A mass spectrometric approach to investigate cardiolipin metabolism in Barth syndrome
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Linoleic acid supplementation of Barth syndrome fibroblasts restores cardiolipin levels: implications for treatment

F. Valianpour, R. J. A. Wanders, H. Overmars, F. M. Vaz, P. G. Barth, and A. H. van Gennip

ABSTRACT The object of this study was to investigate whether the levels of cardiolipin in cultured skin fibroblasts of patients with Barth syndrome (BTHS) can be restored by addition of linoleic acid to growth media. To this end, fibroblasts from controls and BTHS patients were grown in the presence or absence of linoleic acid. High-performance liquid chromatography-electrospray ionization tandem mass spectrometry was used for quantitative and compositional analysis of cardiolipin.

Incubation of cells from both BTHS and controls with different concentrations of linoleic acid led to a dose- and time-dependent increase of cardiolipin levels. The increased levels of cardiolipin in fibroblasts of BTHS patients after treatment with linoleic acid indicate that an increased amount of linoleic acid in the diet might be beneficial to BTHS patients.

INTRODUCTION X-linked cardioskeletal myopathy and neutropenia (Barth syndrome, BTHS) (MIM 302060) is an X-linked recessive disorder characterized by infantile or childhood onset of dilated cardiomyopathy, neutropenia, skeletal myopathy (1), abnormal mitochondrial ultrastructure (2-3), and variable mitochondrial respiratory chain dysfunction not related to a single respiratory chain complex in skeletal muscle (1, 4) and in cultured fibroblasts (5). Biochemical findings include increased urinary excretion of 3-methylglutaconic acid, 3-methylglutaric acid, and 2-ethylhydracrylic acid (6-7), and moderately decreased serum cholesterol levels. The gene mutated in this disorder is the G4.5 gene or Tafazzin (TAZ) gene (8), which is localized on Xq28 (9). In 1997, Neuwald (10) reported that the TAZ gene shares homology with acyltransferases involved in phospholipid biosynthesis and/or remodeling, suggesting the possibility of abnormalities in glycerophospholipid formation in BTHS.

This prompted us to study phospholipid metabolism in cultured skin fibroblasts from BTHS patients. We found reduced levels of cardiolipin (CL) and a disturbance in the remodeling of phosphatidylglycerol (PG) and CL (11). 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. We also showed that the incorporation of linoleic acid into PG and CL in fibroblasts of patients with a variety of mitochondrial disorders different from BTHS was entirely normal. The biosynthesis of CL in BTHS patients was normal, while its pool size was decreased. The biosynthesis of PG, the precursor in de novo synthesis of CL, and its pool size were both slightly increased in BTHS patients.

These results lead to the conclusion that the decreased levels of CL in BTHS patients were due to defective remodeling of CL. In mammals, the biosynthesis of cardiolipin occurs via the cytidinediphosphate-diacylglycerol (CDP-DG) pathway (12). 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 that are observed in mammalian CL (12–16). The aim of the present study was to investigate whether addition of linoleic acid to the growth medium could restore the levels in CL in BTHS fibroblasts. For the analysis of CL and PG molecular species in this study, we used on-line normal-phase high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) (17).

MATERIAL AND METHODS

PATIENTS

Fibroblast cell lines from patients with BTHS and healthy controls were included in this study. Individual patient clinical data are presented in table 1.

MATERIALS

All solutions were of analytical grade and were purchased from Merck (Darmstadt, Germany). (C14:0)₄-CL (internal standard, IS) was purchased from Avanti lipids (Alabaster, AL). (C18:2)₄-CL was purchased from Fluka BioChemika (Fluka, Buchs, Switzerland). Linoleic acid (C18:2) was purchased from Sigma (Sigma, St. Louis, MO). Analytical HPLC LiChrospher Si 60 column (2.1x 250 mm, 5 μm particle size) was purchased from Merck.

