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THE FUNCTIONS OF CARDIOLIPIN IN CELLULAR METABOLISM – POTENTIAL MODIFIERS OF THE BARTH SYNDROME PHENOTYPE

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Abstract

The phospholipid cardiolipin (CL) plays a role in many cellular functions and signaling pathways both inside and outside of mitochondria. This review focuses on the role of CL in energy metabolism. Many reactions of electron transport and oxidative phosphorylation, the transport of metabolites required for these processes, and the stabilization of electron transport chain supercomplexes require CL. Recent studies indicate that CL is required for the synthesis of iron-sulfur (Fe-S) co-factors, which are essential for numerous metabolic pathways. Activation of carnitine shuttle enzymes that are required for fatty acid metabolism is CL dependent. The presence of substantial amounts of CL in the peroxisomal membrane suggests that CL may be required for peroxisomal functions. Understanding the role of CL in energy metabolism may identify physiological modifiers that exacerbate the loss of CL and underlie the variation in symptoms observed in Barth syndrome, a genetic disorder of CL metabolism.

1. Introduction

Cardiolipin (CL) (1,3-diphosphatidyl-sn-glycerol) is the signature phospholipid of the mitochondrial membrane. First isolated from beef heart (Pangborn, 1942), it is ubiquitous in eukaryotes and also present in prokaryotes. Studies in yeast utilizing well-characterized deletion mutants of CL synthesis (Fig. 1) indicate that CL regulates many cellular functions and signaling pathways, both inside and outside of the mitochondria. The ubiquitous association of CL with energy transducing membranes is consistent with the role of this lipid in bioenergetics (reviewed by Joshi et al., 2009). In fact, CL synthesis and mitochondrial bioenergetics are inter-dependent, as CL synthesis is both required for and stimulated by oxidative phosphorylation (Gohil et al., 2004). Within the mitochondria, the effects of CL deficiency extend beyond bioenergetics to decreased mitochondrial protein import and perturbation of mitochondrial fusion (Jiang et al., 2000; Gebert et al., 2009; Joshi et al., 2012). The deleterious effects of CL deficiency outside the mitochondria include perturbation of the PKC-Slt2 cell integrity and high osmolarity glycerol (HOG) signaling

pathways and decreased vacuolar function (Zhong et al., 2005; Zhong et al., 2007; Chen et al., 2008b; Zhou et al., 2009). Perturbation of CL synthesis has long been associated with aging (Paradies et al., 2010), and loss of CL was found to decrease longevity in yeast cells (Zhou et al., 2009). The significance of CL in human health is apparent from clinical findings that perturbation of CL metabolism leads to the life-threatening disorder known as Barth syndrome (BTHS).

In addition to the cellular functions listed above, recent studies indicate that CL is intricately involved in cellular metabolism (Fig. 2). These studies are the focus of the current review. CL interacts with components of the electron transport chain and is required for stabilization of electron transport chain supercomplexes and for optimal respiratory control (Bazan et al., 2013; Pfeiffer et al., 2003; Zhang et al., 2002; Zhang et al., 2005). Perturbation of iron-sulfur (Fe-S) biogenesis has been reported in CL deficient yeast cells, suggesting that iron homeostasis as well as enzymatic activities requiring Fe-S cofactors are dependent on CL biosynthesis (Patil et al., 2013). CL is also required for activities of carrier proteins that transport metabolites for energy metabolism (Kadenbach et al., 1982; Fiermonte et al., 1998; Sedlak et al., 1999; Lange et al., 2001; Hoffmann et al., 1994; Jiang et al., 2000; Bisaccia and Palmieri, 1984), as well as for enzymes in the carnitine shuttle (Pande et al., 1986; Noel and Pande, 1986). In addition, CL might also be required for cellular metabolism outside mitochondria. CL is present in the membrane of peroxisomes (Zinser et al., 1991) and may affect β-oxidation and other metabolic activities of this organelle. The role of CL in mitochondrial protein import is discussed as a potential mechanism underlying the metabolic defects associated with CL deficiency. We speculate that defects in these functions may be physiological modifiers that account for the wide disparity of clinical phenotypes observed in BTHS, and conclude with a discussion of important unanswered questions that are exciting directions for future research.

2. CL and mitochondrial bioenergetics

CL is enriched in the membranes of bacteria, mitochondria, and hydrogenosomes, which play a role in ATP synthesis through the generation of a transmembrane electrochemical gradient (Daum et al., 1985; Dowhan et al., 1997; de Andrade Rosa et al., 2006). The association of CL with energy transducing membranes is consistent with the crucial role of this lipid in cellular bioenergetics (reviewed by Schlame et al., 2000; Hoch, 1992). The physical interaction between CL and mitochondrial respiratory chain complexes and other components of membranes also helps in the formation of a lipid scaffold, which functions to stabilize, tether, and increase the enzyme activity of interacting proteins (Beyer et al., 1985; Beyer et al., 1996; Sedlak et al., 1999). In this light, it is not surprising that perturbation of CL synthesis affects the structure and function of mitochondrial respiratory chain complexes and transporters.

