

## Chapter 2

# Role of Phospholipases in Regulation of Cardiolipin Biosynthesis and Remodeling in the Heart and Mammalian Cells

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**Abstract** Cardiolipin is a key mitochondrial membrane phospholipid involved in the regulation of generation of ATP. Cardiolipin synthesis and remodeling are tightly regulated processes in eukaryotic cells. The role of phospholipases in the regulation of cardiolipin metabolism is becoming much clearer. Cardiolipin is hydrolysed by several classes of phospholipases including calcium-independent phospholipase A<sub>2</sub>, secretory phospholipase A<sub>2</sub>, and cytosolic phospholipase A<sub>2</sub>. Mitochondrial calcium-independent phospholipase A<sub>2</sub> gamma has emerged as a key player not only in the regulated hydrolysis of cardiolipin to monolysocardiolipin, but also in the overall regulation of mitochondrial function and energy production. The purpose of this chapter is to summarize some of the more current findings on the role of phospholipases in the regulation of cardiolipin metabolism in the heart and mammalian tissues. In addition, a brief discussion on the role of exogenous phospholipase-treatment of cells on cardiolipin metabolism is presented.

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## 2.1 Introduction

Phospholipids are important structural and functional components of the cell membrane and alterations in the composition of phospholipids within the heart are linked to alterations in myocardial electrical rhythm [1, 2]. Bis-(1,2-diacyl-*sn*-glycero-3-phospho)-1',3'-*sn*-glycerol or cardiolipin (CL) is the principal polyglycerophospholipid found in the heart and mammalian tissues [3]. CL was initially discovered in beef heart by Mary Pangborn in 1942 and was subsequently shown to comprise approx. 15–20 % of the entire phospholipid phosphorus mass of the heart [3–6]. The heart contains the highest concentration of CL found in any mammalian tissue due to its vast abundance of mitochondria. CL is found within both inner and outer mitochondrial membranes and within their contact sites [7–9]. Both the appropriate content and the fatty acyl molecular composition of CL are critical for the ability to modulate the activity of mitochondrial enzymes involved in the generation of ATP (reviewed in [6, 10]). In fact, CL is the “glue” that holds the mitochondrial respiratory complex together [11]. Hence, maintenance of the appropriate content and fatty acid composition of CL in mitochondria is essential for mammalian cell function.

## 2.2 Cardiolipin: Its Role in Apoptosis, General Mitochondrial Function, and Genetic Disease

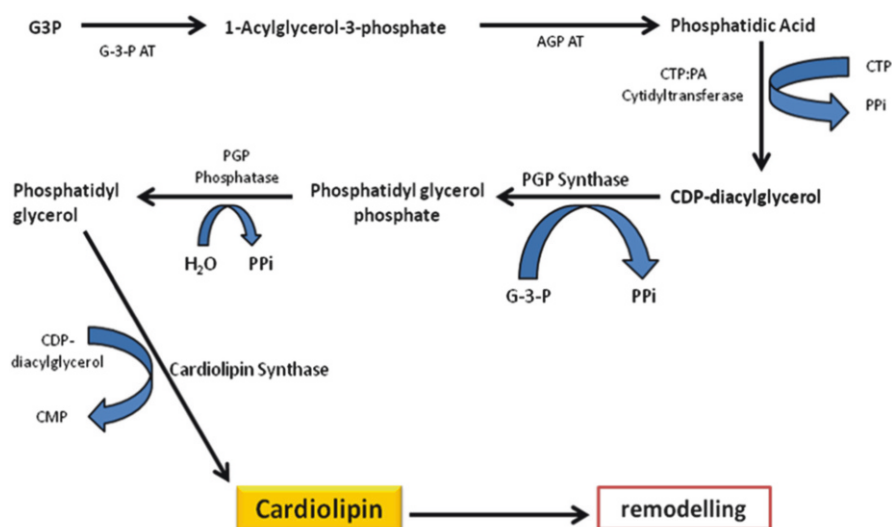
CL has been implicated in the intrinsic pathway of apoptosis [12] and is required for caspase-8 cleavage of Bid at the mitochondrial outer membrane [13]. Stomatin like-2 (SLP-2), a widely expressed mitochondrial inner membrane protein of previously unknown function, expression in T lymphocytes resulted in increased CL content and resistance to apoptosis mediated through the intrinsic pathway [14]. Alteration in the content of CL has been shown to alter oxygen consumption in mitochondria [15, 16]. In rat heart subjected to ischemia and reperfusion the reduction in electron transport chain activity was coupled with reduction in CL [17]. When CL is removed or digested away from mitochondrial respiratory chain proteins by phospholipases, denaturation and complete loss in activity occur (reviewed in [18]). The prohibitins (PHB-1 and PHB-2) are an evolutionarily conserved and ubiquitously expressed family of membrane proteins that are essential for cell proliferation and development in higher eukaryotes [19, 20]. PHB complexes function as protein and lipid scaffolds that ensure the integrity and functionality of the mitochondrial inner membrane and they associate with CL. CL is important for formation of the prohibitin-m-AAA protease complex, the alpha-ketoglutarate

