A mass spectrometric approach to investigate cardiolipin metabolism in Barth syndrome
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CHAPTER 5

Cardiolipin deficiency in X-linked cardioskeletal myopathy and neutropenia (Barth syndrome, MIM 302060): a study in cultured skin fibroblasts

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OBJECTIVE

To determine cardiolipin concentrations in cultured skin fibroblasts of patients with X-linked cardioskeletal myopathy and neutropenia (Barth syndrome, MIM 302060) and in two groups of controls and to evaluate whether specific differences exist between these groups. Method: High Performance Liquid Chromatography-Electrospray Mass spectrometry (HPLC-ES-MS) was used to quantify total cardiolipin and subclasses of cardiolipin molecular species in cultured skin fibroblasts. Groups: normal controls (n=10), patients with various mitochondrial disorders (n=8) and patients with Barth syndrome (n=5). Results were compared in the three groups by using a t-student test for all individual analyses.

RESULTS

Total cardiolipin and cardiolipin subclasses were decreased in BTHS patients as compared to normal controls and disease controls. Conclusion: Barth syndrome patients have a specific decrease of various cardiolipin molecular species, foremost tetratlineoyl-cardiolipin. Therefore the analysis of cardiolipin in fibroblasts offers a specific biochemical approach to detect this disorder.

INTRODUCTION

X-linked cardioskeletal myopathy and neutropenia (Barth syndrome, MIM 302060) is an X-linked recessive dilated cardiomyopathy, neutropenia and skeletal myopathy (1). Biochemical findings include variable mitochondrial respiratory chain dysfunction in skeletal muscle and in cultured fibroblasts, increased urinary excretion of 3-methylglutaconic acid, 3-methylglutaric acid, and 2-ethylhydracrylic acid and moderately decreased serum cholesterol. The gene involved in the disease is the Tafazzin (TAZ) gene, which is localized on Xq28. Mutations have been identified in nearly all Barth syndrome (BTHS) patients.

In 1997 Neuwald (2) reported that the TAZ gene shares homology with acyltransferases, involved in phospholipid biosynthesis and/or remodeling, suggesting that a specific glycerophospholipid might be lacking in BTHS. We studied the biosynthesis and remodeling of the phospholipids phosphatidylglycerol (PG) and cardiolipin, which are mainly confined to mitochondrial membranes, in cultured skin fibroblasts from BTHS patients and controls. These studies revealed reduced levels of CL and a disturbance in the remodeling of PG and CL. In particular the incorporation of linoleic acid into PG and CL was dramatically reduced, whereas the incorporation of other fatty acids into these phospholipids was normal (3). These studies proved defective remodeling of cardiolipin as a characteristic and probably basic disturbance in BTHS patients. The method however is laborious and involves incorporation of radioactive labeled linoleic acid into skin fibroblasts. We therefore set out to
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develop a method that is less laborious and avoids the use of radioactively labeled chemicals.

The quantitative and compositional analysis of CL in cultured skin fibroblasts by normal phase High Performance Liquid Chromatography - Electrospray Mass Spectrometry is a more direct and rapid method and therefore can be useful to identify patients with BTHS.

MATERIAL AND METHODS

PATIENTS

Five patients with BTHS and known mutations in the TAZ gene were included in this study. The group included a patient with ventricular non-compaction, a previously identified subgroup of patients with a TAZ gene mutation. Individual patient data are presented in table 1.

CONTROL GROUPS

1. Fibroblast lines from healthy controls (n=10) and 2. Fibroblast lines from patients with disorders of energy metabolism (n=10) including mitochondrial chain disorders: isolated complex I deficiency (n=1), isolated complex IV deficiency (n=3), MERRF (tRNALys G8344A mutation) (n=1), Leigh syndrome with NARP (T8993C mutation) (n=1), autosomal dominant intergenomic signalling defect (n=1), and a female patient with 3-methylglutaconic aciduria, leukoencephalopathy and lactic acidosis (n=1).