2.1 CL and respiration

Analyses of CL function in yeast have been facilitated by the availability of yeast mutants of each step in CL synthesis (Fig. 1). In particular, the CL synthase null mutant *crd1*Δ, which lacks CL (Jiang et al., 1997; Tuller et al., 1998; Chang et al., 1998), has been the focus of numerous studies. Although *crd1*Δ cells can grow in non-fermentable carbon sources,

indicating that CL is not essential for respiration, the ADP/O and respiratory control ratios (RCR) of *crd1*Δ mitochondria are reduced in these conditions (Koshkin et al., 2002). CL is required for optimal RCR and ADP/O ratios and for maintenance of the mitochondrial membrane potential (Jiang et al., 2000; Claypool et al., 2008), especially during unfavorable conditions such as increased temperature and osmolarity (Koshkin et al., 2002; Koshkin et al., 2000). The role of CL in respiration has been recently reviewed (Joshi et al., 2009; Patil and Greenberg, 2013).

2.2 CL is required for stabilization of supercomplexes

The electron transport chain complexes are organized into supramolecular structures referred to as supercomplexes (Schagger and Pfeiffer, 2000). *S. cerevisiae* lacks complex I (NADH complex) but contains NADH dehydrogenase composed of a single subunit (Ndi1). Yeast supercomplexes are formed by the association of two units of complex III with units of complex IV. Supercomplexes in mammalian cells are formed by the association of complex I with two units of complex III and multiple units of complex IV (Schagger, 2002). The proposed role of supercomplexes is that of efficient substrate channeling between the individual complexes. The *crd1*Δ mutant exhibits destabilization of the supercomplexes (Pfeiffer et al., 2003; Zhang et al., 2002; Zhang et al., 2005). Bazan and co-workers reported the in vitro reconstitution of supercomplexes and showed that supercomplex III_2IV_2 reconstitution is dependent on CL (Bazan et al., 2013). The loss of CL decreases activity of ADP/ATP carrier protein activity and its association with the supercomplexes (Claypool et al., 2008; Jiang et al., 2000). Destabilization of supercomplexes was also reported in tafazzin-deficient human fibroblasts (McKenzie et al., 2006) and, more recently, in tafazzin-deficient induced pluripotent stem cells (Dudek et al., 2013).

2.3 Loss of CL leads to increased generation of reactive oxygen species (ROS)

Destabilization of supercomplexes is expected to result in increased electron leakage and ROS production (Maranzana et al., 2013). Not surprisingly, the absence of CL in yeast cells leads to increased protein carbonylation, a hallmark of increased ROS (Chen et al., 2008a). The primary sites of ROS generation are complexes I and III (Turrens et al., 1985; Barja, 1999; Kushnareva et al., 2002; Grivennikova and Vinogradov, 2006). The CL acyl chains, which are in close proximity to these ROS generating sites, are susceptible to peroxidation (Li et al., 2010; Li et al., 2012; Liu et al., 2012). The superoxides generated by respiratory complex III were shown to cause peroxidation of CL and to reduce the activity of cytochrome c oxidase (Paradies et al., 1998; Paradies et al., 2000; Paradies et al., 2001). The exogenous supplementation of CL, but not peroxidized CL or other phospholipids, rescued both reduced activity of cytochrome c oxidase and increased generation of ROS in reperfused heart (Paradies et al., 2001; Petrosillo et al., 2007). As CL is extensively remodeled by the transacylase tafazzin (Malhotra et al., 2009), we speculate that this may be a mechanism whereby damaged fatty acyl chains are replaced. Under oxidative stress conditions, acyl-CoA:lysocardiolipin acyltransferase-1 (ALCAT1) may also be involved in remodeling of CL. Increased expression of ALCAT1 leads to aberrant remodeling of CL with long polyunsaturated fatty acyl chains, which are sensitive to ROS (Watkins et al., 1998; Hong et al., 2002; Li et al., 2010). Increased expression of ALCAT1 is linked to diabetes and diet-induced obesity in humans and to hyperthyroid cardiomyopathy in mice

(Cao et al., 2009; Li et al., 2010; Li et al., 2012; Liu et al., 2012). In summary, CL deficiency is linked to increased generation of ROS, which in turn damage CL by peroxidation of CL acyl chains.

