Role of cardiolipin alterations in mitochondrial dysfunction and disease

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Chicco AJ, Sparagna GC. Role of cardiolipin alterations in mitochondrial dysfunction and disease. *Am J Physiol Cell Physiol* 292: C33–C44, 2007. First published August 9, 2006; doi:10.1152/ajpcell.00243.2006.—Cardiolipin (CL) is a structurally unique dimeric phospholipid localized in the inner mitochondrial membrane where it is required for optimal mitochondrial function. In addition to its role in maintaining membrane potential and architecture, CL is known to provide essential structural and functional support to several proteins involved in mitochondrial bioenergetics. A loss of CL content, alterations in its acyl chain composition, and/or CL peroxidation have been associated with mitochondrial dysfunction in multiple tissues in a variety of pathological conditions, including ischemia, hypothyroidism, aging, and heart failure. Recently, aberrations in CL metabolism have been implicated as a primary causative factor in the cardioskeletal myopathy known as Barth syndrome, underscoring an important role of CL in human health and disease. The purpose of this review is to provide an overview of evidence that has linked changes in the CL profile to mitochondrial dysfunction in various pathological conditions. In addition, a brief overview of CL function and biosynthesis, and a discussion of methods used to examine CL in biological tissues are provided.

phospholipid; metabolism; heart failure; aging; hypothyroidism; lipid peroxidation; oxidative stress; diet; ischemia

CARDIOLIPIN (CL) is a unique tetra-acyl phospholipid that was first isolated from beef heart in the early 1940s (108). In mammalian cells, CL is found almost exclusively in the inner mitochondrial membrane where it is essential for the optimal function of numerous enzymes that are involved in mitochondrial energy metabolism. Alterations in the content and/or structure of CL have been reported in several tissues in a variety of pathological settings. The purpose of this review is to provide an overview of evidence that has linked CL alterations to widely studied pathologies, and discuss the specific mechanisms and implications of these alterations on mitochondrial function. In addition, a brief overview of CL function and biosynthesis, and published methods for assessing changes in CL content and composition are provided.

CL STRUCTURE AND BIOSYNTHESIS

The biochemistry and metabolism of CL have been extensively reviewed elsewhere (44, 160); therefore only a brief overview is provided here. Unlike most membrane phospholipids that have a single glycerophosphate backbone and two fatty acyl side chains, CL has a double glycerophosphate backbone and four fatty acyl side chains. In eukaryotes, CL is biosynthesized from phosphatidylglycerol (PG) and cytidinediphosphate-diacylglycerol (CDP-DAG) by CL synthase (Fig. 1) on the inner face of the inner mitochondrial membrane (44, 154). The fatty acyl chain composition of CL is highly specific, being predominantly comprised of 18-carbon unsaturated acyl chains, the vast majority of which are linoleic acid (18:2) in most mammalian tissues (52, 159). 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 CL (L_4CL) is the most abundant species (158, 159). The enzymes involved in de novo PG and CL synthesis do not exhibit precise acyl-specificity (59, 149); however, preference for unsaturated fatty acids is indicated by evidence that saturated (dipalmitoyl) PG is a poor substrate for CL synthase in rat neonatal cardiomyocytes (104). The 18:2-rich composition of CL is thought to be achieved by an acyl chain remodeling process that is incompletely understood (44, 161).

Two enzyme-dependent CL remodeling pathways have been proposed to account for the 18:2-enrichment of CL in mammalian mitochondria (Fig. 1). One pathway involves a two-step deacylation-reacylation process (161) whereby nonspecific acyl chains are cleaved from CL, generating monolysocardiolipin [MLCL; $(acyl)_3$-CL], followed by the reacylation of MLCL by an acyl-CoA-dependent MLCL acyltransferase (MLCLAT) (84, 174). The specific enzyme(s) involved in CL deacylation have not been clearly identified, but the role of mitochondrial phospholipase A_2 (PLA_2) has been hypothesized due to abundant evidence of its ability to hydrolyze endogenous and exogenous CL (15, 24, 60). Taylor and Hatch recently purified and characterized a MLCLAT activity corresponding to a 74-kDa protein capable of acylating MLCL with 18:2-CoA in pig liver mitochondria (174). While this MLCLAT exhibited a 10-fold greater preference for unsaturated acyl-CoAs over saturated palmitoyl-CoA, it failed to demonstrate the 18:2-specificity expected to generate the characteristic 18:2-enrichment seen in liver CL (159). Xu et al. (187) demonstrated a CL remodeling pathway in rat liver mitochondria that involves the CoA-independent transfer of 18:2 acyl chains directly from phosphatidylcholine or phosphatidylethanolamine to $(acyl)_4$-CL. This transacylation reaction exhibits a clear specificity for 18:2 acyl chains and was shown to com-