dehydrogenase complex, and mitochondrial respiratory chain supercomplexes [21]. SLP-2 interacts with PHB-1 and -2 and binds to CL to facilitate formation of metabolically active mitochondrial membranes [14]. In T cell-specific SLP-2-deficient mice impaired CL compartmentalization in mitochondrial membranes results in decreased protein and activity of complex I of the mitochondrial respiratory chain [22]. Hence, the function of SLP-2 is to recruit PHBs to CL to form CL-enriched microdomains in which electron transport complexes are optimally assembled. In addition, reduced expression of mitochondrial respiratory complex proteins in right ventricle (RV) of persistent pulmonary hypertension of the newborn (PPHN) piglets provided evidence that PHB complexes may be disrupted in RV cardiac mitochondria of these animals [23].

Barth syndrome (BTHS) is a rare X-linked genetic disorder in young boys characterized by the triad of cardiomyopathy, cyclic neutropenia, and a 3-methylglutaconic aciduria [24–26]. In 50 % of the cases a mild hypocholesterolemia is also observed. The documented hypocholesterolemia observed in at least one BTHS patient may be due to a reduced ability to upregulate mRNA expression and enzyme activity of hydroxymethylglutaryl-Coenzyme A reductase, the rate-limiting enzyme of de novo cholesterol biosynthesis [27]. BTHS is caused by mutations in the tafazzin gene, *TAZ*, localized to chromosome Xq28.12. There are over 100 mutations in *TAZ* identified. However, to date there has been no correlation between genotype and severity of the disease. A reduced ability to resynthesize CL from monolysocardiolipin (MLCL) is the underlying molecular mechanism responsible for BTHS (reviewed in [24, 25]). Hence, BTHS is the only genetic disease identified to date in which the specific biochemical defect is a reduction in mitochondrial CL and accumulation of MLCL. Four *TAZ* mRNA transcripts were shown to be generated in human cells [28]. *Taz* knockdown mice exhibited a dramatic decrease of tetralinoleoyl-CL ( $L_4$ -CL) in cardiac and skeletal muscles, accumulation of MLCL, and pathological changes in mitochondria [29, 30]. Moreover, disruption of *TAZ* alters both assembly and stability of the respiratory chain supercomplexes in the mitochondrial inner membrane [31]. Interestingly, decreased levels of PHB complexes in *TAZ*-deficient mitochondria were shown to be due to a decreased content of CL [21]. Introduction of *TAZ* into yeast with defective *TAZ* or into *TAZ* knockout zebrafish or onto *TAZ* knock out drosophila restored CL levels and mitochondrial function to that of near normal levels [32–34].