3. Perturbation of iron homeostasis in CL deficient cells

A recent report indicates that CL is required for Fe-S biogenesis and iron homeostasis (Patil et al., 2013). Fe-S clusters are present in all kingdoms of life (Lill and Muhlenhoff, 2008; Johnson et al., 2005). They are cofactors for many biochemical reactions, including those required for electron transport and for the TCA cycle (Hausmann et al., 2008; Gerber et al., 2004; Li et al., 1999; Lill and Muhlenhoff. 2008). In eukaryotes, three Fe-S cluster biogenesis systems have been characterized, including the iron-sulfur cluster (ISC) import system in mitochondria (required for the generation of all cellular Fe-S proteins), the cytosolic Fe-S protein assembly (CIA) machinery, and the ISC export apparatus in mitochondria. The latter two processes are also involved in maturation of Fe-S proteins in the cytosol and nucleus (Balk et al., 2004; Ye et al., 2006; Kessler et al., 2005; Balk et al., 2005).

A role for CL in iron homeostasis was first suggested by a microarray analysis of genes exhibiting altered expression in the *crd1*Δ mutant (Patil et al., 2013). Most notably, the genes for iron uptake were greatly upregulated in *crd1*Δ cells, which exhibited increased mitochondrial iron as well as increased sensitivity to iron and to oxidative stress. Perturbation of iron homeostasis is a demonstrated outcome of defective Fe-S biogenesis (Rutherford et al., 2005; Hausmann et al., 2008). Consistent with an Fe-S defect, the *crd1*Δ mutant exhibits decreased activities of both mitochondrial and cytosolic enzymes that require Fe-S co-factors, including mitochondrial enzymes succinate dehydrogenase, aconitase, and ubiquinol cytochrome c oxidoreductase, and cytosolic enzymes sulfite reductase and isopropylmalate isomerase (Patil et al., 2013). The CL deficient mutant also exhibited synthetic interaction with the Fe-S scaffold mutant *isu1*.

The mechanism linking CL to Fe-S biogenesis is not currently understood. We speculate that defective import of proteins required for Fe-S biogenesis may underlie the defect, as several studies have shown that mitochondrial protein import as well as assembly of outer membrane complexes are decreased in yeast CL mutants and in lymphoblasts derived from BTHS patients (Jiang et al., 2000; Gebert et al., 2009). Additional evidence for the role of CL in mitochondrial protein import comes from functional studies of the translocator assembly and maintenance protein Tam41, which is required for the assembly and maintenance of the TIM mitochondrial import complex (Gallas et al., 2006; Tamura et al., 2006). Interestingly, the phenotypes of the *tam41*Δ mutant were found to be similar to those of the CL mutant *crd1*Δ. These include defective protein translocases and respiratory chain supercomplexes, decreased assembly of the ADP/ATP carrier (AAC), and decreased CL levels (Tamura et al., 2006; Gallas et al., 2006; Kutik et al., 2008). These findings suggested that protein import defects in the *tam41*Δ mutant were due to the loss of CL. Consistent with this possibility, Tamura and co-workers demonstrated that Tam41 is the mitochondrial CDP-DG synthase that catalyzes the synthesis of CDP-DG, which is required for CL synthesis (Tamura et al., 2013).

An alternative mechanism is that Fe-S proteins may be damaged by the increase in ROS in CL deficient cells, as the inactivation of Fe-S enzymes by superoxide has been demonstrated (Flint et al., 1993; Gardner, 1997). Increasing antioxidants by overexpression of the *YAP1* gene did not rescue the iron sensitivity phenotypes of the CL mutant (Patil et al., 2013), a finding that might argue against Fe-S damage due to increased ROS. However, it is quite possible that free radicals that are not scavenged by Yap1-induced antioxidants may cause Fe-S damage in CL deficient cells.

In light of the role of Fe-S clusters in a wide variety of cellular functions (Rouault et al., 2012), perturbation of Fe-S biogenesis in CL deficient cells has far-reaching implications.

4. CL and mitochondrial transporters

Soluble molecules and substrates are transferred across organelle membranes via carrier proteins. Transporters that play major roles in energy transfer include the ADP/ATP translocase (AAC), phosphate carrier (PiC), pyruvate carrier, fatty acid transport protein (FATP), tricarboxylate transporter, and 2-oxoglutarate/malate carrier protein, among others (Nury et al., 2006; Klingenberg, 1990; Walker, 1992; Kuan et al., 1993; Nelson et al., 1993; Palmieri, 1994). The reader is referred to an excellent review of the role of CL in the stabilization of mitochondrial carrier proteins (Claypool, 2009). Membrane lipids play an important role in the assembly of carrier proteins (Hunte, 2005). Both the AAC and PiC, which are required for oxidative phosphorylation, interact with CL (Kadenbach et al., 1982; Fiermonte et al., 1998; Sedlak et al., 1999; Lange et al., 2001). Of the three isoforms of AAC that have been identified in yeast and humans, activity of the major isoform, AAC2, requires CL (Hoffmann et al., 1994). Furthermore, activity of AAC purified from the yeast *crd1*Δ mutant is decreased (Jiang et al., 2000). A recent report indicates that AAC also interacts with the TIM mitochondrial protein import complex, and this interaction is dependent on CL (Gebert et al., 2011).

