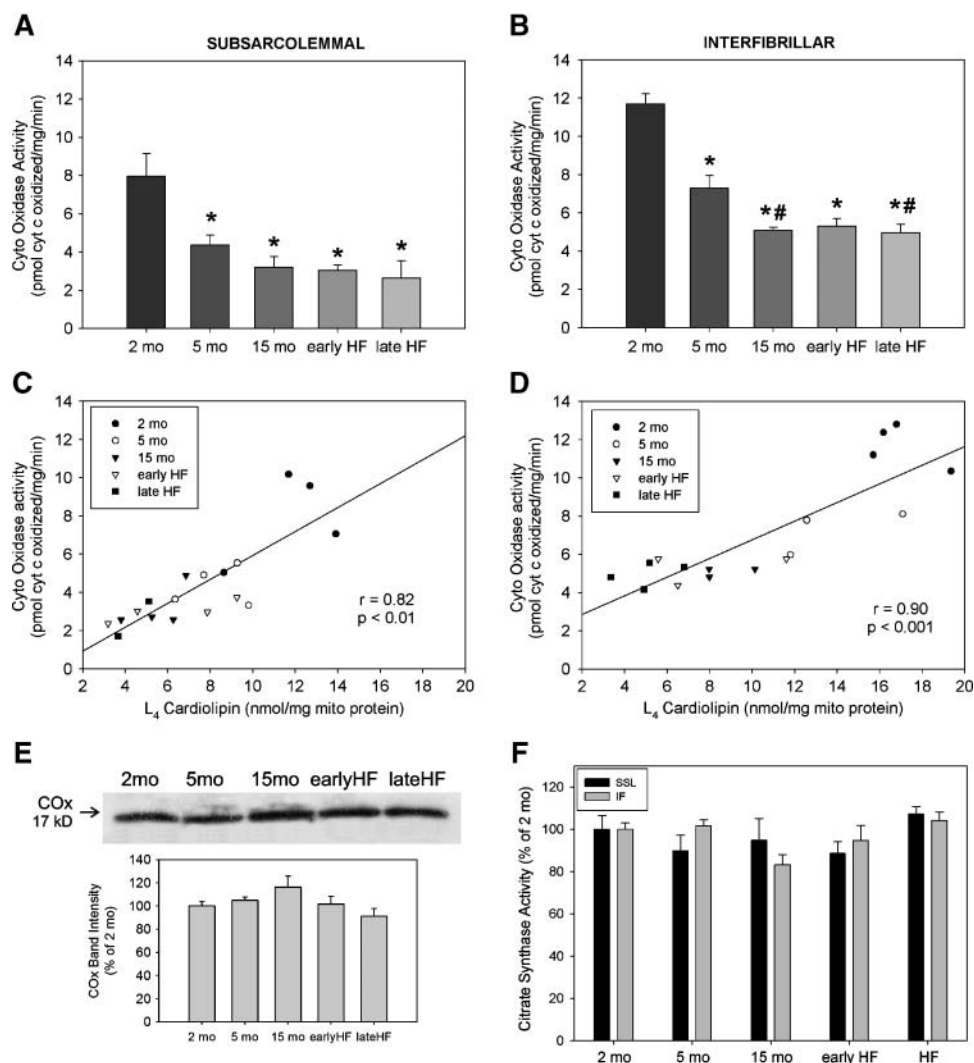

Fig. 2. Cytochrome oxidase (COx) and citrate synthase activity in mitochondria isolated from SHHF rat hearts. COx activity declined in both SSL (A) and IF (B) mitochondria by 5 months of age and remained below 2 month levels until the onset of HF. A strong positive relationship existed between L_4CL content and COx activity in SSL (C) and IF (D) mitochondria. Neither cardiac COx protein expression (E) nor citrate synthase activity (F) was significantly different across time points. * $P < 0.01$ versus 2 months; # $P < 0.05$ versus 5 months. Values shown are means ± SEM; n = 4–6 animals per group.

Isolation of phospholipids from cardiac tissue and mitochondria

Phospholipid fractions were isolated from cardiac mitochondria or LV tissue using a modified method of Bligh and Dyer (34) as described previously by our laboratory (27).

Electrospray ionization mass spectrometry of CL species

Quantification of CL molecular species was conducted by our previously published electrospray ionization mass spectrometry method using $1,1',2,2'$-tetramyristoyl CL as an internal standard (27). Total CL in all cases was determined as the sum of the eight most prevalent CL species in rat, with values of singly ionized CL of m/z 1,422, 1,448, 1,450, 1,470, 1,472, 1,474, 1,496, and 1,498. In human hearts, these same peaks were used with the exception of m/z 1,496 and 1,450, which were undetectable in those samples. These species together constitute >95% of the total CL pool. For the acyl species contained in these peaks, refer to Sparagna et al. (27).

Mitochondrial enzyme activity assays

COx activity was determined in frozen-thawed cardiac mitochondria by monitoring the oxidation of ferrocytochrome c by COx in the sample, detected as a linear disappearance of (reduced) ferricytochrome c at 550 nm over 1 min using standard spectrophotometric methods (35). Citrate synthase activity was determined spectrophotometrically in the same mitochondrial isolates according to the method of Srere (36).