## 2.3 Cardiolipin Biosynthesis and Remodeling

The de novo biosynthesis of CL in the heart occurs via the cytidine-5'-diphosphate-1,2-diacylglycerol (CDP-DG) pathway [35] (Fig. 2.1). Initially, phosphatidic acid (PA) is converted to CDP-DG by CDP-DG synthetase (CDS). The human CDS has been cloned and CDS-2 is the major isoform expressed in mammalian heart [36]. CDS-2 mRNA expression is reduced in AMP-activated protein kinase  $\alpha 2$  null mice and this accounted for the reduction in cardiac CL seen in these animals [37]. Clofibrate-mediated activation of peroxisome proliferator-activated receptor



**Fig. 2.1** Cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol pathway (CDP-DAG). The de novo biosynthesis pathway of Cardiolipin begins with the formation of PA from G-3-P. PA then reacts with CTP to eventually produce CDP-diacylglycerol. Another G-3-P then interacts with CDP-diacylglycerol to produce phosphatidyl glycerol phosphate which is then hydrolysed to yield a Phosphatidyl Glycerol. From this step, the formation of de novo Cardiolipin is catalyzed by an enzyme called cardiolipin synthase. The newly formed cardiolipin is quickly remodeled with specific acyl groups with the help of remodeling enzymes (includes Tafazzin, MLCL AT-1, and/or ALCAT-1). G-3-P, glycerol-3-phosphate; G-3-P AT, glycerol-3-phosphate acyl transferase; AGP-AT, 1-Acylglycerol-3-phosphate acyl transferase; CTP cytidine triphosphate, PA Phosphatidic acid, PPi pyrophosphate, CDP cytidine diphosphate, PGP phosphatidyl glycerol phosphate, CMP cytidine monophosphate, MLCL AT-1 monolysocardiolipin acyl transferase 1, ALCAT-1 Acyl-CoA:Lysocardiolipin acyltransferase-1

$\alpha$  (PPAR $\alpha$ ) in murine heart stimulated CL biosynthesis via an increase in mRNA expression of the CDS-2 isoform of CDS and such an activation was not observed in clofibrate-treated PPAR $\alpha$  knockout mice [38]. In the second step of the pathway, CDP-DG condenses with *sn*-glycerol-3-phosphate to form phosphatidylglycerol (PG) catalyzed by phosphatidylglycerolphosphate (PGP) synthase (PGPS) and PGP phosphatase. The G protein RhoGap plays a key role in controlling PGPS activation and CL synthesis at the transcriptional level [39]. In addition, it is well documented that expression of mitochondrial fusion proteins is altered in heart failure (HF) and expression of the mitochondrial fusion protein, mitofusion-2, may be involved in the regulation of CL de novo biosynthesis through PGPS [40, 41]. In the third step of the pathway PGP is rapidly dephosphorylated by PGP phosphatase [3]. PGP phosphatase was recently identified in yeast and in mammalian cells PGP phosphatase is known as protein tyrosine phosphate localized to mitochondrion-1 (PTPMT-1) and is a member of the protein tyrosine phosphatase superfamily [20, 42]. Fibroblasts from Ptpmt1-deficient mice accumulate PGP and exhibit a decrease in phosphatidylglycerol (PG) and CL [43]. In the last step of the pathway, PG is converted to CL

in the heart by condensation with CDP-DG catalyzed by CL synthase (CLS) [35, 44]. CLS is localized exclusively to the inner mitochondrial membrane [44, 45] and was purified to homogeneity from rat liver [46]. The genes encoding human (hCLS1) and murine CLS (mCLS1) have been identified and the enzyme is highly expressed in heart [47–49]. Loss of CLS mRNA in tissues of lipopolysaccharide-treated mice did not result in loss in CLS activity indicating that the rate of CLS enzyme turnover may be slow in mammalian cells [50].