MATERIAL

All solutions were of analytical quality and were purchased from Merck (Darmstadt, Germany). (C14:0)4-CL (internal standard) was purchased from Avanti lipids (Alabaster, USA). Commercially available (C18:2)4-CL was purchased from Fluka BioChemika (Fluka art No. 71238, Switzerland). Analytical HPLC LiChrospher Si 60 column (2 x 250 mm, 5 μm particle size) was purchased from Merck (Darmstadt, Germany).

CELL CULTURE

Fibroblasts were grown in HAM-F10 medium supplemented with 10% FCS and incubated for 10 days at 37 °C. After trypsinization, cells were collected and centrifuged at 500 g for 5 min and washed twice with 2 ml of phosphate buffered saline (PBS). The pellets were suspended in 1 ml of water and sonificated 2 times during 10 seconds. Ten μL of this solution was used for protein determination.

LIPID EXTRACTION

Lipids were extracted by using the method of Bligh & Dyer (5) and were dissolved in 200 μL chloroform/methanol/water (50:45:5, v/v/v, lipid extract). A portion of this lipid extract was used to measure the total organic phosphorous which is direct reflection of the total amount of phospholipids.

HPLC

The HPLC system consisted of an HP1100 series binary gradient pump, a vacuum
degasser, and a column temperature controller (all from Hewlett Packard) and a Gilson 231 XL autosampler (Gilson). The column temperature was maintained at 22°C. The lipid extract was injected onto a 2.1 x 250 mm silica column, 5 μm particles diameter. The phospholipids were separated from interfering compounds by a linear gradient between solution B (chloroform) and solution A (methanol/water, 9:1 v/v). Both solutions contained 0.01 % of a 25% aqueous ammonia solution. The gradient (0.3 ml/min) was as follows: 0-3 min: 20 % A to 100 % A, 3-8 min: 100% A, 8-8.1 min to 0% A and 8.1-14 min equilibration with 0% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 14 min. A splitter between the HPLC column and the mass spectrometer was used and 30 μl/min of eluant was introduced into the mass spectrometer. An electrically operated valve was used so that only the eluant from 4 to 8 min was introduced into the mass spectrometer.

**MASS SPECTROMETRY**

A Quattro II triple-quadrupole mass spectrometer (Micromass), was used in the negative ESI mode. Nitrogen was used as nebulizing gas. Argon was used as collision gas at a pressure of 2.5 x 10-3 mBar. The capillary voltage used was 3kV. The source temperature was set at 80 °C. Optimal cone voltage energy was 30 V MS and MS/MS (daughter fragments, optimal collision energy of 40 eV) analysis was performed to identify the fatty acid composition of PG and CL molecular species (as indicated in tables 3 and 4).

All CL molecular species including IS were measured by Single Ion Monitoring of their double charged ion in negative mode.

**VALIDATION**

The method was validated using control fibroblasts, commercially available (C18:2)_4-CL as standard analyte and (C14:0)_4-CL as internal standard. A standard procedure for validation of analytical methods was followed.

**STATISTICS**

P values are calculated for each CL molecular species in both control groups versus the same CL molecular species in the BTHS group and were obtained by using the t-student test for all individual analysis.