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Fig. 1. Putative pathways of cardiolipin (CL) biosynthesis, remodeling, and degradation in mammalian cells. CDP-DAG, cytidinediphosphate-diacylglycerol; CDS, CDP-DAG synthase; CS, CL synthase; CMP, cytidinemonophosphate; CTP, cytidinetriphosphate; DLCL, dilyso-CL; FFA, free fatty acid; G3P, glycerol-3-phosphate; MLCL, monolyso-CL; MLCLAT, MLCL acyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PG-P, PG phosphate; PGPP, PG-P phosphatase; PGPS, PG-P synthase; P_i, inorganic phosphate; PLA$_2$, phospholipase A$_2$; TA, transacylase.
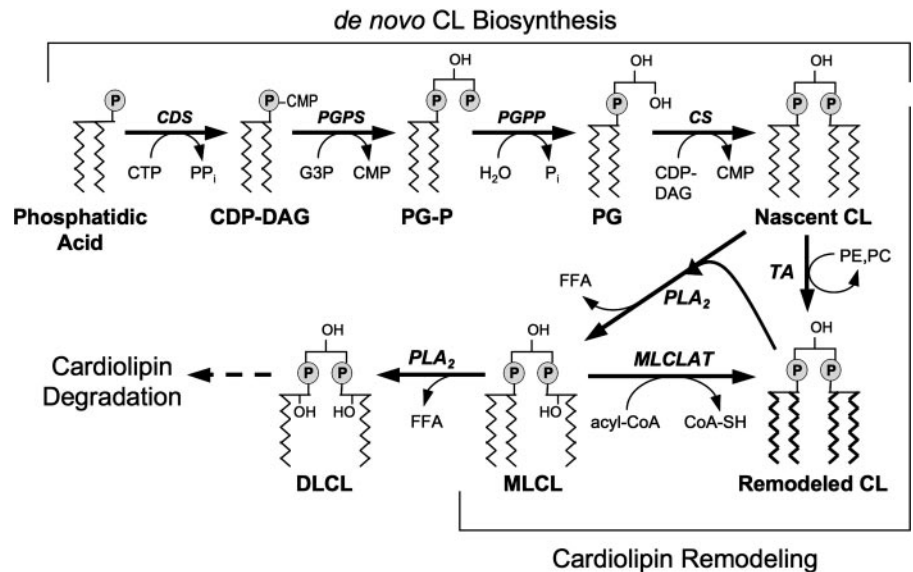
pletely remodel tetraoleoyl-CL to L$_4$CL. While the protein(s) responsible for this transacylase activity remain to be identified, the authors hypothesized that the putative phospholipid aclytransferase tafazzin (further discussed under *Barth Syndrome*) may be involved.

FUNCTIONAL ROLE OF CL

Numerous mitochondrial proteins and processes are known to require the presence of CL for their optimal function (Table 1). The functional importance of CL probably arises from its unique ability to interact with proteins and its role in maintaining inner membrane fluidity and osmotic stability (146, 160). CL is required for the proper structure and activity of several mitochondrial respiratory chain complexes involved in the oxidative generation of ATP (52, 91) and has been proposed to participate directly in proton conduction through cytochrome bc$_1$ (76) and prevent osmotic instability and uncoupling at

higher respiration rates (71). In addition to its role in mitochondrial bioenergetics, CL electrostatically anchors cytochrome *c* to the inner mitochondrial membrane (178) and may therefore play an important regulatory role in cytochrome *c* release (104, 105), which triggers the downstream events in apoptosis (89). CL also plays an essential role in mitochondrial biogenesis (160), and the assembly of respiratory enzyme supercomplexes (138, 192).

Importance of Acyl Composition

Despite knowledge of the highly specific acyl chain configuration of CL for >30 years (67, 140), the precise functional role of its unusual acyl composition is still incompletely understood. While the exact type of fatty acids found in CL varies between organisms and tissues, the major CL species in eukaryotes are comprised of a limited assortment of fatty acids, the vast majority of which contain 18-carbon unsaturated chains (159). Recent work by Schlame et al. (159) suggests that this highly restrictive fatty acid pattern creates a structural uniformity and molecular symmetry common across species, which has stereochemical implications that may be more important for its biological function than the specific type of fatty acid present. Given the focus of this review, attention has been placed primarily on the L$_4$CL species that predominates in healthy mammalian tissues affected by human pathologies. However, it is important to note that much of work that has examined the biochemistry and functional role of CL has been conducted in cell models such as H9c2 cardiac myoblasts and yeast (*Saccaromyces cerevisiae*), where other fatty acid patterns predominate. Therefore, the precise functional importance of the (18:2)$_4$ fatty acid pattern in mammalian cells remains relatively unexplored.

It has been proposed that the (18:2)$_4$ acyl chain configuration is an important structural requirement for the high affinity of CL to inner membrane proteins in mammalian mitochondria (155). However, studies with isolated enzyme preparations indicate that its importance may vary depending on the protein examined. Studies by Yamaoka et al. (188) indicated that rat cardiac mitochondrial oxygen consumption and the enzymatic

Table 1. *CL-dependent mitochondrial proteins and processes*

Protein/Function	Reference No.
Mitochondrial respiration	
State III (ADP dependent)	52
State IV (proton-selective leak)	53
Complex I (NADH:ubiquinone oxidoreductase)	32
Complex III (Ubiquinol:Cyt *c* oxidoreductase)	32, 34, 76
Complex IV (cytochrome *c* oxidase)	31, 146, 164, 190
Complex V (ATP synthase)	25, 151
Adenine nucleotide transporter	11
Mitochondrial creatine kinase	94
Carbamoyl phosphate synthetase I	14
Carnitine/acylcarnitine carrier	62, 99
Pyruvate transporter	98
Phosphate transporter	66
Proton "trapping" for oxidative phosphorylation	42
Respiratory supercomplex formation	191, 192
Mitochondrial GAPDH	9
Cardiolipin synthase	156
Anchoring cytochrome *c* to the IMM	105, 150, 178
Cytochrome P-450SCC	75, 131

CL, cardiolipin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IMM, inner mitochondrial membrane.