Subsequent to its biosynthesis, CL is rapidly remodeled to yield molecular species of CL found in the mitochondrial membrane [24]. In mammalian heart, linoleic acid (18:2) comprises 80–90 % of the acyl chains in CL [51]. The major tetra-acyl molecular species in human heart (approximately 80 % of total) are (18:2-18:2)-(18:2-18:2)-CL or L<sub>4</sub>-CL. Remodeling may occur through the concerted deacylation followed by reacylation (resynthesis) [52]. CL may be hydrolyzed by many different phospholipases A<sub>2</sub> [53] including calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>-VIA) [54, 55], secretory PLA<sub>2</sub> [56], and cytosolic PLA<sub>2</sub> [57]. Resynthesis of cardiac CL from MLCL and linoleate is required to achieve the enrichment in 18:2. CL resynthesis from MLCL occurs via at least three enzymes. A mitochondrial-associated membrane acyllysocardiolipin acyltransferase-1 (ALCAT-1) with specificity for multiple anionic lysophospholipid substrates has been identified [58, 59]. Upregulation of ALCAT-1 by oxidative stress or diet-induced obesity in mice resulted in mitochondrial dysfunction, reactive oxygen species production, and insulin resistance [60]. ALCAT-1 null mice have resistance to diet-induced obesity indicating that this enzyme may be a stress-response enzyme. A decrease in ALCAT-1 mRNA expression was associated with a decrease in CL in AMP-activated protein kinase null mice [37]. However, no alterations in ALCAT-1 mRNA expression were observed in heart explants from humans or spontaneous hypertensive heart failure prone (SHHF) rats in heart failure (HF) in which CL was decreased [51, 61]. A mitochondrial deacylation–reacylation cycle was identified in which newly synthesized CL was rapidly deacylated to MLCL and then reacylated back to CL with linoleoyl-CoA [62]. The mitochondrial activity was characterized and the enzyme purified from pig liver [63, 64] and was shown to be a previously unidentified human protein [65]. An *in vitro* CL transacylase activity that remodels CL was reported in crude mitochondrial fractions from rat liver [66]. This CL transacylase is the BTHS gene product TAZ described above in Sect. 2 [66, 67]. A novel mitochondrial protein, Them5, which exhibits thioesterase activity with long-chain acyl-CoAs and a strong substrate preference for C18 polyunsaturated fatty acids was recently identified [68]. *Them5*<sup>−/−</sup> mice exhibit an increase in MLCL implicating thioesterase activity in the regulation of CL remodeling.

Although evidence indicates that the BTHS gene product TAZ clearly and specifically remodels mitochondrial CL with linoleic acid, the idea that TAZ alone determines the fatty acid profile of CL contradicts experimental evidence. For example, in hearts of AMP-activated protein kinase null mice, cytidine-diphosphate diacyl-*sn*-glycerol synthetase-2, a rate-limiting enzyme of *de novo* CL biosynthesis, and ALCAT-1 mRNA expression were reduced compared to controls and this accompanied reduced levels of CL and linoleic acid in phospholipids within cardiac

mitochondria [37]. *Taz* mRNA expression was unaltered in the hearts of these mice. Moreover, the presence of at least two patients with BTHS and an exon 5 mutation in *TAZ* but with normal CL levels highlight the fact that *TAZ* alone may not be responsible for all mitochondrial CL remodeling (Michael Schlame, personal communication). These data suggest that in addition to *TAZ*, other enzymes may play a key role in mammalian and human mitochondrial CL remodeling [69]. In Epstein–Barr virus-transformed human BTHS lymphoblasts, a 60–80 % reduction in CL levels were observed and transfection of these cells with the CL remodeling enzyme monolysocardiolipin acyltransferase-1 (MLCL AT-1) or the alpha subunit of tri-functional protein restored CL levels to that of control lymphoblasts [65, 70, 71].