Western immunoblotting

To provide an index of cardiac mitochondrial density and to compare changes in protein abundance versus enzymatic activity in isolated mitochondria, relative quantities of COx protein were determined in human and SHHF ventricular tissues by standard SDS-PAGE and immunoblotting methods using a monoclonal antibody against COx subunit IV (1:2,000; Molecular Probes, Eugene, OR). COx blot densities were normalized to calsequestrin to control for potential loading differences (37). Calsequestrin was used as the loading control because it has been shown, unlike some other housekeeping proteins, to remain unaltered during the pathogenesis of HF (38).

Statistical analyses

Data are presented as group means ± SEM. Two-tailed independent-sample t-tests were used when two groups were compared. Time course data in SHHF and SD rats were analyzed by ANOVA with repeated measures. Multiple groups were compared using one-way ANOVA. When appropriate, individual group differences were examined with post hoc Tukey tests. Pearson correlations were conducted to examine the association between L_4CL and COx activity or LV mass. Statistical significance was established at $P \leq 0.05$ for all analyses.

RESULTS

CL alterations in SHHF rat heart mitochondria

To examine the time course of CL alterations in the progression to HF, the CL species profile was examined in cardiac mitochondria obtained at various ages throughout the natural life span of the SHHF rat (**Fig. 1**). Given the potential for different effects of cardiac pathology on the two populations of mitochondria in the heart (6, 23, 33), we examined the CL profile in both SSL and IF mitochondria. A significant loss of L_4CL content became evident in both SSL and IF mitochondria by 5 months of age ($P < 0.05$), well before the development of HF (Fig. 1A, B). Increased content of minor CL species containing alternate acyl side chain compositions was also observed in both mitochondrial populations as animals progressed to HF, indicating that aberrant CL remodeling likely contributed to the loss of L_4CL. Total CL mass remained essentially unchanged in SSL (Fig. 1E) but decreased significantly by 15 months of age in IF mitochondria (Fig. 1F), where it remained until animals developed end-stage HF.

Progressive decline of L_4CL in SHHF rats is associated with decreased COx activity

To examine the potential effect that L_4CL decreases in the SHHF rat heart may have on mitochondrial electron transport chain enzymes, the activity of COx (complex IV) was measured. Cardiac COx activity decreased to 54% (SSL) and 61% (IF) of 2 month levels by 5 months of age in the SHHF rats ($P < 0.05$), well before the development of HF symptoms (**Fig. 2A, B**). Activity further declined by 15 months of age to 38% and 43% of 2 month levels in SSL and IF mitochondria, respectively, where it remained until the animals developed HF several months later. A strong
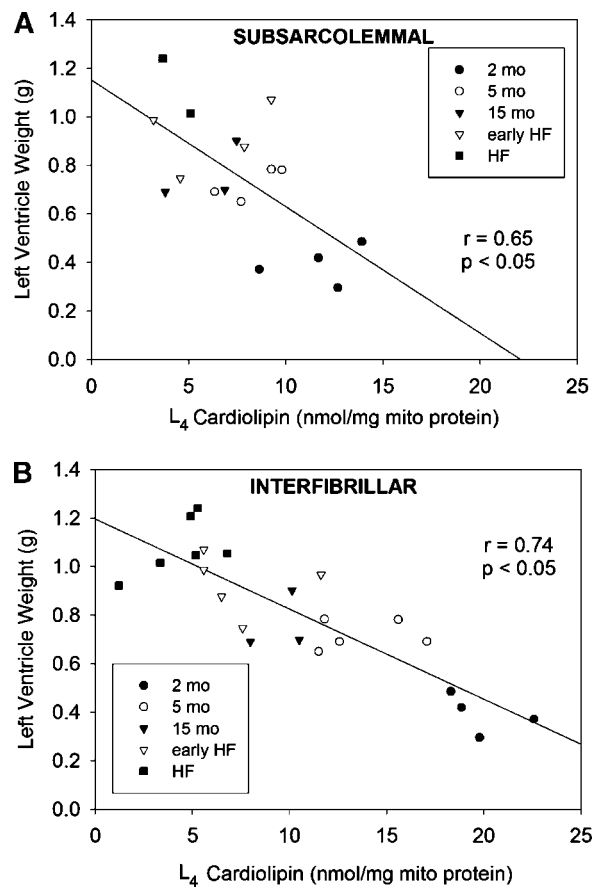

Fig. 3. Relationship between left ventricular (LV) mass and L_4CL. A significant inverse relationship was found between L_4CL content and LV mass in both SSL (A) and IF (B) mitochondria isolated from SHHF rats at various ages. r values shown were calculated using Pearson correlation analyses.

positive correlation existed between L_4CL content and COx activity in both SSL and IF mitochondria (Fig. 2C, D). Interestingly, the substantial decrease in COx activity was not associated with a loss of enzyme protein content, which remained essentially unchanged from 2 months until animals reached late HF (Fig. 2E). The enzymatic activity of the matrix enzyme, citrate synthase, remained constant over the life span of these animals. These data indicate that the progressive loss of COx activity during the pathogenesis of HF may result from changes in the CL-rich inner membrane environment, whereas the activity of non-membrane-associated proteins in the mitochondria is unaffected.