Table 2. *Treatments and pathologies associated with CL alterations and mitochondrial dysfunction*

Pathology/Tissue	Effect on CL	Mitochondrial Dysfunction Attributed to CL Alteration; Comments	Reference No.
Hypothyroidism			
Rat heart	↓ CL content	↓ CAT, ETC IV activity and state 3 respiration; restored with TH	121, 126
	↓ CL content	↓ ETC IV activity; restored with exogenous CL, not PE or PC	117
Rat liver	↓ CL content	↓ phosphate transport; reversed with TH treatment	120
Hyperthyroidism			
Rat heart	↑ CL content	↑ CAT, ETC IV activity, pyruvate transport and state 3 respiration	118, 127, 129
Mouse muscle	↑ CL content, ↑ DBI	↑ tissue lipid peroxidation	36
Ischemia-reperfusion			
Rat heart	↓ CL, ↑ PeroxCL	↓ ETC I, III activity, ↓ state III V_{O_2}; attenuated by ↓ ROS	112, 134, 135
	↓ CL, ↑ PeroxCL	↓ ETC I, III, IV activity; resorted with exogenous CL, not PE, PC or peroxCL	112, 113, 136
Rat brain	↓ CL content	↓ ETC IV and V activity, ↓ V_{O_2}, CL restored by carnitine pretreatment (185)	97, 185
Rat muscle	↓ CL content	↓ ATP, CP, ETC IV activity; ↓ CL and ATP attenuated by ascorbate (74)	74, 170
Ischemia			
Rabbit heart	↓ CL in SSM, not IFM	↓ cytochrome *c* content, ETC IV function; prevented by rotenone (78)	78–80
Rat liver	↓ CL content, ↑ LysoCL	NA	101
Rat muscle	↓ CL content	↓ ETC IV activity	170
Rat kidney	↓ CL content	NA; ↓ CL precedes loss of PC and PE	168
Oxidative stress			
Bovine heart	↓ CL content	↓ ETC I, II, IV activity; restored with exogenous CL, not peroxCL	114–116, 128
Rat brain	↓ CL content	Loss of mitochondrial Δψ and ↓ respiratory function; restored with BHT	165
Rat liver	↓ CL content	↓ Complex I, II, IV activity	106
Heart failure			
Rat heart	↓ 18:2 in CL	NA; cardiac hypertrophy and failure induced by aortic banding	103, 144
Human heart	↓ CL and L_4CL content	CL loss in ischemic and dilated CM; ↓ L_4CL in dilated CM	48
SHHF Rat heart	↓ L_4CL, ↑ minor CL species	↓ ETC IV activity (unpublished data)	171
Aging			
Rat heart	↓ CL content	↓ CAT, ANT, ETC IV activity, ↓ pyruvate carrier activity, ↓ phosphate carrier activity; all resorted with acyl-carnitine	111, 122–125
	↓ CL content	↓ ETC IV activity; resorted with exogenous CL, not PE, PC or peroxCL	125
	↓ 18:2, ↑ 20:4 in CL	NA	77, 82
Human, epidermal	↓ CL content	No effect on mitochondrial Δψ	86
Rat liver	↓ CL content	↓ mitochondrial V_{O_2} and Δψ; resorted with acyl-carnitine	41
Barth syndrome			
Human tissues	↓ CL, L_4CL content	Various respiratory chain defects, dilated cardiomyopathy, neutropenia	6, 7
18:2-deficient diet			
Rat heart	↓ L_4CL	↓ mitochondrial V_{O_2} and ETC IV activity, complex IV activity restored with L_4CL vesicles	188–190

ANT, adenine nucleotide transporter; BHT, butylated hydroxytoluene (inhibitor of lipid peroxidation); CAT, carnitine-acylcarnitine translocase; CM, cardiomyopathy; CP, creatine phosphate; DBI, double bond index; ETC, electron transport complex; NA, no mitochondrial dysfunction attributed to CL alteration; IFM, interfibrillar mitochondria; L_4CL, tetralinoleoyl CL; PC, phosphatidylcholine; PE, phosphatidylethanolamine; peroxCL, peroxidized CL; SHHF, spontaneously hypertensive heart failure; SSM, subsarcolemmal mitochondria; TH, thyroid hormone; V_{O_2}, oxygen consumption; 18:2, linoleic acid; 20:4, arachidonic acid; Δψ, membrane potential.

activity of cytochrome oxidase decreased as the quantity of L_4CL was reduced by varying the fatty acid composition of the diet. The same group later demonstrated that the activity of delipidated bovine cytochrome oxidase was effectively restored only by reconstitution with L_4CL-rich vesicles, whereas other CL molecular species were far less effective (190). However, despite the 18:2-rich acyl composition of CL tightly associated with the ADP/ATP carrier in vivo, reconstitution studies indicate that this specific acyl composition is not required for high affinity binding of CL to the protein (153). Therefore, while the abundance of the L_4CL species suggests some biological role, the importance of this acyl composition on the function of other CL-dependent proteins and processes in mammalian tissues requires further investigation.

PATHOLOGICAL ROLE OF CL ALTERATIONS

The importance of CL in mitochondrial function suggests that any disturbance of the CL profile may result in mitochondrial dysfunction. Indeed, alterations in the content and/or composition of CL have been well documented in a variety of pathological settings associated with mitochondrial dysfunction (Table 2). In

addition, the changes in CL discussed below have been shown to initiate the mitochondrial apoptotic cascade by resulting in a detachment of cytochrome *c* from the inner membrane (see Ref. 89 for review). Recently, it was discovered that the primary molecular manifestation of the lethal cardioskeletal myopathy known as Barth syndrome is an alteration in CL metabolism that results in a loss of CL content and defective CL remodeling (157, 163, 184). This discovery provides direct evidence for a pathological role of CL abnormalities in human disease and underscores the importance examining the mechanisms and functional importance of CL changes in health and disease.