## 2.4 Role of Phospholipases in the Regulation of Cardiolipin Metabolism

The observation that elevated CL remodeling occurs as a compensatory mechanism for increased hydrolysis of CL mediated by phospholipase activation is supported by several studies. Elevated  $\text{PLA}_2$  activity has been seen in various models of stimuli-induced apoptosis. Addition of the proapoptotic factor  $\text{TNF-}\alpha$  to H9c2 cardiac myoblast cells stimulated mitochondrial  $\text{PLA}_2$  activity towards mitochondrial phospholipids [72]. In addition, MLCL accumulates during Fas-mediated apoptosis as a by-product of CL degradation by mitochondrial  $\text{PLA}_2$  [73]. Furthermore, MLCL generated by  $\text{PLA}_2$  hydrolysis of CL during induction of apoptosis was shown to enhance t-Bid binding to membranes [73–75]. 2-Deoxyglucose (2-DG) has been shown to induce apoptosis by stimulating intracellular reactive oxygen species production, CL oxidation, and the release of cytochrome *c* from mitochondria in several cell lines. The effect of apoptosis mediated by metabolic hypoxia on phospholipase  $\text{A}_2$  activity and CL metabolism was examined in the surviving population of H9c2 cells exposed to 2-DG [76]. Treatment of these cells with 100 mmol/L 2-DG for 16 h stimulated caspase-3 and PARP cleavage, indicating that apoptosis occurred in this cell population. Mitochondrial  $\text{PLA}_2$  activity towards mitochondrial phospholipids was elevated indicating the potential for enhanced CL hydrolysis in these cells. However, the pool size of CL and incorporation of [ $1\text{-}^{14}\text{C}$ ]linoleic acid as a precursor into CL was unaltered due to an increase in expression and activity of mitochondrial MLCL AT activity. These results indicated that there was an elevation in the resynthesis of CL from MLCL in the surviving population of H9c2 cells treated with 2-DG likely as a compensatory mechanism for elevated mitochondrial  $\text{PLA}_2$  activity. Interestingly, the activity of ALCAT-1, the mitochondrial-associated membrane protein capable of resynthesizing CL from MLCL and unsaturated fatty acid, was reduced in 2-DG-treated cells supporting the observation that ALCAT-1 and MLCL AT-1 are reciprocally regulated [60]. If the accumulation of MLCL indeed plays a role in mitochondria-mediated apoptosis, it is possible that rapid CL resynthesis from MLCL is required in response to proapoptotic stimuli-mediated CL degradation to restore cellular homeostasis and thus prevent the

apoptotic cascade. The expression of group VIA calcium-independent PLA<sub>2</sub> has been shown to play a role in the protection of mitochondrial function from damage caused by mitochondria-generated reactive oxygen species during apoptotic induction by staurosporine [55]. 2-DG addition to cells was shown to result in the generation of reactive oxygen species [77, 78]. Since cell viability of the 45 % surviving population of H9c2 cells exposed to 2-DG was greater than 95 %, as assessed by Trypan blue exclusion, it is possible that increased mitochondrial MLCL AT activity and its expression, and hence, elevated CL resynthesis, may work in concert with elevation in mitochondrial PLA<sub>2</sub> activity to be a protective mechanism against MLCL-mediated apoptosis [76].

Mitochondrial PLA<sub>2</sub> activity towards CL may also be regulated by an intracellular ceramide-regulated process not directly related to cell killing [39]. Mitochondrial PLA<sub>2</sub> activity was examined in a novel Chinese hamster ovary (CHO) cell line resistant to ceramide-induced apoptosis. A promoter trap mutagenesis approach was used to isolate this etoposide-resistant CHO cell line. The resistant cell line, named E91, showed cross-resistance to *N*-acetylsphingosine. The promoter trap retrovirus was found integrated into intron 1-2 of the Dlc-2 (Stard13) RhoGap gene. The E91 cells showed elevated guanosine triphosphate (GTP)-bound RhoA levels compared to parental cells, suggesting that the retrovirus integration had inactivated one of the Dlc-2 RhoGap alleles. The parental cells showed elevated PLA<sub>2</sub> activity after treatment with *N*-acetylsphingosine. Intracellular ceramide-signaling was defective in the E91 cells due to increased levels of active GTP-bound RhoA. This study was the first report for the regulation of a mammalian PLA<sub>2</sub> through RhoGap expression [39].