L_4CL loss is associated with pathologic LV hypertrophy

To relate L_4CL changes to the degree of cardiac pathology in the SHHF rat, correlation analyses were conducted between L_4CL and the progressive pathological LV hypertrophy observed in the SHHF rat (**Fig. 3**). A strong inverse

relationship was found between LV mass and L_4CL content in both SSL ($r = 0.65$, $P < 0.05$) and IF ($r = 0.74$, $P < 0.05$) mitochondria, indicating that changes in mitochondrial L_4CL are associated with the progression of pathologic LV hypertrophy.

CL changes do not occur progressively during aging in SD and FBN rats

Prior studies have suggested that total CL levels are markedly decreased in cardiac SSL mitochondria of male aged (26 months) Fisher 344 rats (22, 30). However, Pacher et al. (39) demonstrated that Fisher rats develop marked systolic and diastolic HF by 24–26 months of age, suggesting that the previously reported CL alterations in this model may be attributable, at least in part, to cardiac pathology. To contrast the pathology associated with the progression of HF in the SHHF rat to aging in another widely used rat model, CL and mitochondrial enzyme data from SHHF rats were compared with those obtained from
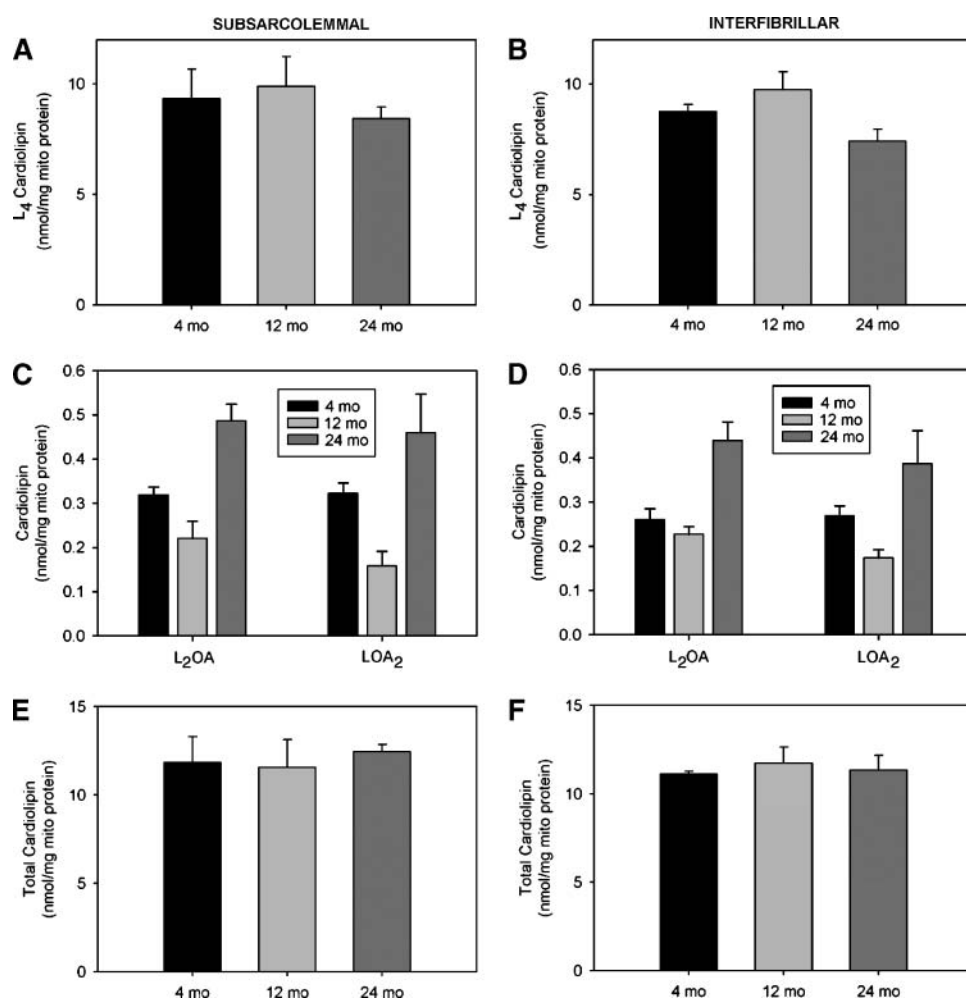

Fig. 4. CL molecular species in cardiac mitochondria isolated from Sprague-Dawley (SD) rats. CL subspecies were quantified in 4, 12, and 24 month old SD rats. ANOVA analyses indicated that L_4CL (A, B) and total CL (E, F) levels are preserved with aging in the SD rat heart. However, a statistically significant increase in CL species containing oleic (O) and arachidonic (A) acid side chains was observed at 24 months (C, D). * $P < 0.05$ versus the 4 month group; [#] $P < 0.05$ versus the 4 and 12 month groups. Values shown are means ± SEM; n = 4 animals per group.

SD rats. The L_4CL and total CL profile remained essentially unchanged in both SSL and IF mitochondria from 4 to 24 months of age in the SD rat (**Fig. 4A, B, E, F**), although a significant increase in minor L_2AO and LOA_2 species was seen at 24 months of age (Fig. 4C, D). There were no significant age-related changes in the enzymatic activity of COx (**Fig. 5A, B**) or citrate synthase (Fig. 5C) in either SSL or IF mitochondria in the SD rats.