LINOLEIC ACID SUPPLEMENTATION STUDIES

Fibroblasts were grown at 37 °C under standard conditions in HAM-F10 medium supplemented with 10% FCS. This medium contains a final concentration of 5–6 μM of linoleic acid (standard growth medium). For dose-dependency experiments, the standard growth medium was supplemented with linoleic acid ranging from 3.5–50 μM.

For the time-dependency experiments, fibroblasts were grown for a time period up to 5 days using the standard growth medium in the presence or absence of 50 μM of additional linoleic acid. Growth media were refreshed every 48 h. Cells were collected after trypsinization, centrifuged at 500 g for 5 min, washed twice with 2 ml of PBS, and stored at -80°C until analysis.

HPLC-MS/MS ANALYSIS

The compositional and quantitative analysis of CL and compositional analysis of PG were performed using the method described by Valianpour et al. (17). In order to investigate whether the differences in the sample matrix could affect the results, the extraction efficiency, recovery of the method, and ion suppression were determined for fibroblasts.

STATISTICS

The results of the experiments in BTHS patients and controls were compared by a Student's t-test for all individual analysis.
RESULTS The method used here was originally developed and validated for the quantitative and compositional analysis of CL in platelets (17). In order to establish whether the method can also be used successfully in fibroblasts, we determined the extraction efficiency, recovery of the method, and ion suppression in fibroblast samples to verify whether the analysis was matrix dependent. The extraction efficiency of (C18:2)₄-CL was 78.1 +/- 2.4% (n=10). The recovery after HPLC was 92.8% +/- 3.8 (n=5). The total recovery of the method was 75.6% +/- 3.1. Both (C18:2)₄-CL (analyte) and (C14:0)₄-CL (IS) ions were suppressed by 23.1 +/- 2.9% and 25.9 +/- 2.1% (n=5), respectively, indicating that (C14:0)₄-CL can be used for the quantitative analysis of CL in fibroblasts.

Control fibroblasts and fibroblasts from a BTHS patient were grown in the presence or absence of additional linoleic acid (50 µM). Mass spectra revealed markedly reduced levels of the different CL molecular species in fibroblasts of BTHS patients compared with controls when the cells were grown in standard medium (Figure 1B, C). In the presence of 50 µM of linoleic acid, however, the levels of the most abundant CL species were markedly increased in fibroblasts from the BTHS patient (Figure 1A).

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age at onset and presenting symptom</th>
<th>Age in years at time of study</th>
<th>Dilated cardiomyopathy</th>
<th>Mild peroximal muscle weakness</th>
<th>Neutropenia</th>
<th>Staturnal growth -SD</th>
<th>Organic asiduria</th>
<th>TAZ gene mutation</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-mgca, 3-mgra, 2-eha</td>
<td>C441G premature</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-mgca, 3-mgra, 2-eha</td>
<td>G327 ins 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-mgca, 3-mgra, 2-eha</td>
<td>428del13</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-mgca</td>
<td>G877T</td>
</tr>
<tr>
<td>5</td>
<td>1st month non-specific illness, abnormal cardiac echocromam</td>
<td>1.7</td>
<td>+</td>
<td>ventricular noncompaction</td>
<td>+</td>
<td>-2</td>
<td>3-mgca, 3-mgra</td>
<td>exon2, AG &gt; AC splice acceptor site</td>
</tr>
</tbody>
</table>

Reference sequence Genebank Nr. NM_000116, Tafazzin, TAZ. Abbreviations: 3-mgca 3-methyl-glutaconic acid; 3-mgra 3-methyl-glutaric acid; 2-eha 2 ethyl-hydracrylic acid

The fatty acid composition of the most abundant CL molecular species at m/z 723.7, 724.6, and 725.7 was established by MS/MS (Figure 1D–1F). The CL molecular species at m/z
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723.7 exclusively contains C18:2 (Figure 1D), while the CL species at m/z 724.6 and 725.7 contain both C18:2 as well as C18:1 (Figure 1E, F). The contribution of the isotope peaks, however, is likely the reason that the ratio of C18:2/C18:1 in Figure 1E and F is not, as one would expect, equal to three for \([(\text{C18:2})_3/(\text{C18:1})_1-2\text{H}]^{2-}\) or one for \([(\text{C18:2})_2/(\text{C18:1})_2-2\text{H}]^{2-}\). The possible fatty acid composition of CL molecular species in fibroblasts of control and BTHS patients grown in linoleic acid enriched medium are summarized in Table 2. As indicated in this table, CL molecular species, except the one with m/z 738.6, have one or more C18:2 moieties, indicating that linoleic acid is the predominant fatty acid in the CL fraction.