There is evidence for at least three general categories of pathological alterations in the CL profile (Fig. 2): *1*) a loss of CL content, *2*) CL peroxidation, and *3*) a change in CL acyl chain composition leading to a loss of 18:2-rich species.

Loss of Content

A decrease in the mitochondrial content of CL is the most frequently reported pathological alteration of the CL profile (see Table 2). A selective loss of CL content in mitochondria could arise from enhanced CL degradation (e.g., hydrolysis by
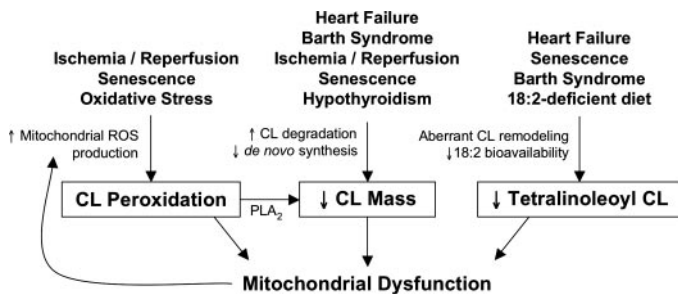
Fig. 2. CL alterations associated with various pathological conditions. ROS, reactive oxygen species.

endogenous phospholipases) (97, 101) or by decreased de novo CL synthesis resulting from impaired function of enzymes involved in CL synthesis (175) or decreased bioavailability of CL precursors (18, 102). However, in studies where CL is assessed in whole tissue homogenates, a loss of CL content may simply reflect a decrease in mitochondrial mass. It is impossible to know whether there is an isolated effect on mitochondrial CL content in such studies unless measures were taken to control for variations in mitochondrial density.

Peroxidation

Mitochondrial oxidative stress and lipid peroxidation have been associated with a wide variety of diseases and disorders, including most of those listed in Table 2 (see Refs. 27 and 29 for review). CL may be particularly susceptible to peroxidation because of the abundance of double bonds in its structure (100), and its close association with respiratory chain proteins, which are known to be a major source of reactive oxygen species (ROS) in mitochondria (116, 137). Several studies indicate that peroxidized CL is unable to support the reconstituted activity of mitochondrial respiratory enzymes (95, 112–116, 125, 128). Furthermore, CL peroxidation may lead to an overall loss of detectable CL content, either by preferential hydrolysis of peroxidized acyl chains by PLA_2 (88), direct decomposition of lipid peroxides (100), or the formation of CL-protein complexes that would no longer be detected as phospholipids (176). Evidence for the role of oxidative stress in a loss of CL comes from studies where CL content was preserved by reducing ROS in ischemic heart and skeletal muscle (74, 78, 134, 135).

Changes in Composition

A pathological consequence of CL compositional changes has been primarily confined to a loss of the L_4 species in the heart, where it is generated by the acyl chain remodeling pathway illustrated in Fig. 1. Aberrant CL remodeling may result from a decreased bioavailability of 18:2 or alterations in the activity or acyl-specificity of putative CL remodeling enzymes. The acyl composition of CL is known to be sensitive to FA composition of the diet (reviewed in Ref. 10), and restricting the dietary intake of 18:2, an essential fatty acid, results in a loss of L_4CL and mitochondrial respiratory dysfunction in the heart (189). Decreases in L_4CL or the 18:2 content of CL have been reported in the aged (77) and failing rat heart (171) despite sufficient provision of 18:2 in the diet, suggesting a loss of endogenous 18:2 available for CL remodeling or defects in the remodeling pathway. The cardioskeletal

myopathy known as Barth syndrome (discussed below) was recently found to be caused by a mutation in the tafazzin (*TAZ*) gene, which results in aberrant CL remodeling (157, 184) and a specific loss of L_4CL species (163). The precise role and identity of *TAZ* gene products involved in CL metabolism remain unidentified, thus any role *TAZ* defects may play in other pathologies associated with altered CL composition awaits future investigation.

Methodological Considerations

When determining the specific role of CL alterations in disease, it is essential that the preparation and type of tissue examined be considered. As mentioned above, a loss of CL content in whole tissue homogenates may simply reflect a loss of mitochondrial density and not a selective depletion of mitochondrial CL content. Indeed, tissue CL content has been recently used as a measure of tissue mitochondrial mass (69). Moreover, structural changes in CL (e.g., peroxidation or altered acyl patterns) may coincide with global changes in the cellular phospholipid pool, the consequences of which could be erroneously attributed to a selective alteration in CL. Therefore, assessment of CL content and composition should be conducted in mitochondrial fractions of consistent purity, and ideally be distinguished from changes occurring in other mitochondrial phospholipid species.

Given the abundance of studies that have examined CL in the heart (Table 2), it is important to note that cardiac myocytes have two functionally distinct populations of mitochondria, the subsarcolemmal, located beneath the plasma membrane and interfibrillar, located between the myofibrils (107). There is evidence that these populations are differentially affected by pathological conditions such as aging (56), ischemia (80) and cardiomyopathy (57). Isolation of mitochondria from polytron homogenized tissues using standard differential centrifugation methods will yield primarily subsarcolemmal mitochondria, whereas the use of proteases during the isolation will likely yield a mixed population. Therefore, distinguishing between these two populations may avoid discrepant results and provide a more thorough examination of the effect and consequences of CL changes in pathological states.