In comparing the levels of L_4CL in the young SHHF rats (Fig. 1A, B) and the SD rats (Fig. 4A, B), it appears that the amount of L_4CL is higher in the 2 month old SHHF rats than in the SD rats per unit of mitochondrial protein. To resolve this apparently counterintuitive situation (the less functional mitochondria in the SHHF rat having more functional CL), the fraction of L_4CL in each type of mitochondria was calculated for SHHF, SD, and FBN rats. We included the FBN rat because it represents a well-established animal model of nonpathological aging (40), exhibiting little evidence of age-related cardiac pathology at the ages examined in this study (41) and no development of the marked cardiac hypertrophy observed in the aged SHHF rats (Table 1). In contrast to the strong negative correlation found between L_4CL and LV mass in the

SHHF rats (Fig. 3), no significant relationship was observed between these two factors in the FBN rats ($r = 0.07$; data not shown).

When the fraction of L_4CL in these three rat strains was compared (**Fig. 6**), a striking difference became apparent between the progressive decline of the L_4CL fraction in the SHHF rat heart and the maintenance of the previously published ratio of 0.77 (10) in both SD and FBN (SSL mitochondria only) rat hearts. Only in the oldest age of SD and FBN rats did this ratio decrease below this 77% threshold, shown in Fig. 6 by the dotted line.

To more precisely isolate the effect of cardiac pressure overload from the aging process in the SHHF rat, CL species were examined in 8 week old SHHF rats exposed to TAB followed by a high-sodium diet (TAB+HS) to induce rapid LV hypertrophy and failure by 9–12 weeks of age. Significant cardiac hypertrophy was evident at 1 week after TAB in the young SHHF rats, and the addition of an 8% sodium diet resulted in marked hypertrophy and classic HF symptoms by 4 weeks after TAB (**Table 3**). CL data from TAB and TAB+HS rats are presented in **Fig. 7**. One week after TAB, cardiac L_4CL content was 63% of sham-operated control values, although this decrease was not
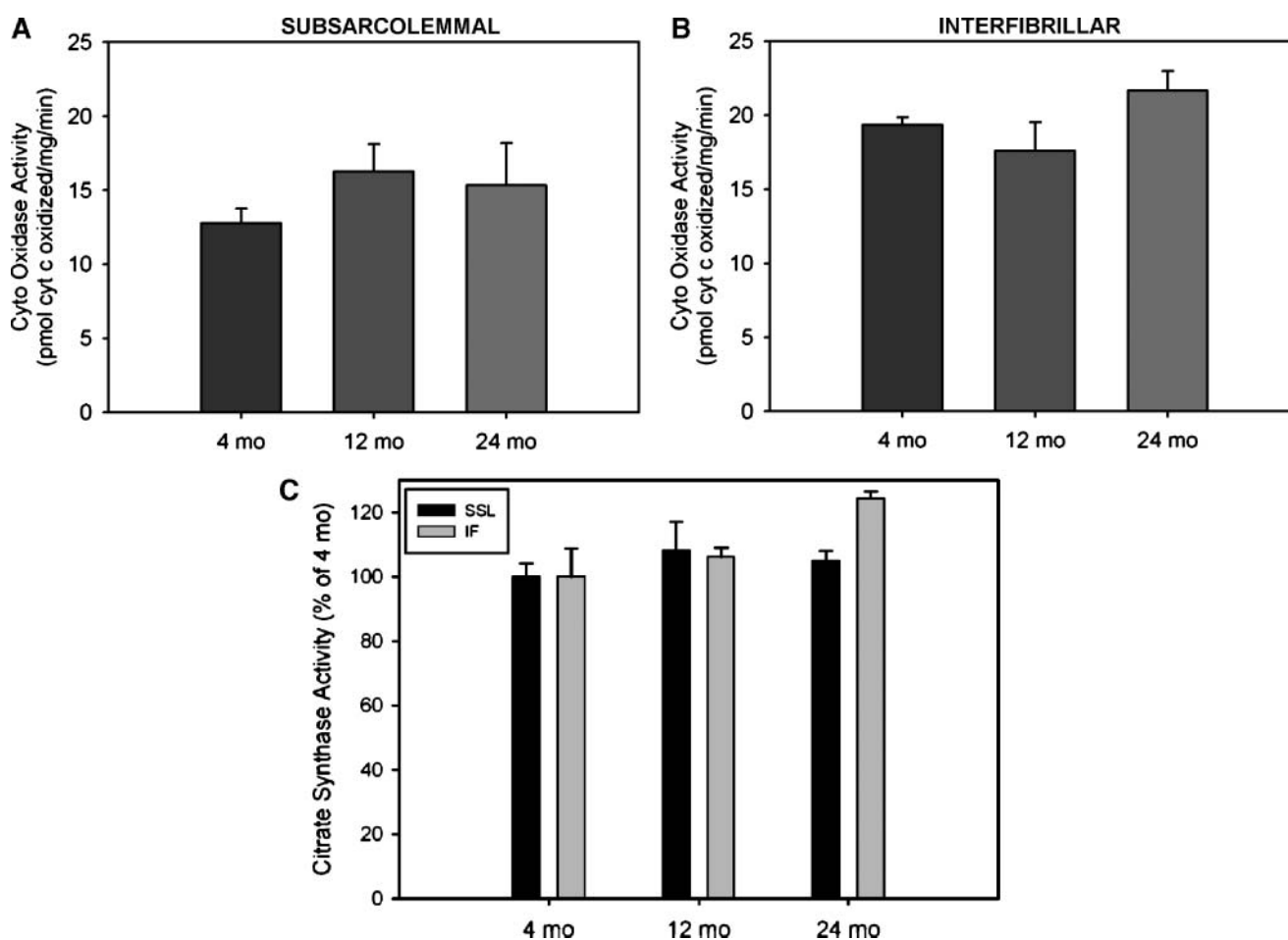