*Chlamydia trachomatis* is a prevalent sexually transmitted bacterial disease and is the leading cause of infectious blindness in developing nations [79]. *C. trachomatis* is an intracellular parasite and obtains its phospholipids from the host cell. However, no PLA<sub>2</sub> homologues have been identified in chlamydial genomes. It was previously demonstrated that endogenous host cell-derived phospholipids are trafficked to *C. trachomatis* and that the phospholipid composition of *C. trachomatis* mimics that of the eukaryotic host cell in which it was grown [18, 80–82]. In these studies, *C. trachomatis* infection of each mammalian cell type investigated resulted in an increase in host cell PLA<sub>2</sub> activity resulting in hydrolysis of host cell phospholipids, including CL, to their respective lysophospholipid. This was followed by trafficking of the lysophospholipid to the intracellular chlamydial inclusion where it was rapidly remodeled with a bacterial-specific branched chain fatty acid to form the chlamydial-specific parent phospholipid. Activation of the host Raf-MEK-ERK-cPLA<sub>2</sub> signaling cascade was required for this chlamydial uptake of host glycerophospholipids [57]. Both the MAP kinase pathway (Ras/Raf/MEK/ERK) and calcium-dependent cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) are activated in chlamydia-infected cells. Inhibition of cPLA<sub>2</sub> activity blocked chlamydial uptake of host glycerophospholipids and resulted in impairment in chlamydial growth. In addition, attenuation of either c-Raf-1 or MEK1/2 activity prevented the chlamydial activation of ERK1/2, leading to the suppression of both chlamydial activation of the host cPLA<sub>2</sub> and the uptake of glycerophospholipids from the host cells.



The role of PPAR $\alpha$ -stimulated PLA $_2$  in cardiac mitochondrial CL biosynthesis was examined in both in vivo and in vitro models [38]. Treatment of rat heart H9c2 cells with clofibrate increased the expression and activity of 14 kDa mitochondrial PLA $_2$ , but did not affect the pool size of CL. Clofibrate treatment stimulated de novo CL biosynthesis via an increase in PGPS activity, accounting for the unaltered CL content. Cardiac PLA $_2$ , PGPS, and CDS-2 activities and CDS-2 mRNA levels were elevated in mice-fed clofibrate for 14 days compared with controls. In PPAR $\alpha$ -null mice, clofibrate feeding did not alter cardiac PLA $_2$ , PGPS activities, or CDS-2 activity and mRNA level, confirming that these enzymes are regulated by PPAR $\alpha$  activation. This study was the first to demonstrate that CL de novo biosynthesis is regulated by PPAR $\alpha$  activation through PLA $_2$  activation.

Eukaryotic cell reproduction involves duplication of cellular components, including biological membranes and DNA content, resulting in a doubling in size and then division into two components. In the absence of growth factors (e.g. serum starvation) cells will not divide, but enter into a quiescent state known as G $_0$ . Cells depleted of serum in G $_0$  may be triggered to enter into the S-phase by the addition of serum. Since CL plays an important role in generation of ATP required for the human cell cycle, the role of PLA $_2$  in CL metabolism was investigated in quiescent HeLa cells induced to enter into the S-phase of the cell cycle [70]. HeLa cells were serum starved for 24 h, then incubated for up to 24 h in the absence or presence of serum. CL mass was doubled by 16 h of incubation and this was accompanied by dramatic increases in the expression and activities of the CL de novo biosynthetic enzymes. In addition, an increase in mitochondrial PLA $_2$ , MLCL AT-1, and ALCAT-1 activities were observed. It was suggested that the elevated activities of the CL remodeling enzymes PLA $_2$ , MLCL AT-1, and ALCAT-1 were required to support remodeling of the increased newly synthesized CL required during S-phase of the human cell cycle.