**RESULTS** The recovery of the method was 92.8% +/- 3.8. Both (C18:2)_4-CL and (C14:0)_4-CL (IS) ions were suppressed by 23.1 +/- 2.9% and 25.9 +/- 2.1. The intra-assay variation and inter-assay variation were 8.6% and 12.1% respectively. The response factor was calculated as the ratio of (C18:2)_4-CL / (C14:0)_4-CL from the analysis of calibration mixture before each set of analyses. The method was not validated for a quantitative measurement of PG.
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<table>
<thead>
<tr>
<th>Patients</th>
<th>Age at onset and presenting symptom</th>
<th>Age</th>
<th>Dilated cardiomyopathy</th>
<th>Mild proximal muscle weakness</th>
<th>Neutropenia</th>
<th>Statural growth -SD</th>
<th>Organic asiduria</th>
<th>TAZ gene mutation and effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3 y muscle weakness</td>
<td>11.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-3</td>
<td>3-mcga, 3-mgra, 2-eha</td>
<td>C441G premature stopcodon exon 2</td>
</tr>
<tr>
<td>2</td>
<td>0.5 y failure to thrive</td>
<td>Died 1.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-2</td>
<td>3-mcga, 3-mgra, 2-eha</td>
<td>G527-1 G&gt;C</td>
</tr>
<tr>
<td>3</td>
<td>1.1 y cardiac decompensation</td>
<td>14.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-3</td>
<td>3-mcga, 3-mgra, 2-eha</td>
<td>428de113 frameshift exon2</td>
</tr>
<tr>
<td>4</td>
<td>6 wk cardiac decompensation</td>
<td>10.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-2</td>
<td>3-mcga</td>
<td>G877T glycine &gt;valine exon8</td>
</tr>
<tr>
<td>5</td>
<td>1st month non-specific illness, abnormal cardiac echochram</td>
<td>1.7</td>
<td>+</td>
<td>ventricular noncompaction</td>
<td>+</td>
<td>+</td>
<td>-2</td>
<td>3-mcga, 3-mgra</td>
</tr>
</tbody>
</table>

Table 1: Clinical, biochemical and genetic findings. Reference sequence Genebank Nr. NM_000116. Tafazzin, TAZ. Abbreviations: 3-mcga 3-methyl-giutaconic acid; 3-mgra 3-methylglutaric acid; 2-eha 2 ethyl-hydracrylic acid

CL and PG were successfully separated from the non-polar lipids and other phospholipids by HPLC resulting in a more pure fraction. The purity of this fraction was not established by other methods. However the MS spectra showed some co-eluting components in the mass range lower than m/z 500. After separation by HPLC, individual CL and PG molecular species were measured by scanning of respectively negatively double and single charged ions. The levels of the most abundant CL species (C18:2)4-CL, (C18:2)3/(C18:1)-CL and (C18:2)2/(C18:1)2-CL in cultured fibroblasts from the individual samples belonging to BTHS patients and control groups were then quantified (table 2).

Table 2 shows the median and the range of the concentrations (nmol/mg protein) of different CL molecular species for each group. The levels of CL species were decreased in BTHS patients while there were no significant differences between the normal control group and the group of mitochondrial disorders. The fatty acid composition of various CL molecular species was determined by MS/MS in fibroblasts of all groups. No differences were observed in the fatty acid compositions of molecular species with the same mass in the different groups used in this study. Linoleic acid forms the predominant fatty acid in the CL fraction (table 2). The fatty acid composition of various PG molecular species was established by MS/MS analysis of each PG compound. In comparison to CL, the fatty acids of the PG molecular species contain more C18:1, C16:0, C20:4 and C20:2. Although the PG molecular
species were not quantified in this study, we detected qualitative differences between the control groups and BTHS group. The relative levels of PG molecular species containing linoleic acid compared to PG molecular species containing other fatty acids were very low. One PG compound (at m/z 797.6) had the fatty acid composition C18:1/C20:3 in controls while the same mass in BTHS patients had both C18:1/C20:3 (major) and C18:2/C20:2 (minor). The median, lowest and the highest concentrations (nmol/mg protein) of different CL molecular species in controls (n=10), disease controls (n=8) and BTHS patients (n=5) are shown. A significant difference is found between both controls groups and BTHS patients’ fibroblasts.

**Table 2: Concentrations (nmol/mg protein) of the most abundant CL molecular species in cultured fibroblasts from all groups.**

<table>
<thead>
<tr>
<th>Normal control patients (n=10)</th>
<th>Mitochondrial disease control patients (n=8)</th>
<th>BTHS (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C18:2)4-CL</td>
<td>(C18:2)4-CL</td>
<td>(C18:2)4-CL</td>
</tr>
<tr>
<td>Median</td>
<td>0.70</td>
<td>0.09</td>
</tr>
<tr>
<td>Range</td>
<td>0.63-0.94</td>
<td>0.05-0.14</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>0.66-1.45</td>
<td>0.06-0.42</td>
</tr>
<tr>
<td></td>
<td>1.92</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>0.81-2.13</td>
<td>0.89-1.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vs BTHS group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>.0003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>.0006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>.0003</td>
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<td>.0008</td>
<td></td>
</tr>
<tr>
<td></td>
<td>.00009</td>
<td></td>
</tr>
</tbody>
</table>