In order to study whether the effect of linoleic acid was restricted to CL, we also studied the levels and fatty acid composition of other phospholipids. As shown in Figure 2, different molecular species of PG, the precursor in the biosynthesis of CL, were detected in fibroblasts of BTHS patients. No differences were observed in the PG molecular species between the control subjects and BTHS patients when fibroblasts were grown in standard or enriched medium (data not shown). In the presence of linoleic acid, (C18:2)_{2}-PG (m/z 769.6) considerably increased compared with the other PG molecular species in both patient and control cell lines (Figure 2A, B; shown only for the BTHS patient). The fatty acid composition of the observed molecular species of PG was established as shown in Figure 2C–2F. The mass at m/z 769.6 contains C18:2 (linoleic acid) exclusively. The mass at 797.6 represents two different fatty acid compositions. The most abundant one is C18:1/C20:3 as shown in Figure 2D, where the other composition, C18:2/C20:2, is less abundant.

<table>
<thead>
<tr>
<th>m/z</th>
<th>CL Molecular species</th>
<th>m/z</th>
<th>PG Molecular species</th>
</tr>
</thead>
<tbody>
<tr>
<td>710.6</td>
<td>(C18:2)/w(C16:1)-CL</td>
<td>745.6</td>
<td>(C16:1/C18:1)-PG</td>
</tr>
<tr>
<td>711.7</td>
<td>(C18:2)/w(C16:0)-CL</td>
<td>747.6</td>
<td>(C18:0/C18:1)-PG</td>
</tr>
<tr>
<td>723.7*</td>
<td>(C18:2)-CL</td>
<td>769.6</td>
<td>(C18:2)-PG</td>
</tr>
<tr>
<td>724.6*</td>
<td>(C18:2)/w(C18:1)-CL</td>
<td>771.6</td>
<td>(C18:2/C18:1)-PG</td>
</tr>
<tr>
<td>725.7*</td>
<td>(C18:2)/w(C18:1)-2CL</td>
<td>773.6</td>
<td>(C18:1)-PG</td>
</tr>
<tr>
<td>736.6</td>
<td>((C18:2)/w(C18:1/C20:4)-CL</td>
<td>775.6</td>
<td>(C18:0/C18:1)-PG</td>
</tr>
<tr>
<td>737.7</td>
<td>(C18:2)/(C18:1)/w(C20:4)-CL</td>
<td>795.7</td>
<td>(C18:1/C20:4)-PG</td>
</tr>
<tr>
<td>738.6</td>
<td>((C18:1)/w(C20:4)-CL</td>
<td>797.6</td>
<td>(C18:1/C20:3)-PG and (C18:2/C20:2)-PG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>799.7</td>
<td>(C18:1/-C20:2)-PG</td>
</tr>
</tbody>
</table>

CL, cardiolipin; PG, phosphatidylglycerol. (a): The fatty acid composition was established using MS/MS analysis.
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The peaks at m/z 747.6 and m/z 773.6 correspond to the fatty acid compositions C16:0/C18:1 and C18:1/C18:1, respectively, as shown in Figure 2E and F. Compositional analysis of other phospholipid major classes showed that these all contained a higher amount of linoleic acid when cells were grown in the presence of enriched medium (data not shown), indicating that the effect of linoleic acid was not restricted to CL and PG. The results of the linoleic acid supplementation studies are shown in figure 3 and table 3. In order to study the dose-dependent effect of linoleic acid on the CL levels in fibroblasts, cells from controls and patients were grown for 3 days in the presence of increasing concentrations of linoleic acid in the growth medium (Figure 3A). Figure 3A shows a dose-dependent increase in the levels of CL in controls and patients. In the presence of 50 μM of added linoleic acid, the CL levels in the patients approached the normal range of CL in non-treated controls.