In models of cerebral stroke, the activity, mRNA expression, and immunoreactivity of cPLA $_2$  and the activity and mRNA expression of secretory PLA $_2$  (sPLA $_2$ ) were shown to be elevated and may be involved in CL degradation leading to mitochondrial dysfunction and subsequent reactive oxygen species generation [56]. MLCL was shown to be generated through cleavage of mycobacterial CL by a lysosomal type calcium-independent PLA $_2$  present in macrophage lysosomes [83]. Finally, group VIA calcium-independent PLA $_2$  beta (iPLA $_2$  $\beta$ ) localizes in and protects beta-cell mitochondria from oxidative damage during staurosporine-induced apoptosis [84]. In that study, islets isolated from iPLA $_2$  $\beta$  null mice are more sensitive to staurosporine-induced apoptosis than those from wild-type littermates and that 2 weeks of daily intraperitoneal administration of staurosporine to iPLA $_2$  $\beta$  null mice impairs both the animals' glucose tolerance and glucose-stimulated insulin secretion by their pancreatic islets. iPLA $_2$  $\beta$  was expressed only at low levels in islet beta-cells from obesity- and diabetes-prone *db/db* mice. Hence, the low iPLA $_2$  $\beta$  expression level observed in *db/db* mouse beta-cells may render them vulnerable to injury by reactive oxygen species.



## 2.5 Role of Calcium-Independent PLA<sub>2</sub> in CL Metabolism in Mammalian Models

Alterations in calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) have been shown to contribute to diminished cardiac function in failing hearts due to myocardial infarction [85]. In cardiac myocytes prepared from normal rats and rat with SHHF, it was demonstrated that CL remodeling was performed singly with respect to each fatty acyl moiety, was attenuated in heart failure (HF) relative to non-HF, and was partially sensitive to iPLA<sub>2</sub> inhibition suggesting that CL remodeling occurs in a step-wise manner, that compromised 18:2 incorporation contributes to a reduction in L<sub>4</sub>-CL in the failing rat heart, and that mitochondrial iPLA<sub>2</sub> plays a role in the remodeling of CL acyl composition in the heart [86]. Genetic ablation of iPLA<sub>2</sub> gamma (iPLA<sub>2</sub>γ) in mice resulted in decreased L<sub>4</sub>-CL and abnormal mitochondrial function and a deficient mitochondrial bioenergetic phenotype including a mitochondrial neurodegenerative disorder characterized by degenerating mitochondria, autophagy, and cognitive dysfunction in mice [54, 87]. In iPLA<sub>2</sub> null mice impairment of iPLA<sub>2</sub>γ caused mitochondrial dysfunction and increased oxidative stress, leading to the loss of skeletal muscle structure and function [88]. These authors found that the composition of CL and other phospholipid classes were altered and that the levels of myoprotective prostanooids were reduced in skeletal muscle of iPLA<sub>2</sub>γ null mice. Thus, in addition to maintenance of homeostasis of the CL within the mitochondrial membrane, iPLA<sub>2</sub>γ may contribute to modulation of lipid mediator production in vivo.

Mice null for iPLA<sub>2</sub>γ are also completely resistant to high fat diet-induced weight gain, adipocyte hypertrophy, hyperinsulinemia, and insulin resistance, which occur in wild-type mice after high fat feeding [89]. Notably, iPLA<sub>2</sub>γ null mice were lean, demonstrated abdominal lipodystrophy, and remained insulin-sensitive despite having a marked impairment in glucose-stimulated insulin secretion after high fat feeding. Respirometry of skeletal muscle mitochondria from iPLA<sub>2</sub>γ null mice demonstrated marked decreases in state 3 respiration using multiple substrates whose metabolism was uncoupled from ATP production. Shotgun lipidomics of skeletal muscle revealed a decreased content of CL with an altered molecular species composition, thereby identifying the mechanism underlying mitochondrial uncoupling in the iPLA<sub>2</sub>γ null mice. Collectively, these results identify iPLA<sub>2</sub>γ as an obligatory upstream enzyme that is necessary for efficient electron transport chain coupling and energy production through its participation in the alterations of cellular bioenergetics that promote the development of the metabolic syndrome.