In addition to oxidative phosphorylation, transporters play a role in two other sources of energy production, the oxidation of pyruvate and β-oxidation of fatty acids. Pyruvate is transported into the mitochondria of yeast, Drosophila, and humans by the pyruvate carrier proteins MPC1 & MPC2 (Bricker et al., 2012). The purification of pyruvate carrier proteins requires phospholipids, especially CL (Bisaccia and Palmieri, 1984). In the absence of CL, transport activity of the pyruvate carrier protein was not observed (Nalecz et al., 1986). Fatty acids are taken up by fatty acid transport proteins (FATP1 – FATP 6) in the plasma membrane (Van der Vusse et al., 2000; Gimeno, 2007; Jia et al., 2007). While a role for CL in FATP activation has not been reported,Mitchell et al. (2009) demonstrated that knockdown of FATPs in HEK 293 cells inhibits de novo CL synthesis, suggesting that fatty acid transport and CL synthesis may be interdependent. In summary, CL is required for the transport of metabolites utilized in the major cellular energy producing metabolic pathways.

5. Inter-relationship between CL and carnitine

Activated fatty acids (fatty acyl-CoAs) are metabolized to acetyl-CoA by the enzymes of β-oxidation. As membranes are impermeable to activated acyl-CoAs, their transport into mitochondria is facilitated by the quaternary ammonium compound L-carnitine (3-

hydroxy-4-N-trimethylaminobutanoate) (Reuter and Evans, 2012). The transfer of acyl residues from CoA to carnitine is catalyzed by carnitine acyltransferase (van der Leij et al., 2000; Jogl and Tong, 2003; Franken et al., 2008). Carnitine/acylcarnitine translocase catalyzes the transport of acylcarnitine across the mitochondrial membrane (Murthy and Pande, 1984; Van Roermund et al., 1995). The carnitine shuttle is conserved throughout the eukaryotic kingdom (Bremer, 1983).

A role for CL in the carnitine shuttle is suggested by reports that CL is required for the activities of the carnitine shuttle enzymes. Carnitine acyltransferase (carnitine palmitoyltransferase) purified from rat liver cells was shown to contain CL (Fiol et al., 1984), and enzymatic activity of the enzyme was stimulated by CL (Pande et al., 1986). Carnitine/acylcarnitine translocase activity was also shown to require CL (Noel and Pande, 1986). Furthermore, the stimulating effect of carnitine on state 2 respiration in rat liver mitochondria was abolished by doxorubicin, which binds CL, and activity was restored by adding CL (Battelli et al., 1992).

In addition to its function in the transport of acyl groups, carnitine also plays a role in oxidative stress and aging. In *S. cerevisiae*, carnitine was shown to improve growth in the presence of oxidative stress (Franken et al., 2010). Several features of aging in rats are reversed by supplementation with carnitine, including both decreased CL and mitochondrial dysfunction. Decreased levels and pathological remodeling of CL with polyunsaturated fatty acids (arachidonic and docosahexaenoic acids) have been described in old rats (Lee et al., 2006; Sparagna et al., 2009; Maftah et al., 1994; Lewin et al., 1984; Paradies et al., 1993; Paradies et al., 1997; Lenaz et al., 1997; Paradies et al., 1990). Mitochondrial dysfunction in old animals is also associated with defects in ATP synthesis and oxidative phosphorylation (Maftah et al., 1994; Hoch, 1992; Hagen et al., 1998; Sen et al., 2006; Sen et al., 2007). Dietary supplementation of old rats with acylcarnitine significantly improved cellular respiration and mitochondrial membrane potential and, interestingly, also increased CL levels (Hagen et al., 1998).

These studies suggest an inter-relationship between CL and carnitine in regulating mitochondrial functions. CL is required for optimal activity of the carnitine shuttle enzymes, with implications for the generation of energy from β-oxidation and mitochondrial functions associated with aging. In addition, CL levels may be affected by carnitine supplementation.

6. CL, peroxisomes, and β-oxidation

The peroxisome is a unique, versatile, single membrane bound organelle found in all eukaryotes. The number, size, and function of peroxisomes vary among cell types and in response to physiological conditions (Van den Bosch et al., 1992; Veenhuis et al., 1988). Studies of peroxisomal biogenesis have been hampered by the fragility and low abundance of the organelle (Platta et al., 2007). Peroxisomal enzymes carry out β- oxidation of fatty acids, ether lipid synthesis, and reactions of the glyoxylate cycle (Van den Bosch et al., 1992). In eukaryotes, fatty acids are degraded by α-, β-, and ω- oxidation (Wanders et al., 2003). The major pathway, β-oxidation, is conserved from yeast to higher eukaryotes (Houten et al., 2010; Wanders et al., 2010), although localization of the pathways differs. In

yeast, peroxisomes are the sole site of β- oxidation. In mammals, β-oxidation takes place in both mitochondria and peroxisomes (Barlett and Eaton, 2004; Van der Klei and Veenhuis, 1997). β-oxidation of fatty acids plays a key role in energy homeostasis in liver, heart, and skeletal muscle (Houten et al., 2010). The depletion of glucose during fasting is compensated by β-oxidation of fatty acids in many tissues (but not brain) to generate energy (Houten et al., 2010). Fatty acids are also converted to ketone bodies, an additional source of energy that can be utilized by all tissues, including the brain (Houten et al., 2010). The reader is referred to excellent recent reviews of β-oxidation in peroxisomes and mitochondria (Houten et al., 2010; Wanders et al., 2010).