ROLE OF CL IN SPECIFIC PATHOLOGIES

Barth Syndrome

Barth syndrome is an X-linked cardioskeletal myopathy characterized by infantile or childhood onset of dilated cardiomyopathy and neutropenia associated with mitochondrial respiratory chain dysfunction (6, 7) and high mortality rates (5). Barth syndrome is believed to result from a mutation in the *TAZ* gene, which encodes proteins homologous to phospholipid acyltransferases (12). The *TAZ* gene product, while still unidentified, is believed to be involved in CL remodeling, as the *TAZ* mutation in Barth syndrome is associated with marked alterations in CL metabolism (reviewed in Ref. 47). Barth syndrome patients exhibit a loss of CL content (157), impaired incorporation of 18:2 into CL (184), and a specific loss of L_4CL (163) in a variety of tissues. Incubation of fibroblasts from Barth syndrome patients with 18:2 led to a dose- and time-dependent increase of CL levels (180), but the effect of this treatment on mitochondrial function and overall prognosis awaits further investigation.

Recent studies in *taz*1Δ mutant yeast indicate that inactivation of *Taz*1 protein, an orthologue of human *TAZ*, results in a decrease in total CL content, an accumulation of MLCL, and a loss of unsaturated acyl chains in CL (13, 85). These findings are consistent with the notion that *TAZ* is at least partially responsible for the remodeling and reacylation of CL into its characteristic acyl pattern which, in yeast, is represented by species containing oleoyl (18:1) and palmitoleoyl (16:1) acyl chains. *Taz*1Δ mutant yeast mitochondria also exhibit defective energetic coupling (13) and destabilization of respiratory chain complexes (85), further supporting the role of aberrant CL remodeling in mitochondrial dysfunction.

Ischemia and Reperfusion

A loss of CL in the heart following ischemia and reperfusion (I/R) has been known since the early 1980s (177, 181), and has since been well documented in a variety of tissues (Table 1). While widespread phospholipid damage and hydrolysis are well known to occur during I/R (23), CL losses appear to precede and exceed losses in other phospholipids (80, 113). Interestingly, the CL loss following myocardial I/R appears to be limited to subsarcolemmal mitochondria, while interfibrillar mitochondria are largely unaffected (80). Studies by Lesnefsky et al. (78, 79) indicate that a 20–25% loss of CL results from ischemia in isolated rabbit hearts, with no further loss during reperfusion, whereas others have reported additional losses of CL during reperfusion in the isolated rat heart (112, 113) and brain (97). No change in the 18:2 content of the CL fraction, or in the levels of major CL molecular species seem to occur with I/R (79, 80).

Several studies have provided a strong association between CL loss and decreased maximal activity of mitochondrial respiratory enzymes (see Table 2). A causal role of CL loss, while it is difficult to establish unequivocally, is supported by the finding that the associated loss of cytochrome oxidase activity occurs despite no change in enzyme protein content (113). A series of studies by Paradies et al. (112, 113, 136) have demonstrated that reconstitution of delipidated respiratory proteins with exogenous CL liposomes restores activity to preischemia levels, while phosphatidylcholine, phosphatidylethanolamine, and peroxidized CL liposomes were ineffective.

Prevailing hypotheses suggest that the loss of CL following I/R results from oxidative injury by ROS generated from the mitochondrial respiratory chain, which may render the phospholipid more susceptible to degradation by PLA$_2$ or otherwise result in undetectable CL (88, 100, 176). The loss of CL following I/R has been attenuated by quenching (74) or preventing the production of mitochondrial ROS (112, 134, 135) in skeletal muscle and heart. Increased levels of peroxidized CL have been reported in the heart following I/R in some (112, 134, 135), but not all studies (79), possibly due to the unstable nature of peroxidized acyl chains and methodological variations.

ROS and CL peroxidation may lead to PLA$_2$-mediated CL hydrolysis in I/R, given evidence that PLA$_2$ may preferentially hydrolyze peroxidized phospholipids over nonoxidized lipids (88), and the mitochondrial PLA$_2$ isoform may be activated by ROS during I/R (1). Phospholipid hydrolysis by PLA$_2$ is known to be involved in a global phospholipid loss following I/R (see Ref. 23 for a review), and has been implicated in the CL loss observed following I/R in the heart (177), brain (1, 97), and liver (101). However, while PLA$_2$ enzymes are capable of hydrolyzing and degrading CL in vitro (50), exactly which PLA$_2$(s) are involved in CL hydrolysis during I/R, or its preference for peroxidized CL, is unknown.

Finally, an inhibition of de novo CL biosynthesis may contribute to CL loss during prolonged I/R. Cheng and Hatch(18) indicated that CDP-DAG formation was attenuated during hypoxia in the isolated rat heart, possibly due to a depletion of ATP and CTP, the latter of which is known to be required for de novo CL synthesis (46).

Aging

A loss of CL content has been hypothesized to contribute to the age-related decline in mitochondrial function reported in a variety of tissues (4). While the majority of aging studies have focused on the heart (see Table 1), decreased CL content has also been observed in rat hepatocytes (41), rat brain mitochondria (147), guinea pig kidney (49), and in human epidermal cells (86). Studies from two laboratories have reported a selective loss of CL content in isolated rat cardiac mitochondria (111, 132). However, Moghaddas et al. (92) reported no age-related decline in CL levels in either population of rat heart mitochondria, which is supported by recent work in our laboratory indicating that mitochondrial CL levels are maintained in the heart throughout a 30-mo lifespan in the Fisher Brown Norway rat (unpublished data), a rat known to age in the absence of cardiac pathology (83). These disparate findings may be due to differences in the methods used to assess CL content and/or the presence of varying degrees of age-related cardiac pathology in different rat strains.