Reductions in L<sub>4</sub>-CL and alterations in CL biosynthetic and remodeling processes have been observed in left ventricular (LV) hypertrophy and subsequent HF in SHHF rats and in LV human heart explants isolated from HF patients [61]. PPHN results in right ventricular (RV) hypertrophy followed by right heart failure and an associated mitochondrial dysfunction [90, 91]. iPLA<sub>2</sub>γ mRNA expression was decreased in the LV and RV of PPHN piglets compared with control animals [23].

In addition, a decrease in [ $1\text{-}^{14}\text{C}$ ]linoleoyl-CoA incorporated into MLCL in the LV and RV of PPHN piglets was observed indicating that iPLA $_2\gamma$  may be reduced in PPHN. This was confirmed by the decreased mRNA expression of iPLA $_2\gamma$  observed in the LV and RV of these PPHN animals. The above data clearly support iPLA $_2\gamma$  as the enzyme in the remodeling and the metabolism of CL.

## 2.6 Role of Exogenous Phospholipase-Treatment of Cells on CL Metabolism

Controlled and limited treatment of H9c2 cardiac myoblast cells with *Naja mocambique mocambique* PLA $_2$  reduced the pool sizes of PC and PE and resulted in elevation of LPC and LPE, whereas the pool size of CL and other phospholipids were unaltered [92]. Pulse radiolabeling and pulse-chase radiolabeling experiments with [ $1,3\text{-}^3\text{H}$ ]glycerol in cells incubated or preincubated in the absence or presence of PLA $_2$  resulted in reduced radioactivity incorporated into CL indicating attenuated de novo biosynthesis of CL. The mechanism for the reduction in CL appeared to be a decrease in the activity of phosphatidic acid:cytidine-5'-triphosphate cytidyltransferase, a rate-limiting enzyme of de novo CL biosynthesis in H9c2 cells, mediated by elevated cellular LPC levels. The results indicated that de novo CL biosynthesis in H9c2 cells may be regulated by the cellular level of the PLA $_2$  product LPC.

Treatment of H9c2 cardiac myoblast cells with PC-specific *Clostridium welchii* phospholipase C (PLC) was shown to reduce the cellular pool size of PC without altering cellular CL levels [93]. Pulse radiolabeling and pulse-chase radiolabeling experiments with [ $1,3\text{-}^3\text{H}$ ]glycerol demonstrated that radioactivity incorporated into CL was reduced in PLC-treated cells with time compared with controls indicating attenuated de novo biosynthesis of CL. Addition of 1,2-dioctanoyl-*sn*-glycerol, a cell permeable 1,2-diacyl-*sn*-glycerol analog, to cells mimicked the inhibitory effect of PLC on CL biosynthesis indicating the involvement of 1,2-diacyl-*sn*-glycerol. The mechanism for the reduction in CL biosynthesis in PLC-treated cells appeared to be a decrease in the activities of phosphatidic acid:cytidine-5'-triphosphate cytidyltransferase and PGPS, mediated by elevated 1,2-diacyl-*sn*-glycerol levels. These data indicated that de novo CL synthesis may be regulated by 1,2-diacyl-*sn*-glycerol and may be coordinated with PC biosynthesis in H9c2 cardiac myoblast cells.

## 2.7 Conclusions

It is clear that CL may be hydrolyzed by several different classes of PLA $_2$  including iPLA $_2$ , sPLA $_2$ , and cPLA $_2$ . Important questions remain to be addressed including whether there is indeed a coordination between CL de novo synthesis and the remodeling of CL mediated by the hydrolysis of these PLA $_2$ 's and subsequent resynthesis in vivo and whether the by-products of CL degradation themselves play

a role in cellular metabolism. The generation of the TAZ knock down mouse is likely to provide more concrete evidence surrounding the role that the iPLA<sub>2</sub> $\gamma$  plays in the regulation of CL metabolism in mammalian tissues.

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