Zinser et al. (1991) reported that the peroxisomal membrane of *S. cerevisiae* grown in rich media (yeast extract-peptone-dextrose) contains considerable levels of CL (7% of total phospholipid). This amount of CL is slightly more than half the level observed in mitochondria (13% of total phospholipid). The CL content of peroxisomal membranes of *Pichia pastoris* cells grown under conditions that induce peroxisomes (methanol or oleic acid as the sole source of carbon) was reported to be about a third of the level observed in mitochondria (Wriessneggar et al., 2007).

It is unclear how CL, which is synthesized in mitochondria, is transported to peroxisomes. Neuspiel et al. (2008) showed that, in mammalian cells, mitochondria derived vesicles (MDV) fuse to a fraction of pre-existing peroxisomes. The MDVs formed are of two types, single-membrane MDVs (mitochondrial outer membrane derived vesicles) and double-membrane MDVs (mitochondrial inner and outer membrane derived vesicles). Both types of MDVs fuse to form single membrane enclosed peroxisomes (Neuspiel et al., 2008). MDVs are thought to be involved in vesicular trafficking of membrane lipids (possibly including CL) and proteins from the intermembrane space of mitochondria to peroxisomes (Schumann et al., 2008).

In summary, while there is currently no direct evidence for a role of CL in peroxisomal metabolism, CL is clearly present at significant levels in the peroxisomal membrane. As discussed above, it is now well established that CL affects numerous mitochondrial functions by interacting with proteins and complexes in the mitochondrial membrane. We speculate that this structurally unique lipid also affects peroxisomal function as a consequence of interacting with peroxisomal membrane enzymes and transporters.

7. Implications for Barth syndrome

Barth syndrome (BTHS) is a severe X-linked disorder characterized by a decrease in total CL, an accumulation of MLCL, and the absence of the predominant unsaturated CL species due to the loss of the CL remodeling enzyme tafazzin (Schlame and Ren, 2006). The phenotypes observed in BTHS patients include dilated cardiomyopathy, neutropenia, skeletal myopathy, growth retardation, 3-methylglutaconic aciduria, defective oxidative phosphorylation, hypercholesterolemia, arrhythmia, and exercise intolerance (Clarke et al., 2013). The molecular basis underlying the pathology observed in BTHS patients is not understood. Interestingly, tafazzin mutations do not correlate with the extent of clinical abnormalities in BTHS, and substantial phenotypic variation occurs even for a single

tafazzin mutation. The high degree of variation in the symptoms of BTHS patients with the same tafazzin mutation suggests that physiological factors influence the outcome of defective tafazzin (Johnston et al., 1997). We suggest that the functions of CL discussed above may be potential physiological modifiers of the BTHS phenotype.

7.1 Fatty acid metabolism, peroxisome biogenesis and the carnitine shuttle

In a landmark study, Spencer et al. (2011) observed that impaired oxygen utilization directly contributes to exercise intolerance in BTHS patients. It is well-established that mild or moderate exercise is driven by a significant increase in fat oxidation. This suggests the possibility that BTHS patients exhibit exercise intolerance due to the inability to adequately metabolize fat.

The clinical outcomes observed in many human disorders of β-oxidation are similar to those seen in BTHS. Perturbation of β-oxidation of fatty acids in mitochondria was shown to cause cardiomyopathy and arrhythmia (Bonnet et al., 1999; Saudubray et al., 1999). The role of CL in the carnitine shuttle suggests that the loss of CL may lead to defects in β-oxidation and or carnitine mediated transport of acyl-CoA. Consistent with this possibility, carnitine mutations lead to clinical outcomes similar to those seen in BTHS (Wanders et al., 2010). Exercise intolerance has been demonstrated in carnitine palmitoyltransferase deficiency (Ørngreen et al., 2003). Carnitine-acylcarnitine translocase deficiency results in cardiomyopathy with arrhythmia (Longo et al., 2006). Cardiomyopathy and skeletal myopathy are observed in deficiencies of the *OCTN2*- encoded carnitine transporter and very long chain acyl-CoA dehydrogenase (Rinaldo et al., 2002).