Fig. 5. COx and citrate synthase activity from mitochondria isolated from SD rats. Mitochondrial enzyme activities were quantified in 4, 12, and 24 month old SD rats. No significant changes were seen in COx activity in SSL (A) or IF (B) mitochondria or in citrate synthase activity (C) in 4 to 24 month old SD rats. Values shown are means ± SEM; n = 4 animals per group.
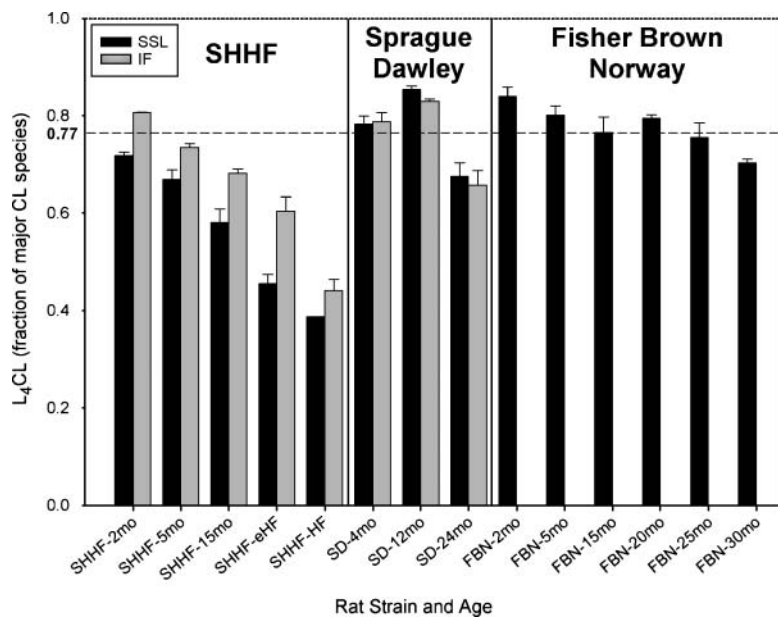
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Fig. 6. L_4CL as a fraction of total CL. L_4CL is expressed as a fraction of total CL in mitochondrial samples from three different rat strains: SHHF, SD, and Fisher Brown Norway (FBN). The dashed line indicates the previously published value (77%) of this fraction in rat cardiac ventricles. The value of this ratio steeply declines in the SHHF rats but does not go below the 77% level until 24 months in SD rats and 30 months in FBN rats. IF mitochondrial samples were not available for the FBN rats. Values shown are means ± SEM; n = 4–6 animals per group.

statistically significant ($P = 0.13$). L_4CL content declined further to 50% of sham levels after 3 weeks of a high-sodium diet (TAB+HS), which was accompanied by a significant increase in CL species containing alternate acyl side chain configurations. No significant differences in total CL mass (Fig. 7C) or COx protein content (Fig. 7D) were observed among the three groups, indicating that the loss of L_4CL likely resulted from aberrant CL remodeling rather than from a loss of total CL mass or decreased mitochondrial density.

L_4CL deficiency in the failing human heart

To determine whether the changes in cardiac L_4CL seen in SHHF rats are also present in human IDC patients, CL was measured in IDC and NF heart samples (**Fig. 8**). L_4CL content was significantly lower in LV samples obtained from IDC hearts compared with NF hearts ($P < 0.05$). A significant increase in minor CL species containing alternate acyl side chains was also found in the IDC versus NF hearts ($P < 0.05$), indicating that alterations in CL compo-

sition may have contributed to the observed loss of L_4CL. This decrease may be explained by an overall decrease in CL mass, which was also seen in the IDC versus NF hearts (Fig. 8C). Importantly, COx protein expression was not significantly different between these groups, indicating that the loss of CL mass in IDC samples was not attributable to a decrease in mitochondrial density.

DISCUSSION

The primary findings of this study are as follows: *1*) a progressive decline in cardiac L_4CL occurs in both SSL and IF mitochondria before the development of overt HF in SHHF rats; *2*) L_4CL deficiency is associated with an increase in minor CL species containing aberrant acyl composition in the failing SHHF rat and failing human heart; *3*) the activity of COx decreases in concert with L_4CL in both populations of cardiac mitochondria in the SHHF rat, despite no loss of mitochondrial COx protein content, but it does not decrease in SD rats where L_4CL does not decrease; *4*) these changes in COx activity are specific for this membrane-associated protein and not for a matrix protein, citrate synthase; and *5*) progressive cardiac CL alterations and changes in the fraction of L_4CL occur during the natural (with aging) and accelerated (TAB+HS) development of HF in the SHHF rat, but not until the end of the life span when cardiac pathology is absent or mild (SD and FBN rats). Collectively, these data indicate that progressive changes in the CL profile of cardiac mitochondria are associated with pathology, precede the development of HF, and may contribute to mitochondrial respiratory dysfunction early during the progression from hypertension to HF.