The median, lowest and the highest concentrations (nmol/mg protein) of different CL molecular species in controls (n=10), disease controls (n=8) and BTHS patients (n=5) are shown. A significant difference is found between both controls groups and BTHS patients’ fibroblasts.

**DISCUSSION** The method applied in this paper is a fast and reproducible method for quantitative measurement of CL and compositional analysis of PG in fibroblasts. In the present study we show that various cardiolipin molecular species are deficient in fibroblasts of BTHS patients. Because cardiolipin is normally synthesized in BTHS cells, as previously shown (3), this deficiency is most probably the result of its deficient fatty acid remodeling. In mammalian tissues the biosynthesis of cardiolipin occurs via the cytidinediphosphate-diacylglycerol (CDP-DG) pathway (6). Newly formed CL undergoes extensive remodeling by deacylation and reacylation in order to produce the specific C18:2-C18:2 and C18:1-C18:2 diacyl combinations which are observed in mammalian CL (6-10).

These studies indicate that tetraineoyl-cardiolipin is the most important cardiolipin species. Most of the cardiolipin is found in the inner mitochondrial membrane. Cardiolipin
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is needed for proper functioning of respiratory chain components, especially cytochrome C. Loss of cardiolipin from the inner mitochondrial membrane causes loss of cytochrome C into the cytosol and represents a crucial step in cellular apoptosis (11). Variable involvement of several respiratory chain complexes has been reported in some publications dealing with mitochondrial function in BTHS (1).

The significance of CL for respiratory chain functioning has been shown previously in yeast cells deficient in cardiolipin (12-13) and Chinese hamster ovary (CHO) cells genetically deficient in cardiolipin (14). The precise role of cardiolipin in the functioning of the inner mitochondrial membrane is yet to be determined. Other, less well known roles for cardiolipin are suggested by papers reporting the presence of free cardiolipin in plasma (15), surface expression on apoptotic cells (16) and a bound form occurring together with chromatin (17).

We observed differences within the patients' group with respect to CL levels. Patient 4 has the lowest CL level (total CL level: 0.20 nmol/mg protein) while patient 1 has the highest total CL level of 0.53 nmol/mg protein. Patient 1 has a mutation in exon 2 resulting in a premature stop codon. This mutation causes loss of the first of the two ATG-sites from which 5'-translation starts which causes loss of the membrane anchoring (N-terminal) end of tafazzin protein, but still allows gene translation starting from the second ATG-site on exon 3. Patient 4 has a missense mutation in exon 8, leading to structural changes in all 10 tafazzin proteins, that can be predicted on the basis of the gene structure.

The clinical involvement of patients 1 and 4 is approximately of the same degree. It is therefore not possible to relate absolute levels of CL in fibroblasts to specific TAZ mutations or to severity of clinical involvement. We did not quantify the PG molecular species. We observed only some minor changes in fatty acid composition of PG molecular species in one of BTHS patients. The relative levels of PG molecular species containing linoleic acid compared to PG molecular species containing other fatty acids were very low while CL pool has a high content of linoleic acid. These data indicate that the newly formed CL from PG has to be remodeled to form CL molecular species with high content of linoleic acid.

We conclude that analysis of cardiolipin in cultured skin fibroblasts by HPLC-ESI-MS is a fast and simple method to identify patients with BTHS which is less laborious and avoids the use of radioactively labeled chemicals. However, there is no evidence yet that the decreased level of CL is a primary effect of deficiency of one or more tafazzin proteins. Thus at this time a gene mutation analysis has to be performed to complete the diagnosis. Compared to our earlier published method (3), the method presented here gives more information about the compounds involved.

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REFERENCES