Oxidative injury of mitochondria is widely believed to play an important role in the mitochondrial decay and dysfunction seen in aging (3, 4, 166), and may contribute to an age-associated decline in CL (52). Recently, a strong correlation was found between CL losses and the age-related increase in hydrogen peroxide in rat heart mitochondria, both of which were abolished by chronic L-carnitine and α-lipoic acid supplementation (152). Alterations in CL composition, favoring a loss of 18:2 and an increase in longer chain polyunsaturated acyl chains, have also been reported in the aged rat heart (77, 82) and liver (73), leading to an increased unsaturation index in the CL fraction. These changes would theoretically render CL more susceptible to peroxidation by ROS, which may promote CL degradation or hydrolysis by PLA$_2$ (discussed above). A role of PLA$_2$-mediated CL hydrolysis in aging has recently been suggested by the increased levels of MLCL in the aged guinea pig kidney (49).

In a series of studies by Paradies et al., an age-dependent loss of cardiac mitochondrial CL was closely associated with decreased activity of the mitochondrial phosphate transporter (122), pyruvate carrier (111), adenine nucleotide transporter (124), cytochrome oxidase (124), and the carnitine-acylcarnitine transporter (123). In each of these studies, CL levels and enzyme activities were restored to those of young rats by acyl-carnitine supplementation. These authors suggested that acyl-carnitine increased the function of these CL-dependent proteins by restoring levels of CL, as no changes in enzyme protein were observed. Interestingly, acyl-carnitine had no

effect on CL levels in young or mature rats, suggesting that its effect is limited to preservation of "normal" CL levels, rather than directly stimulating CL biosynthesis. The precise mechanisms by which acyl-carnitine restores CL content in the aged heart are unknown, but may involve improved mitochondrial fatty acid import and a preservation of cellular high energy phosphate content (166).

Thyroid Status

Thyroid hormone is a major regulator of mitochondrial biogenesis, respiratory function and lipid metabolism (54), and has been shown to directly modulate CL content by influencing the activity of CL biosynthesis enzymes. Hyperthyroidism induced by chronic thyroxin treatment evokes increases in mitochondrial CL content in the rat heart (16, 118, 129) and liver (119), and increases the activity of multiple CL-dependent mitochondrial proteins and processes (see Table 2). Thyroxin induces de novo CL synthesis by increasing the activities of CL synthase and PG phosphate synthase (16, 58), and promotes CL remodeling via increasing MLCLAT activity (96). Conversely, hypothyroidism induced by chronic 6-*n*-propyl-uracil (PTU) treatment decreases the activities of CL synthase and MLCLAT (175), and results in a marked loss of CL content and CL-dependent protein function (Table 2). PTU-induced decrements in CL content and protein function in rat heart mitochondria are completely restored by treating animals with thyroid hormone (126), further establishing the role of thyroxin on CL metabolism and mitochondrial function. The specific role of CL loss in the PTU-induced decrease in complex IV activity was demonstrated in a study by Paradies el al. (117), where enzyme activity was completely restored by reconstituting hypothyroid cardiac mitochondria with exogenous CL liposomes.

Despite its effect on MLCLAT activity and CL remodeling, the effect of thyroid hormone on CL composition is not entirely clear. While two early studies reported a modest decrease in the 18:2/20:4 ratio of the total mitochondrial phospholipid fraction of hyperthyroid rat heart and liver (118, 148), subsequent studies by the same group indicated that no significant alterations in the fatty acid composition of CL occur in the heart following hyperthyroidism (127) or hypothyroidism (121, 126, 130). More recently, Gredilla et al. (36) found that hyperthyroidism induced a marked loss of 18:2 in mouse skeletal muscle, while levels of highly unsaturated fatty acids (20:4 and 22:6) were markedly increased. The authors noted that these changes resulted in a 27% increase in the CL double bond index, which equated to a 266% increase in the CL peroxidizibility index. CL peroxidation was not examined in this study, however, hyperthyroidism significantly increased skeletal muscle lipid peroxidation, which the authors ascribed to increased content of highly unsaturated CL. PTU treatment significantly increased 18:2 content in CL, but reduced the double bond index by decreasing levels of 18:3 and 18:4.

Heart Failure

Alterations in mitochondrial energy metabolism have been widely observed in human and animal models of heart failure (HF) and are hypothesized to play an important role in the development and/or progression of the disease (63, 182). Early evidence for a role of CL alterations in HF came from O'Rourke and Reibel (103, 144), who reported a reduction in the 18:2 content of CL fractions isolated from rat hearts induced to rapid pressure-overload hypertrophy and failure by chronic aortic banding. A loss of cardiac CL content was reported during the progression of HF in cardiomyopathic hamsters, which the authors ascribed to a loss of cardiac diacylglycerol content and impaired phospholipid biosynthesis (102). Recently, a loss of cardiac CL content was found in failing hearts from patients with dilated or ischemic cardiomyopathies (48). While Heerdt et al. (48) did not rule out the effect of decreased mitochondrial density on CL content, they did report changes in the relative quantities of CL species in ischemic HF, which were further modified by support with a left ventricular assist device.

Recent data from our laboratory indicate that progressive changes in cardiac CL composition occur in both interfibrillar and subsarcolemmal mitochondria during the pathogenesis of HF in spontaneously hypertensive HF rats. These changes are characterized by a marked loss of the L_4 species and an increase in minor species containing highly unsaturated acyl chains, e.g., 20:4 and 22:6, with a loss of CL mass occurring only in interfibrillar mitochondria (Ref. 171; Chicco and Sparagna). Decreases in L_4CL preceded the development of HF in the spontaneously hypertensive HF rats by several months and correlated closely ($r = 0.8 - 0.9$) with a loss of cytochrome oxidase activity in both populations of cardiac mitochondria, despite no change in enzyme protein expression.