Early evidence of CL alterations in the failing heart come from studies conducted by O'Rourke, Reibel, and colleagues (42, 43), who reported a reduction in the

TABLE 3. Morphological data from young SHHF rats induced to rapid LV hypertrophy

Group	Age	n	BW	Heart Weight	Heart/BW	LV Weight
	weeks		*g*	*mg*	*mg/g*	*mg*
Sham	12	4	223 ± 5	850 ± 25	3.8 ± 0.1	536 ± 12
TAB	9	4	213 ± 23	1,343 ± 77[a]	1,343 ± 77[a]	813 ± 38[a]
TAB+HS	12	4	179 ± 7	1,534 ± 59[a]	8.6 ± 0.3[a]	872 ± 35[a]

BW, final body weight; sham, animals euthanized at 4 weeks after sham TAB surgery; TAB, animals euthanized at 1 week after thoracic aortic banding surgery; TAB+HS, animals fed an 8% NaCl diet for 3 weeks after a 1 week recovery from TAB surgery. Data are presented as means ± SEM.

[a] Both TAB and TAB+HS animals exhibited substantially greater relative (mg/g) and absolute (g) heart and LV weights compared with sham-treated rats ($P < 0.01$).
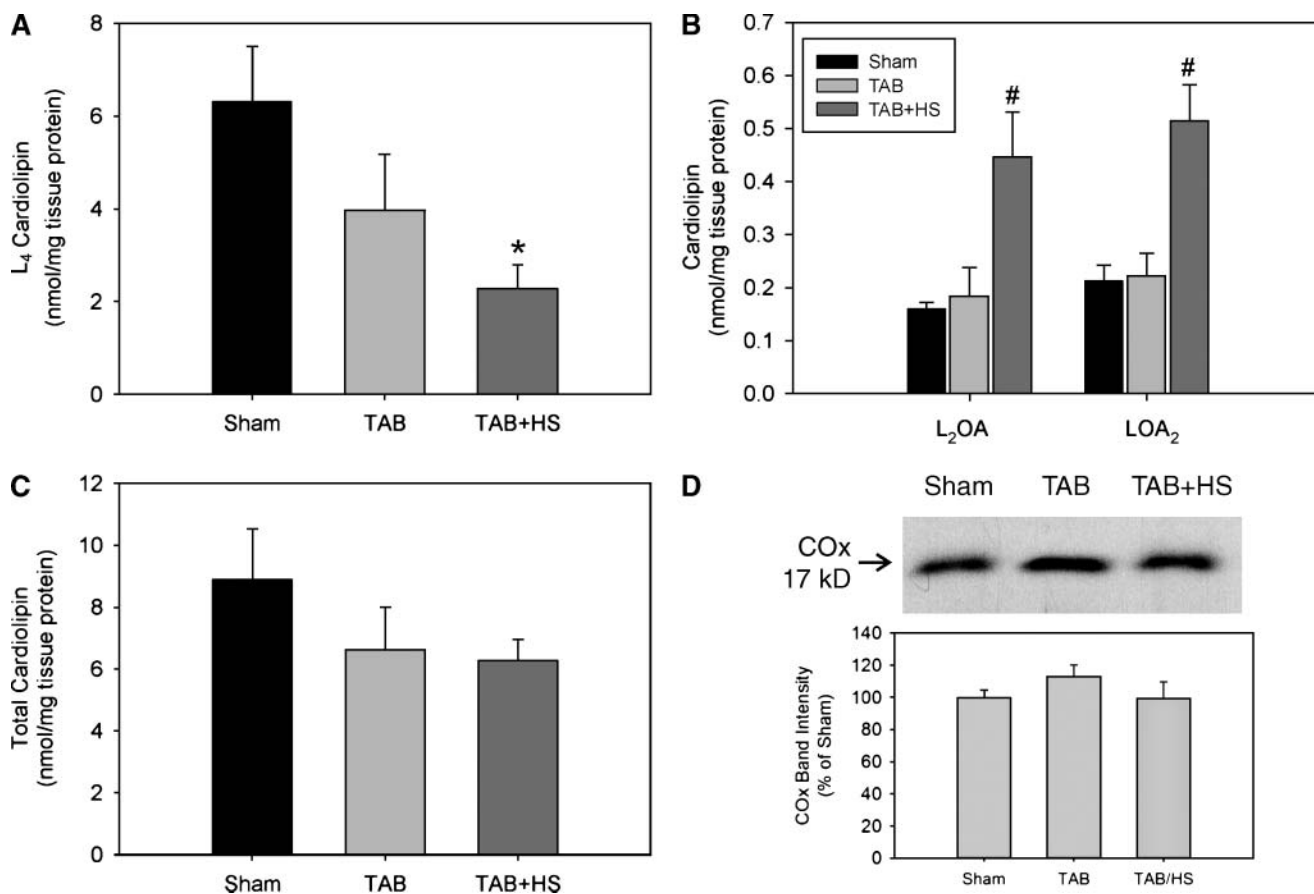
Fig. 7. Induction of HF in young SHHF rats. CL molecular species in young SHHF rats at 1 week after thoracic aortic banding (TAB) surgery and after an additional 3 weeks of a high-sodium (8% NaCl) diet (TAB+HS). TAB+HS rats had significantly lower L_4CL content than sham-operated controls (A) and significantly greater levels of CL species containing oleic (O) and arachidonic (A) acid side chains (B). Total CL (C) and COx content (D) were not altered significantly by the TAB or TAB+HS treatment. * $P < 0.05$ versus sham treatment; [#] $P < 0.05$ versus sham treatment and TAB. Values shown are means ± SEM; n = 4 animals per group.

