Journal of Molecular and Cellular Cardiology 74 (2014) 251–259

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

# Dysregulation of cardiolipin biosynthesis in pediatric heart failure

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ARTICLE INFO

*Article history:*
Received 4 November 2013
Received in revised form 5 June 2014
Accepted 7 June 2014
Available online 14 June 2014

*Keywords:*
Cardiolipin
Pediatric
Heart failure
Dilated cardiomyopathy
Mitochondria

ABSTRACT

Cardiolipin, a unique phospholipid in the inner mitochondrial membrane, is critical for optimal mitochondrial function. CL abnormalities have been demonstrated in the failing rodent and adult human heart. The aim of this study was to determine whether abnormalities in CL content and the CL biosynthesis and remodeling pathways are present in pediatric idiopathic dilated cardiomyopathy (IDC).

A cross-sectional analysis of myocardial tissue from 119 IDC and non-failing (NF) control samples was performed. Electrospray ionizing mass spectrometry was used to measure total CL and CL species content in LV tissue. RT-PCR was employed to measure gene expression of the enzymes in the CL biosynthesis and remodeling pathways in both the adult and pediatric heart. Significantly lower total and $(18:2)_4$CL (the beneficial species) content was demonstrated in myocardium from pediatric patients with IDC compared to NF controls. Analysis of mitochondrial gene transcripts was used to demonstrate that there is no decrease in mitochondrial content. Expression of two biosynthesis enzymes and one remodeling enzyme was significantly lower in pediatric IDC compared to NF controls. Expression of two phospholipases involved in CL degradation were also altered, one up- and one down-regulated. Except for one remodeling enzyme, these changes are unique from those in the failing adult heart.

Similar to what has been seen in adults and in a rat model of IDC, total and $(18:2)_4$CL are lower in pediatric IDC. Unique CL species profiles are seen in heart tissue from children with IDC compared to adults. Differences in CL biosynthesis and remodeling enzyme expression likely explain the differences in CL profiles observed in IDC and implicate unique age-related mechanisms of disease.

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## 1. Introduction

Idiopathic dilated cardiomyopathy (IDC) is a common form of cardiomyopathy and indication for cardiac transplantation in children and adults. The incidence of dilated cardiomyopathy in adults is about 5.5 per 100,000 people per year and between 0.34 and 1.09 per 100,000 people per year in children [1–4]. While the disease is less common in children than adults the clinical consequences are similarly devastating with 1- and 5-year rates of death or transplantation of approximately 30% and 40%, respectively [2,5]. Although the prevalence of heart failure in children is less than in adults, pediatric IDC represents a significant burden of disease and health care cost in the pediatric population [6,7]. IDC is the leading indication for transplantation in children over 1 year of age; additionally these patients are among the highest risk among children for cardiopulmonary resuscitation [2,8]. Evidence-based medical therapy for adults with heart failure (HF) is well established and has improved clinical outcomes [9]. In contrast, modern medical regimens have not significantly improved outcomes in children with HF over those from the 1970s digoxin and diuretic era therapy [5,10,11]. While primary disorders of mitochondrial respiration are a known cause of myocardial dysfunction in children and adults, secondary mitochondrial dysfunction may be a down-stream effect contributing to heart failure [12–15]. Whether mitochondrial dysfunction is a primary cause or consequence of heart failure, improving myocardial energy utilization is an attractive target for new therapies to improve outcomes in the pediatric population [16].

Cardiolipin (CL) is a major cardiac phospholipid found almost exclusively in the mitochondrial inner membrane where it is essential for the optimal function of key energy producing enzymes in the electron transport chain (ETC) [17–20]. A known genetic defect in the CL pathway leads to decreased expression of the CL remodeling enzyme, tafazzin, in X-linked Barth syndrome that is characterized by cardiomyopathy, skeletal myopathy and developmental delay in boys [21]. Evidence from Barth syndrome and other models suggests that CL

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http://dx.doi.org/10.1016/j.yjmcc.2014.06.002
0022-2828/Published by Elsevier Ltd.

must be in the tetralinoleoyl form [i.e. 4 linoleic acid side chains, or $(18:2)_4$CL)] in order to allow optimal function of numerous mitochondrial systems related to energy production [11–13]. Nascent CL is synthesized by conjugation of two di-acylglycerols with non-specific fatty acid side chain incorporation, then remodeled into $(18:2)_4$CL via a process where linoleoyl moieties are incorporated onto the di-phosphatidylglycerol backbone via tafazzin and other remodeling transacylases. Calcium-independent phospholipases iPLA2-G6 and iPLA2-γ have both been implicated in removal of specific CL fatty acid side-chains in the remodeling process [22,23]. Proper synthesis, remodeling, and degradation of CL species are essential to maintain mitochondrial electron flux and metabolic integrity of the ETC, preserving the ATP content, and permitting normal myocardial performance [17,24–27].