The mechanism(s) of CL alterations in the hypertrophied and failing heart are unknown, and are currently being explored in our laboratory. Prior studies indicate that CL compositional changes following aortic banding are unaffected by α- or β-adrenergic blockade (103), arguing against a role of chronic adrenergic stimulation. In fact, CL content was elevated relative to other phospholipids in spontaneously hypertensive rat hearts (19), and isoproterenol has been shown to increase CDP-DAG synthase activity (183), suggesting that heightened sympathetic drive and hypertension may actually increase CL synthesis.

Neurodegenerative Disease

Oxidative stress and lipid peroxidation are believed to be important contributing factors leading to neuronal loss and mitochondrial dysfunction in the substantia nigra in Parkinson's disease (8, 65), and may play an early role in the pathogenesis of Alzheimer's disease (55). Decreases in CL have been reported in the brain with aging (147), and have recently been shown to result from lipid peroxidation in rat brain mitochondria exposed to free radical stress (165). While there appears to be no change in the content or composition of brain CL in patients with Alzheimer's disease (38), a recent study by Ellis et al. (26) provides intriguing evidence for a potential role of CL alterations in Parkinson's disease. Mutations in the gene coding for the presynaptic protein α-synuclein have been implicated in Parkinson's disease. Ellis et al. found that mice lacking α-synuclein exhibited a 22% reduction in CL mass in the brain, a 25% reduction in CL n-6 polyunsaturated fatty acids (including 18:2), and a 51% increase in saturated fatty acids in CL. There was also a 23% reduction in PG, a CL precursor (Fig. 1), without any changes in the content of other brain phospholipids or mitochondrial density, suggesting that

the CL biosynthesis pathway may be selectively impaired. These alterations were associated with a 15% reduction in linked complex I/III activity of the electron transport chain, which is thought to be a critical factor in the development of Parkinson's disease (21).

Dietary 18:2 Deficiency

Manipulating the fatty acid composition of diet is known to modify CL composition in heart (190), brain (87), liver (141) and kidney (186), and has been reviewed elsewhere (10). Incorporation of dietary 18:2 into liver CL occurs within 4 h of feeding in the rat (51). Since 18:2 is an "essential" fatty acid, sufficient quantities must be consumed in the diet for the 18:2 enrichment of CL to occur. Studies by Yamaoka et al. (190) demonstrated that 30 days of an 18:2-deficient diet significantly reduces L_4CL content and mitochondrial oxygen consumption in the rat heart (188, 189). In addition to providing 18:2-CoA for CL remodeling by MLCLAT (Fig. 1), 18:2 supplementation may enrich CL precursors, as indicated by restoration of L_4CL in cultured fibroblasts from Barth syndrome patients, where CL remodeling is believed to be defective (180). Besides underscoring the importance of sufficient 18:2 dietary intake, these studies indicate that altering CL composition by manipulating dietary fatty acid composition may represent an efficient method for assessing the role of CL composition on other CL-dependent mitochondrial proteins and processes.

Chronic Ethanol Consumption

Chronic ethanol (EtOH) consumption has been shown by several investigators to decrease the 18:2 content of CL, and lead to a more saturated cardiolipin profile in liver mitochondria (17, 20, 173). These authors proposed that these alterations may render the mitochondrial membrane resistant to disordering by ethanol. The mechanism(s) by which EtOH alters CL composition are not known, but may involve defective remodeling of nascent CL or decreased 18:2 bioavailability for CL remodeling resulting from EtOH-induced changes in the activities of microsomal fatty acid desaturases (20). Elucidating the precise effects of EtOH consumption on CL metabolism and the consequences of EtOH-induced CL saturation on mitochondrial enzyme function requires further investigation.

Diabetes

Diabetes mellitus is a common metabolic disease characterized by insulin deficiency (type 1) or insensitivity (type 2), resulting in chronic hyperglycemia, altered substrate utilization, and a variety of systemic complications, including neuropathy and cardiomyopathy. To our knowledge, CL has not been examined in any tissues from diabetic humans, and studies that have examined CL in animal models of diabetes have yielded conflicting results. In streptozotocin-treated rats (a model of type 1 diabetes), mitochondrial CL content has been reported to decrease in the rat brain (93), increase in the rat liver (30), or remain unaltered in the rat heart (39, 45, 175). Taylor et al. (175) found no effect of streptozotocin-induced diabetes or hyperinsulinemia on cardiac CL content or the activities of CL synthase and MLCLAT in the rat heart. Therefore, while it appears that diabetes may have no effect on CL content and metabolism in the rat heart, further study is

needed to clarify the role, if any, CL changes might play in other organs affected by diabetes.

METHODS OF SEPARATION AND DETECTION OF CL SPECIES

Mary Pangborn discovered CL in 1941 using serum from syphilis patients to bind beef heart CL (109). She went on to develop a method for the isolation of CL from beef heart, parts of which are still used today (110). Several techniques have since been developed to separate CL from other phospholipids and, more recently, to separate and quantify the various molecular subspecies of CL. These techniques generally take advantage of four unique properties of CL: *1*) it is the most acidic of mitochondrial phospholipids, *2*) it is the only anionic phospholipid in significant quantities in the inner mitochondrial membrane, *3*) its mass is double that of other phospholipids, and *4*) it mostly has at least one 18:2 acyl side chain. Nevertheless, separation and quantitation of CL has been challenging. Unless heart tissue is used or mitochondria are isolated, the proportion of CL is small compared with the quantities of other phospholipids. Another problem is that, with the exception of mass spectrometry, phospholipids are difficult to detect online due to their lack of conjugated double bonds and unreactive aliphatic functional groups (143). For that reason, detection of CL often involves modification by attachment of a chromophoric group or separation with offline assessment of phosphate groups.