linoleic acid content of CL fractions isolated from rat hearts induced to rapid pressure overload hypertrophy and failure by chronic aortic banding. A loss of L_4CL content has been reported previously in the failing human heart (44), but those authors did not rule out the possibility that this loss may simply reflect a decrease in tissue mitochondrial density in the failing heart. In this study, we have shown that a significant decline in total CL mass and L_4CL content occurs in the failing human heart despite no change in mitochondrial density (indicated by stable COx protein expression; Fig. 8). In addition, we demonstrate that a progressive loss of L_4CL occurs in both populations of cardiac mitochondria, beginning early during the transition from hypertension to HF in the SHHF rat model. The loss of L_4CL in the human heart and the SHHF rat heart corresponded to a decrease in total CL mass and/or an increased content of CL species with alternate acyl chain configurations, suggesting that decreases in L_4CL may result from inadequate CL biosynthesis, CL degradation, and/or aberrant acyl chain remodeling. Importantly, these data indicate that marked changes in the CL-rich inner membrane environment of cardiac mitochondria precede the development of HF in the SHHF rat, which

may have important metabolic consequences for the heart in the early pathogenesis of hypertensive heart disease.

Metabolic abnormalities and ATP deficiency have been widely hypothesized to play an important role in the etiology of HF (1, 45, 46). In particular, defective respiratory chain function has been suggested to play a causative role in the development of HF in some inherited mitochondrial cardiomyopathies (47) and in mitochondrial respiratory chain-deficient mice (48). A specific loss of COx activity has been reported in the failing human heart (2, 3) and in animal models of HF (4, 49, 50) and has been shown to correlate with LV dysfunction in human HF (2). However, the majority of the evidence of COx deficiency in HF has come from patients in end-stage HF or animal models exposed to severe cardiac overload and is most often attributed to a loss of COx protein secondary to a downregulation of COx subunit gene expression (4, 50, 51). Using the SHHF rat, a model that closely mimics the pathogenesis of human IDC (31), we have provided novel evidence for a decrease in myocardial COx activity early during the progression from hypertension to overt HF that is independent of changes in protein expression. We propose that the loss of COx activity results from a loss of
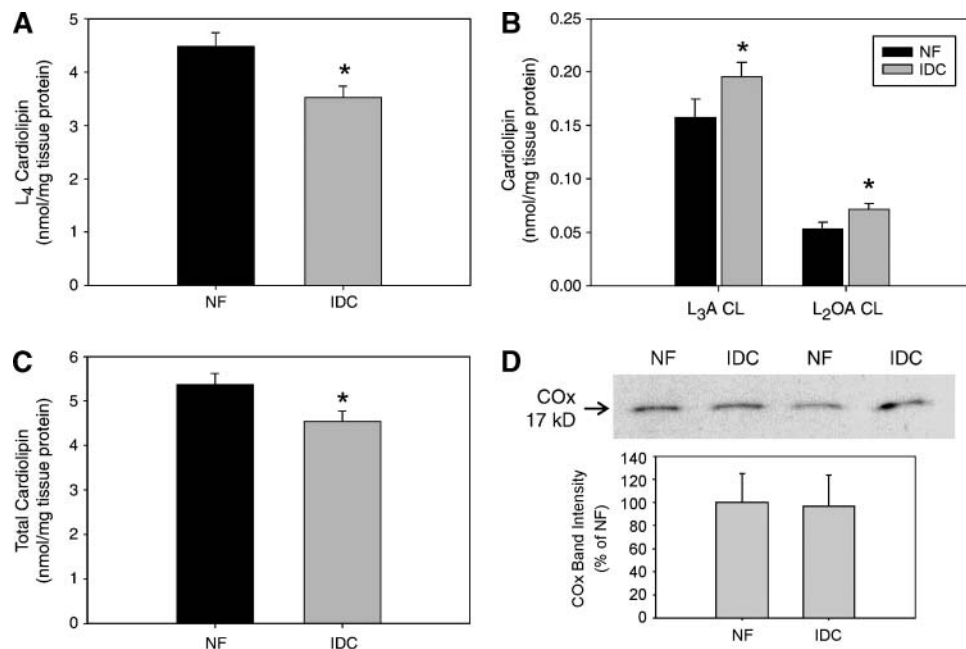
Fig. 8. CL changes in LV samples obtained from patients diagnosed with idiopathic dilated cardio-myopathy (IDC). L_4CL levels were lower in failing (IDC) compared with nonfailing (NF) hearts (A), which was matched by an increase in CL species containing oleic (O) and arachidonic (A) acid side chains (B), suggesting aberrant CL remodeling in IDC. Total CL mass (C) and COx protein (D) were also significantly lower in the IDC versus NF hearts, suggesting a decrease in mitochondrial density. $* P < 0.05$. Values shown are means $\pm$ SEM; n = 10–11 per group.

L_4CL, which is known to be required for optimal COx activity in the heart (14, 52).