Figure 1: Mass spectrum of cardiolipin (CL) molecular species from fibroblasts of a Barth syndrome (BTHS) patient grown in enriched medium (A), grown in standard medium (B), and control cells grown in standard medium (C). Increased levels of CL in BTHS cell line were observed when the cells were grown in enriched medium (A). Daughter fragments of the three most abundant CL molecular species, m/z 723.7 (D), m/z 724.6 (C), and 725.7 (F), are also shown.
In order to study the time-dependent effect of linoleic acid on the CL levels in fibroblasts, cells were grown in the absence (Figure 3B) or presence (Figure 3C) of 50 μM of added linoleic acid in growth medium during a period of up to 5 days. As shown in Figure 3B, the levels of CL remained virtually stable when the cells were grown in the absence of added linoleic acid, both in control and the patients' cell lines, although the levels of CL in patient cells were considerably lower. In contrast, supplementation with linoleic acid resulted in a time-dependent increase of CL levels in both BTHS and control cell lines (Figure 3C).

To establish whether the effect of linoleic acid on the levels of CL was reversible, cells from controls and patients were first grown in the presence of supplemented linoleic acid for 3 days. Subsequently, the medium was replaced by standard medium and cells were grown for another 5 days. After the removal of the supplemented medium, the levels of CL returned to their pre-treatment values in both BTHS and control cell lines, showing that the linoleic acid effect is reversible (Figure 3D). Table 3 depicts the results of the CL levels (mean +/- SD) in fibroblasts of both control and BTHS patient groups after a 3-day incubation in the presence or absence of added linoleic acid. The linoleic acid supplementation resulted in a highly significant increase of the three most abundant molecular species in BTHS and control cells (P < 0.001).

**DISCUSSION** Since CL and PG are required for proper mitochondrial functioning (18–23) and CL levels are markedly reduced in BTHS patients (11, 17), we studied the effect of linoleic acid supplementation on CL levels in cultured skin fibroblasts of BTHS patients and healthy controls. The main question in this study was whether the levels of CL in BTHS patients could be restored when the fibroblasts were grown in the presence of relatively high amounts of linoleic acid. Comparison of our validation data for the CL analysis in fibroblasts with the CL analysis in platelets (17) shows that this method is suitable for the compositional and quantitative analysis of CL in fibroblasts.

As shown in this paper, there was a time- and dose-dependent increase of the CL levels when the cells were grown in the presence of supplemental linoleic acid. This leads to the restoration of the CL levels to near-normal values in skin fibroblasts of patients suffering from BTHS. We observed that the linoleic acid content of all major phospholipids, including PG, which is the precursor in de novo synthesis of CL, increased when cells were grown in the presence of supplemental linoleic acid. The levels of (C18:2)_{2}-PG increased considerably in treated cells, indicating that the increased levels of CL result from de novo synthesis of CL from (C18:2)_{2}-PG. This would lead to the formation of CL molecular species with a higher content of linoleic acid, which is indeed observed after treatment. Based on this
mechanism, one would expect that both control and BTHS cells would reach the same CL values upon linoleic acid supplementation. In contrast, the CL levels in BTHS fibroblasts after linoleic acid treatment were considerably lower than those in controls. This observation might be explained by the remodeling mechanism of CL (15). CL remodeling takes place via a deacylation step, which is catalyzed by phospholipase A, followed by a reacylation step, which is catalyzed by specific acyltransferase. Our hypothesis is that the TAZ gene product(s) are responsible for the acyltransferase step.

![Mass spectrum of phosphatidylglycerol (PG) molecular species in fibroblasts of a BTHS patient grown in standard medium (A) and medium enriched with 50 M of linoleic acid (B). The peak at m/z 769.6 (C18:2)-PG is markedly increased after treatment with linoleic acid. Daughter fragments of PG molecular species, (C18:2)-PG (m/z 769.6), C18:1/C20:3-PG, and C18:2/C20:2-PG (m/z 797.6, D), C16:0/C18:1-PG (m/z 747.6, E), and (C18:1)2-PG (m/z 773.6, F), are also shown.]