Separation of CL from Other Phospholipids

The first popular method employed for separation of CL from other phospholipid classes was thin-layer chromatography (TLC) on silica plates. TLC is an effective and inexpensive method still employed today. Various improvements to one dimensional TLC were made, including the use of two-dimensional TLC for assessing CL content (139). Iatroscan TLC complexed to flame ionization detection enabled researchers to automate phospholipid detection (22), and various sprays were developed for the quantitation of peroxidized phospholipids (70, 72). Methods of separation and quantitation of phospholipids have been developed using ^{31}P-nuclear magnetic resonance (NMR) (90, 169) or high-field ^1H-NMR (2). The authors claim NMR is just as effective as chromatographic methods in quantitation of phospholipids; however, it does not allow physical separation for further analysis of phospholipid classes. High pressure liquid chromatography (HPLC) has been widely used for separation of phospholipid classes, usually in the normal phase using silica based columns and hexane-alcohol mixtures with small amounts of acids or bases as the mobile phase (35, 37). Reverse-phase HPLC can then be used to separate out the lysophospholipids and different molecular species (81). Smaal (167) published an HPLC-based method intended for large scale isolation of CL. Other chromatographic techniques such as solid phase extraction (172) and capillary electrophoresis (40) have also been used to separate phospholipid classes.

The fluorescent mitochondrial indicator, nonyl acridine orange (NAO) was introduced in 1982 (28), and was later found to target mitochondria by binding to CL (133). Several studies were subsequently published utilizing NAO both as a quantitative mitochondrial indicator and an indicator of CL content in

mitochondria. Three studies were then published that all concluded that NAO was influenced by membrane potential and/or the spatial arrangement of CL (33, 64, 68), rendering it useless for the quantitation of CL or mitochondria in studies of intact respiring mitochondria. However, in studies where membrane potential or spatial arrangement is not an issue, such as in capillary electrophoresis of mitochondrial membrane extracts (143), NAO still represents a simple method of assessing CL content.

Separation of CL Subspecies

Given accumulating evidence that compositional changes in the CL profile (e.g., peroxidation and aberrant acyl chain remodeling) may play an important role in mitochondrial dysfunction and disease, it is has become increasingly important to examine individual CL subspecies. The most powerful and sensitive tool available for determination of the composition of CL is electrospray ionization-mass spectrometry (ESI-MS) and similar mass spectrometric methods discussed below (see Ref. 142 for review). However, ESI-MS requires specialized and expensive equipment, leading two laboratories to develop methods for observing CL acyl chain composition in an alternate manner. Schlame's group has developed two methods for analysis of CL subspecies without mass spectrometry. Their early method (158) employed head group and phosphate labeling using methylation and benzoylation of the hydroxyl group which facilitated separation by reverse phase HPLC and detection by UV absorbance. CL was isolated and derivitized, after which it was run though a reverse phase column. Eleven different subspecies of CL were identified by gas chromatography, but could be identified by retention time (assuming the appropriate standards were available) since there was a linear relationship between the number of acyl double bonds and the retention time. Schlame developed another method which requires very little starting material. The technique, using methylated and fluorescently labeled CL, is a time-consuming, but inexpensive, alternative to ESI mass spectrometry (162). In an attempt to simplify Schlame's techniques, Kelley's laboratory recently developed an HPLC-based method where the derivitization was done in one step and direct detection could be accomplished with fluorescent HPLC (145). These last two methods can easily detect CL species containing linoleate and oleate groups, but are much less useful for detecting other CL molecular species.

Recently, several articles have been published on the use of ESI-MS for CL analysis. Valianpour et al. (179) and Sparagna et al. (171) used product ion analysis of doubly charged or singly charged CL, respectively, to identify and quantify CL molecular species using normal phase HPLC coupled to ESI-MS. Both of these methods use an internal standard [(14:0)$_4$CL] to quantify the amount of CL present. Hsu et al. (61) took it a step further by analysis of the configuration of regioisomers of CL using both triple quadrupole and quadrupole ion trap mass spectrometry. Perhaps the most elegant, yet complex, study of this type is one in which "shotgun lipidomics" is used to directly assess all the CL molecular species in three tissue types (some having as many as 40 different species) without using any type of chromatographic separation (43). This method utilizes properties of CL such as linoleic acid content and its large size to screen extracts for the presence of CL molecular species.

SUMMARY

In summary, changes in the content and structure of CL occur in a variety of pathologies and have been implicated in mitochondrial dysfunction in several tissues. The loss of CL and associated mitochondrial protein function in the aged, hypothyroid, and ischemic rat heart have been effectively restored by reconstituting mitochondria with exogenous CL liposomes in vitro, providing evidence for an important role of CL loss in the mitochondrial dysfunction in these pathologies. Treatment with thyroxin, acyl-carnitine and antioxidant therapy in vivo restore CL levels and CL-dependent protein function in several tissues in the same pathologies, but may affect numerous other cellular functions, making it difficult to isolate the independent therapeutic benefits of CL restoration. Evidence that aberrant CL metabolism is the primary mechanism responsible for Barth syndrome indicates that changes in CL content and composition alone may have serious pathological consequences, particularly in the heart. Highly sensitive methods for analyzing the CL profile have recently been developed that will allow investigators to assess CL compositional changes in unprecedented detail. Future studies should be conducted to further elucidate the importance of CL acyl chain composition and configuration on the function of CL-dependent proteins and processes in health and disease.

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