The essential role of CL in maintaining the structural and functional integrity of mitochondrial respiratory complexes in the inner mitochondrial membrane is well established (52–54). Moreover, cardiac CL deficiencies have been reported in several cardiac pathologies associated with mitochondrial respiratory dysfunction and have been linked specifically to decreased COx activity in hypothyroidism (55), aging (22), and free radical stress (56). Interestingly, Yamaoka-Koseki, Urade, and Kito (52) found that delipidated bovine COx activity was stimulated only by reconstitution with L_4CL-enriched CL fractions, and not L_4CL-deficient fractions. The same authors demonstrated a loss of mitochondrial oxygen consumption as the linoleic acid content of CL fractions was decreased by restricting dietary linoleic acid intake in rats (13, 14). Therefore, it appears that the L_4CL species, in particular, may be essential for maintaining COx activity and mitochondrial function. In this study, a strong positive correlation was found between COx activity and L_4CL content in cardiac SSL and IF mitochondria in SHHF rats of varying ages, despite the absence of any significant age-related change in COx protein expression. These data suggest that a progressive decrease of L_4CL may impair COx function during the progression of hypertensive heart disease. This could contribute to mitochondrial respiratory dysfunction and metabolic stress, which has been hypothesized to play a critical role in the pathogenesis of HF. However, further study is required to establish a causal relationship between CL alterations, mitochondrial dysfunction, and overall prognosis in HF.

Recently, direct evidence for a pathological consequence of L_4CL deficiency and altered CL remodeling came from the discovery that the mechanism responsible for the X-linked cardioskeletal myopathy known as Barth syndrome (characterized by childhood onset of severe dilated cardiomyopathy) is a mutation of the tafazzin gene, which encodes proteins homologous to phospholipid acyl-transferases (57). Tafazzin mutations result in defective CL remodeling (26, 58) and a specific loss of L_4CL species (25) in a variety of tissues from Barth syndrome patients, which is believed to result in destabilization of the mitochondrial respiratory chain supercomplex and mitochondrial dysfunction (18–20). Recently, Xu et al. (59) demonstrated a tafazzin-associated CL transacylase activity whereby linoleoyl acyl chains are transferred from phosphatidylcholine to CL. Interestingly, the CL transacylation activity was ∼10-fold higher for 18:2 groups than for 18:1 groups and was nearly undetectable for 20:4 groups. To our knowledge, there are currently no published studies to determine whether alterations of tafazzin activity are responsible for altered CL remodeling in disease states such as HF.

Loss of L_4CL is not associated with aging in the absence of cardiac pathology

Several studies published in the last decade have indicated that the total amount of cardiac CL decreases with

aging, with reports ranging from a 23% to 37% loss of total cardiac CL in aged (24–30 months) Fisher 344 and Wistar rats (22, 30, 60, 61). To our knowledge, only one previous study (62) reported no age-related alterations in total CL content in cardiac mitochondria from Fisher 344 rats. Compositional changes in cardiac CL were recently reported by Lee et al. (28), who demonstrated a loss of linoleic acid content in cardiac CL from aged (24 months) CDF-344 rats paralleled by increases in arachidonic and docosahexaenoic acids. However, it is important to note that significant age-related cardiac pathology is well documented in Fisher 344 (39, 63) and Wistar (64, 65) rat strains by 23–26 months of age. The FBN rat exhibits only mild manifestations of cardiac aging through 30 months of age, with more pronounced age-related pathology becoming evident by 36 months (40, 41). In this study, we have shown that, unlike in the SHHF rats, the CL content and molecular species profile of the SD rat heart remains essentially unaltered from 4 to 24 months of age. The fraction of total CL containing L_4CL begins to decrease at 24 months in the SD rat and at 30 months in the FBN rat, in contrast to the early and steady decline of this ratio in the SHHF rat (Fig. 6). Our use of a highly sensitive electrospray ionization mass spectrometry method for the quantitative assessment of cardiac CL species in SD and FBN rats argues against a substantial effect of "non-pathological" aging on cardiac CL. Therefore, the conflicting results of this study with previous reports may be attributable to differences in the methods used to assess CL content and/or the presence of varying degrees of age-related cardiac pathology in the different rat strains.

To further distinguish the effect of cardiac overload from aging in the SHHF rat, the progression of cardiac hypertrophy and failure was dramatically accelerated in young SHHF rats exposed to TAB surgery followed by a high-sodium diet at 8 weeks of age (Fig. 7). Similar to aged SHHF rats in HF, TAB+HS in young SHHF rats led to a pronounced loss of cardiac L_4CL and an increase in linoleoyl-deficient CL species. These data provide additional support for the notion that a loss of L_4CL in the SHHF rat heart is associated with chronic cardiac pressure overload rather than a rat strain-specific aging effect.

In summary, this study indicates that a selective loss of cardiac L_4CL occurs in human and experimental HF but not with aging in the absence of cardiac pathology. We have demonstrated for the first time that a marked loss of L_4CL occurs in cardiac mitochondria early during the progression from hypertension to HF, which is closely associated with the pathologic increase in LV mass and decreased COx activity (with no change in the activity of citrate synthase, a matrix enzyme) in the SHHF rat heart. Decreases in L_4CL are paralleled by a decrease in the total mass of major CL species and increases in CL species containing alternate acyl chain configurations, suggesting that impaired CL biosynthesis, enhanced degradation, and/or aberrant acyl chain remodeling may contribute to the L_4CL deficiency. This progressive decline in cardiac L_4CL may lead to impaired mitochondrial energy metabolism and contribute to the complex etiology of HF.

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