In summary, numerous disorders of fatty acid metabolism lead to clinical phenotypes similar to those found in BTHS, including cardiomyopathy, skeletal myopathy, arrhythmia, and exercise intolerance. Elucidating the role of CL in β-oxidation and carnitine-mediated transport of fatty acids may identify potential avenues for treatment of BTHS.

7.2 Bioenergetics and Fe-S biogenesis

Three human genetic disorders of Fe-S biogenesis have been described (Rouault and Tong, 2008). These include defects in the iron chaperone frataxin, the iron sulfur cluster assembly protein ISCU, and glutaredoxin. Mutations in frataxin in the disorder Friedreich ataxia lead to hypertrophic cardiomyopathy, heart failure, and deficiencies in enzymes requiring Fe-S cofactors, including aconitase and complex I-III enzymes. Interestingly, mitochondrial deficiencies similar to those seen in CL deficient cells can be rescued by overexpression of frataxin, including decreased mitochondrial membrane potential and ATP levels, sensitivity to oxidative stress, and reduced life span (Ristow et al., 2000; Runko et al., 2008). Cardiomyopathy and cardiac failure in mice were also shown to be relieved by overexpression of frataxin (Schulz et al., 2010). A mutation in the Fe-S scaffold protein ISCU has been described in an inherited skeletal muscle disorder characterized by muscle weakness, exercise-induced lactic acidosis, deficiencies in aconitase and succinate dehydrogenase, and iron overload (Mochel at al., 2008; Kollberg et al., 2009). This finding was consistent with earlier reports of aconitase and succinate dehydrogenase deficiencies in muscle disorders (Haller et al., 1991; Hall et al., 1993). In light of the role of CL in Fe-S

biogenesis, we speculate that the clinical presentation in BTHS may be exacerbated by additional deficiencies in Fe-S metabolism.

7.3 Mitochondrial protein import

The clinical presentation of a disorder known as dilated cardiomyopathy with ataxia (DCMA) syndrome is very similar to BTHS despite normal CL metabolism in DCMA. Symptoms include cardiomyopathy, elevated 3-methylglutaconic acid, and neutropenia. DCMA syndrome is due to a mutation in a gene thought to function in protein import, TIM14 (which shares homology with yeast Tim14p) (Davey et al., 2006). The similarity in the clinical outcomes of DCMA and BTHS suggests that BTHS may be caused or exacerbated by defective mitochondrial import of specific proteins, the identities of which may shed light on the pathology of the disorders. We speculate that proteins required for Fe-S biogenesis may be likely candidates.

8. Future directions and unanswered questions

While it is clear that CL is required for numerous bioenergetic and metabolic pathways, many questions remain to be addressed to fully understand the role of CL in energy metabolism:

What are the implications of Fe-S perturbation in CL deficient cells? Fe-S clusters are essential for numerous metabolic pathways, including those of electron transport, the TCA cycle, heme synthesis, and amino acid synthesis, among others. To what extent are these pathways impaired during CL deficiency?

What is the mechanism underlying defective Fe-S biogenesis in CL deficient cells? The integrity and stability of the protein complexes in the mitochondrial membrane that drive protein import are altered in CL deficient cells, which could lead to defective import of proteins required for Fe-S biogenesis. The loss of CL also leads to increased ROS production (Chen et al., 2008a), which can result in damage to Fe-S proteins. The contributions of these (or other) potential mechanisms remain to be elucidated.

Significant levels of CL in the peroxisomal membrane suggest that this lipid plays a role in peroxisomal function. The many roles of CL in the mitochondrial membrane result from the direct interactions of CL with proteins, as well as from indirect effects on membrane function and membrane curvature (Acehan et al., 2011). Based on the physical properties of CL that underlie these interactions, we speculate that this lipid is also involved in peroxisomal activities, including import of peroxisomal proteins and β- oxidation of fatty acids, among other functions. It is also important to note that the mechanism underlying transport of CL from the mitochondria to the peroxisome is not known. Understanding the role of MDVs might address this question.

To what extent can other lipids compensate for CL deficiency? Studies in yeast indicate that cells that cannot synthesize CL are viable, although they exhibit numerous defects, especially when stressed. However, mutants blocked in the synthesis of the precursor lipid phosphatidylglycerol exhibit dramatically diminished growth and cannot respire (Chang et

al., 1998). These findings suggest that phosphatidylglycerol can compensate for many functions of CL. At least some functions of CL may be compensated by phosphatidylethanolamine, which is also a non-bilayer forming lipid capable of forming hexagonal structures. This is supported by findings that the loss of both CL and mitochondrial phosphatidylethanolamine is lethal in yeast cells (Gohil et al., 2005) and that at least one common function of these lipids is in mitochondrial fusion (Joshi et al., 2012). The identification of compensating lipids may shed light on BTHS, as it is not clear if the pathology results from decreased CL, increased MLCL, and/or aberrant acylation of CL.