Since the first step of CL remodeling is still active in BTHS cells, this might lead to degradation of newly formed CL, which cannot be converted back into CL because of the deficient acyltransferase. This might cause the differences in CL levels in control versus BTHS cells after supplementation with linoleic acid. The higher levels of CL in both cell lines returned to the pretreatment values when the enriched medium was replaced by standard growth medium, indicating that the observed increased levels of CL were directly caused by the supplementation of linoleic acid.

The results described in this report suggest that a treatment based on dietary supplementation of linoleic acid may lead to an increase of CL levels in different tissues and that this may be beneficial to BTHS patients. We have initiated a clinical trial to investigate whether the treatment described for fibroblasts in vitro can also be reproduced under in vivo conditions and whether this treatment is beneficial for the patients' condition.
**Table 3: CL-levels in fibroblasts of control and BTHS patients after 3 days of growth in standard or linoleic acid enriched medium.**

<table>
<thead>
<tr>
<th>CL molecular species</th>
<th>Standard growth medium*</th>
<th>Enriched growth mediuma,b</th>
<th>Pvalues*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=10)</td>
<td>Control (n=5)</td>
<td></td>
</tr>
<tr>
<td>(C18:2),-CL</td>
<td>0.78 ± 0.14</td>
<td>3.88 ± 0.41</td>
<td>0.000000000002</td>
</tr>
<tr>
<td>(C18:2),(C18:1),-CL</td>
<td>0.96 ± 0.32</td>
<td>2.32 ± 0.51</td>
<td>0.00001</td>
</tr>
<tr>
<td>(C18:2),(C18:1),-CL</td>
<td>1.74 ± 0.59</td>
<td>4.32 ± 0.28</td>
<td>0.0000001</td>
</tr>
<tr>
<td></td>
<td>Barths Syndrome (n=5)</td>
<td>Barths Syndrome (n=5)</td>
<td></td>
</tr>
<tr>
<td>(C18:2),-CL</td>
<td>0.09 ± 0.04</td>
<td>1.28 ± 0.21</td>
<td>0.00000002</td>
</tr>
<tr>
<td>(C18:2),(C18:1),-CL</td>
<td>0.18 ± 0.14</td>
<td>1.16 ± 0.17</td>
<td>0.0000002</td>
</tr>
<tr>
<td>(C18:2),(C18:1),-CL</td>
<td>0.22 ± 0.11</td>
<td>1.59 ± 0.19</td>
<td>0.0000001</td>
</tr>
</tbody>
</table>

*a Mean ± SD.

b Fifty micromoles of linoleic acid was added to the standard medium.

c P values of standard medium versus enriched medium were obtained using all individual data in Student's t-test.

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**Figure 3: Effect of linoleic acid supplementation on the total levels of CL in cultured skin fibroblasts.** In order to investigate the dose-dependent effect of linoleic acid, cells were grown for 3 days in the presence of different concentrations of linoleic acid (total concentration in the growth medium). The total level of CL defined as the sum of the three most abundant species (see legend Figure 1) versus the total linoleic acid concentration in growth medium is shown (A). Fibroblasts also were grown in the absence (B) or presence (C) of 50 M of added linoleic acid for a period of 0-5 days. The reversibility of the effect of linoleic acid is shown in (D). Fibroblasts from controls (n=2) and patients (n=2) were grown in enriched medium with 50 μM linoleic acid for 3 days. On day 3, the enriched growth medium was replaced with standard medium and the cells were grown for another 5 days. Cells were collected after 3 and 8 days. As shown, in all experiments the levels of CL in both control and patient groups were increased as a consequence of the added linoleic acid. This effect was time and dose dependent.
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To this end, the patients received a linoleic-acid enriched diet and the CL content of platelets was measured in time that revealed a definite increase in CL levels. The in vivo effect of the increase in CL will be monitored by evaluation of the cardiac output and neutrophils count.

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