Previous work has shown that decreases in the linoleoyl content of CL are dramatic in adult idiopathic dilated cardiomyopathy (IDC) and in a rat model of heart failure [28]. Additionally, in the spontaneously hypertensive HF rat model (SHHF), a well-established congenital model of IDC, a high linoleic acid diet can restore cardiac $(18:2)_4$CL levels and markedly increase survival [29,30]. This dietary intervention also improves mitochondrial function (measured by cytochrome C oxidase activity), arrests the usual decline in systolic cardiac function, and improves survival in the SHHF rat. Changes in expression of enzymes in the CL biosynthesis pathway have been shown in cardiac tissue from SHHF rats and adults with IDC corresponding with these changes in CL composition [31]. Specifically, we have previously shown that in adult IDC, the biosynthetic enzyme cytidinediphosphatediacylglycerol synthetase (CDS-2) and the remodeling enzyme tafazzin (TAZ) are significantly down-regulated when compared to nonfailing controls, which parallels findings in the rat model. Cardiolipin synthase (CLS), the last step in the biosynthetic pathway, was down-regulated in the failing rat heart as well, although a significant difference was not observed in CLS expression between normal and IDC in human tissue. This work demonstrated that derangements in CL content and composition are observed in human heart failure, likely related to changes in both biosynthesis and remodeling of this mitochondrial phospholipid. The aim of the current study was to directly assess whether CL compositional abnormalities are present in ventricular tissue from children with IDC, compare these to CL composition changes in adults with IDC, and identify changes in expression of biosynthetic and remodeling enzymes associated with changes in CL content and composition.

## 2. Methods

### 2.1. Subjects

Subjects were males and females of all races and ethnic backgrounds that donated their heart to the COMIRB approved pediatric and adult transplant tissue banks at the University of Colorado. All non-failing (NF) hearts were donor hearts with normal left ventricular ejection fraction (LVEF) not transplanted for technical reasons and all in HF were idiopathic dilated cardiomyopathic (IDC) hearts (i.e. not ischemic cardiomyopathic hearts or cardiomyopathies from congenital heart disease) with LVEF <30. Pediatric contents were between the ages of 0 and 18 years, 54 IDC (39% male), and 23 NF controls (54% male). Post-hoc analysis was performed to compare pre-pubertal children with adolescent subjects. Adult samples included tissue from individuals aged 20–66 years in the IDC group (27 samples, 56% male) and 44–69 years in the NF group (15 samples, 47% male).

### 2.2. Left ventricle tissue from pediatric patients with IDC and non-failing controls

At the time of cardiac transplantation, the explanted hearts were immediately cooled in ice cold oxygenated Tyrodes in the operating room. Left ventricle tissue was rapidly dissected flash frozen and stored at −80 °C.

### 2.3. CL molecular species quantification

Lipid was extracted from pediatric LV tissue homogenates (pediatric group *n*: NF 20, IDC 44, adult group *n*: NF 10, IDC 15) for quantification by normal phase high pressure liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) as described by Sparagna *et al.* [32]. Using 1,1′,2,2′-tetramyristoyl CL as an internal standard ESI-MS was employed for quantification of total CL from the 7 most prevalent molecular species present in human heart tissue (mass/charge or *m/z* 1186, 1422, 1424, 1448, 1450, 1472, and 1474) measured individually (Table 1). These species comprise >95% of CL present in human myocardium. CLs were extracted from LV tissue from IDC and non-failing control samples, and quantitated by ESI-MS. Total (absolute) amounts of detectable CL species were quantitated in nmol per milligram of protein extracted. The fractional content of each CL species was calculated based on the total CL content for each sample as a percentage. Mass/charge species are defined using abbreviations for monolyso (M), palmitic (P), palmitoleic acid (Po), linoleic (L), oleic (O), and arachidonic (Ar) acid side chains.

### 2.4. Real-time quantitative PCR (RT-qPCR)

RNA extracted from adult (*n*: NF 15, IDC 27) and pediatric (*n*: NF 23, IDC 54) LV tissue (Ambion mirVana isolation kit, manufacturer's protocol) was reverse-transcribed to cDNA using the Qiagen miScript II RT kit (per manufacturer's protocol). RNA quality was verified using NanoDrop® ND-1000 UV-Vis Spectrophotometer analysis prior to RT-qPCR, performed at the University of Colorado Denver Genomics and Microarray Core. The SYBR Green method was used to quantify enzyme expression using 10 ng cDNA per reaction using the AB StepOne Rapid RT-PCR protocol. All reactions were performed in duplicate with melting curves to ensure specificity of PCR product, and normalized to 18S expression. No difference in expression of 18S between groups was appreciated. RT expression was measured using the delta delta CT method as previously described (values compared to non-failing controls) [33]. Primer sets for target genes are listed in Table II (supplement).

### 2.5. Statistical analysis

Data is expressed as means +/− SEM. The difference between two groups was evaluated by Students *t*-test. Comparisons were considered to be significant for $p$ values <0.05 unless otherwise noted.

## 3. Results

### 3.1. Total CL and $(18:2)_4$CL content is depleted in left ventricular myocardium from pediatric patients with IDC

To determine if total and $(18:2)_4$CL content is altered in pediatric IDC, similar to what has been demonstrated in adults, CL species were

**Table 1**
Major cardiolipin species detected in human heart.

| *m/z* | Fatty acid side chain composition | Abbreviation |
|---|---|---|
| 1186 | $(18:2)_3$ CL | $L_3$ |
| 1422 | $(18:2)_3(16:1)_1$ CL | $L_3Po_1$ |
| 1424 | $(18:2)_3(16:0)_1$ CL | $L_3P_1$ |
| 1448 | $(18:2)_4$ CL | $L_4$ |
| 1450 | $(18:2)_3(18:1)_1$ CL | $L_3O_1$ |
| 1472 | $(18:2)_3(20:4)_1$ CL | $L_3Ar_1$ |
| 1474 | $(18:2)_2(18:1)_1(20:4)_1$ CL | $L_2O_1Ar_1$ |

Ar, arachidonic acid; L, linoleic acid; O, oleic acid; P, palmitic acid; Po, palmitoleic acid.