One of the enigmas of BTHS (and other monogenic disorders) is that the disorder is characterized by a wide disparity of symptoms ranging from asymptomatic to newborn death, even in the presence of identical tafazzin mutations. This indicates that physiological modifiers affect the clinical outcome in BTHS. We suggest that deficiencies in specific CL functions may act as physiological modifiers that exacerbate the loss of CL. In this light, the elucidation of mechanisms underlying CL functions may lead to new treatment options for BTHS, and for other disorders in which CL plays a role.

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Abbreviations

CL	cardiolipin
Fe-S	iron-sulfur
PKC-Slt2	Protein kinase C-Slt2 mitogen activated protein kinase
HOG	high osmolarity glycerol
BTHS	Barth syndrome
RCR	respiratory control ratio
ROS	reactive oxygen species
ALCAT1	acyl-CoA:lysocardiolipin acyltransferase-1
ISC	iron-sulfur cluster
CIA	cytosolic Fe-S protein assembly
TIM	translocase of the inner membrane
CDP-DG	CDP-diacylglycerol
AAC	ADP/ATP translocase
PiC	phosphate carrier
FATP	fatty acid transport protein
MDV	mitochondrial derived vesicles

DMCA	dilated cardiomyopathy with ataxia
PGP	phosphatidylglycerolphosphate
PGPS	phosphatidylglycerolphosphate synthase
PG	phosphatidylglycerol
MLCL	monolysocardiolipin
TOM	translocase of the outer membrane
SAM	sorting and assembly machinery
CAT	carnitine acyltransferase
CRC	carnitine/acylcarnitine translocase

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Highlights

- Barth syndrome, a disorder of cardiolipin (CL) remodeling caused by mutations in tafazzin, is characterized by a wide range of phenotypes.
- Loss of CL-dependent functions may exacerbate phenotypes associated with tafazzin deficiency.
- The requirement for CL in metabolism is reflected in its role in mitochondrial bioenergetics, transporter activity, and iron-sulfur biogenesis.
- The presence of CL in peroxisomes suggests that it may also be required for peroxisomal function and the carnitine shuttle.
- Perturbation of these metabolic functions may exacerbate the loss of tafazzin and other conditions of CL deficiency.

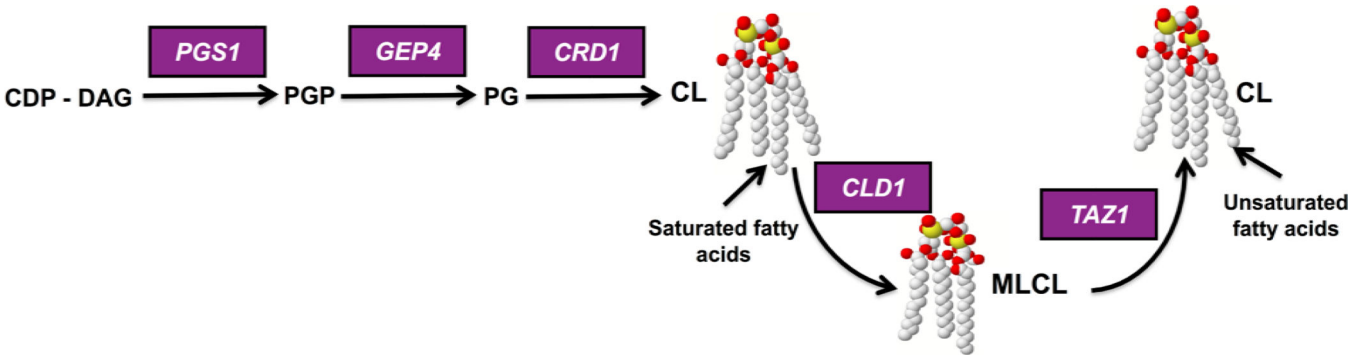

Figure 1. Synthesis and remodeling of cardiolipin (CL) in yeast

CDP-DAG is converted to phosphatidylglycerolphosphate (PGP) by phosphatidylglycerolphosphate synthase (PGPS), encoded by *PGS1* (Chang et al., 1998; Dzugasova et al., 1998). PGP phosphatase (Gep4) catalyzes the conversion of PGP to phosphatidylgylcerol (PG) (Osman et al., 2010). PG is converted to cardiolipin (CL) by CL synthase (Crd1) (Jiang et al., 1997; Chang et al., 1998; Tuller et al., 1998). CL is remodeled in a two-step process in which the CL specific deacylase encoded by *CLD1* removes a fatty acyl group, forming monolysocardiolipin (MLCL) (Beranek et al., 2009), and tafazzin (Taz1) reacylates MLCL to form a generally more unsaturated CL (Xu et al., 2003). In mammalian cells, CL is deacylated by more than one enzyme (Kiebish et al., 2013). Tafazzin is the enzyme that is mutated in Barth syndrome.

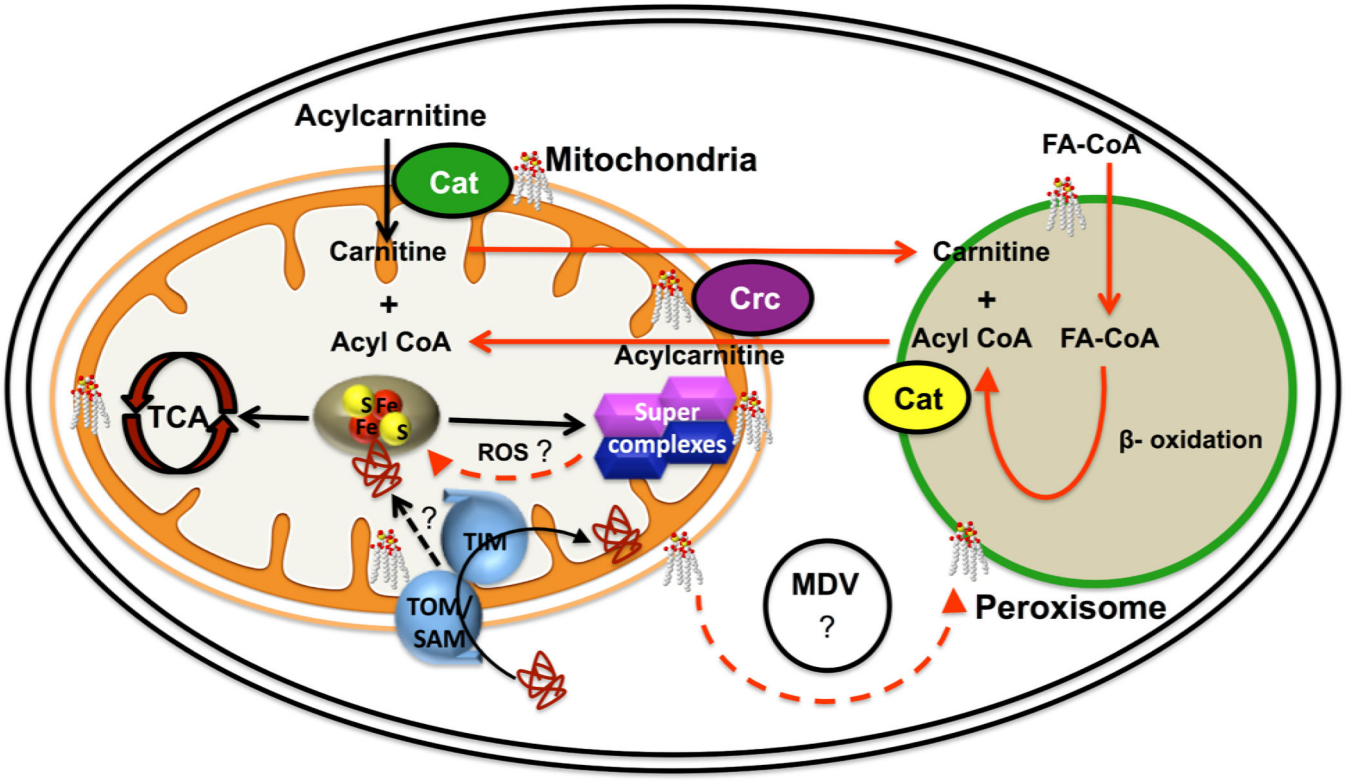

Figure 2. Functions of cardiolipin (CL) in metabolic pathways

CL is most abundant in the inner membrane and is also present in the outer membrane of mitochondria. It is required for activities of transporters and electron transport chain enzymes and for stabilization of electron transport supercomplexes. Loss of CL leads to perturbation of Fe-S biogenesis, resulting in decreased activity of Fe-S enzymes in the TCA cycle, electron transport, and other pathways. The mechanism linking CL and Fe-S biogenesis is unknown. Because CL is required for the import of proteins through mitochondrial import complexes (TOM, SAM and TIM), it is possible that import of specific proteins required for Fe-S synthesis is defective in CL deficient cells. Alternatively, increased ROS generated by inefficient electron transport in CL deficient cells may damage Fe-S proteins. CL is also present in the membrane of the peroxisome, an organelle that carries out β-oxidation of fatty acids, ether lipid synthesis, and reactions of the glyoxylate cycle. The route whereby CL is transported from mitochondria to peroxisomes is unclear, but may involve mitochondria derived vesicles (MDVs). Acyl CoA produced by β-oxidation of long chain fatty acids in peroxisomes is transported to the mitochondria via the carnitine shuttle. The acyl CoA is transferred to carnitine in the peroxisome by carnitine acyltransferase (Cat). Acylcarnitine from the peroxisome crosses the mitochondrial membrane, facilitated by the carnitine/acylcarnitine translocase (Crc). CL is required for efficient activity of both mitochondrial carnitine enzymes in mammalian cells.