A mass spectrometric approach to investigate cardiolipin metabolism in Barth syndrome
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CHAPTER 7

Monolysocardiolipins accumulate in Barth syndrome but do not lead to enhanced apoptosis

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Chapter 7

SUMMARY Barth syndrome is an X-linked recessive disorder, which is biochemically characterized by low cellular levels of the mitochondrial phospholipid cardiolipin. Previously, we and others discovered that the yeast disruptant of the TAZ orthologue in Saccharomyces cerevisiae not only displays cardiolipin deficiency but also accumulates monolysocardiolipins, which are intermediates in cardiolipin remodeling. We therefore set out to investigate whether monolysocardiolipin accumulation also occurs in Barth syndrome. Indeed, we observed monolysocardiolipin accumulation in heart, muscle, lymphocytes and cultured lymphoblasts of Barth syndrome patients, however, no accumulation of these lysophospholipids was found in platelets and fibroblasts of these patients.

Although the fatty acid composition of the monolysocardiolipins was different depending on the tissue source, it did parallel the fatty acid composition of the (remaining) cardiolipins. Based on these observations and those of others we suggest that the two cardiolipin remodeling systems that exist, the deacylation/reacylation cycle and the transacylation mechanism, work in concert to generate a tissue-specific cardiolipin composition and that TAZ likely plays a role in the transacylation process. Because monolysocardiolipins have been proposed to be involved in the initiation of apoptosis-mediated cell-death by the sequestration of the pro-apoptotic protein (t)Bid to the mitochondrial membrane, we used control and Barth syndrome lymphoblasts to investigate whether the accumulation of monolysocardiolipins in these Barth syndrome cells results in higher levels of apoptosis.

Based on the cellular distribution of Bid and cytochrome c and other parameters, our results indicate that monolysocardiolipin accumulation does not lead to enhanced apoptosis in cultured Barth syndrome lymphoblasts.

INTRODUCTION Barth Syndrome 1 (BTHS; MIM 302060) is an X-linked recessive disorder, which clinically is characterized by cardiomyopathy, skeletal myopathy, growth retardation and neutropenia (1). Additional laboratory findings include intermittent lactic acidemia, low blood cholesterol and increased urinary excretion of 3-methylglutaconic acid, 3-methylglutaric acid and 2-ethylhydracrylic acid (2). Moreover, mitochondria of BTHS patients have an abnormal ultrastructure and several different respiratory chain defects in muscle and fibroblasts have been reported (3;4). The disease can be fatal in childhood, due to cardiac failure or sepsis. The clinical expression of the disease, however, is quite variable in severity, and may show profound intrafamilial variability.

Mutations in the TAZ gene, which is located at Xq28, are responsible for BTHS (5). Because of the homology of the predicted TAZ gene product(s) with acyltransferases involved in phospholipids metabolism, it is suggested that TAZ is involved in remodeling of phos-
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Figure 1: Representative mass spectra of MLCL molecular species in control and BTHS lymphoblasts. The same amount of cellular protein (2 mg) was extracted for both control and BTHS sample. The peak at mlz 619.5 corresponds to the (14:0)_{4} CL, the internal standard. In both control (A) and BTHS (B) lymphoblasts, three different MLCL clusters are present, however, MLCL levels are highly elevated in BTHS lymphoblasts. A semi-quantitative representation of the levels of selected MLCLs, including their fatty acid composition, is depicted in (C). These values are the average ± SD of three different control and BTHS lymphoblast cell lines.

pholipids (6). This suggestion was supported by our finding that the incorporation of linoleic acid into cardiolipin (CL) was defective in patients suffering from BTHS although their biosynthetic capacity to synthesize CL was entirely normal (7). Additionally, the CL levels have been shown to be markedly decreased in platelets (8), fibroblasts (9) and tissues (10) of patients suffering from BTHS.

CL is a unique polyglycerophospholipid, which has a dimeric structure consisting of two phosphatidyl moieties linked by a glycerol bridge (11). Therefore, CL has four fatty acyl side-chains and two negatively charged phosphate groups. CL is found almost exclusively in the inner mitochondrial membrane (12) and plays a central role in mitochondrial function, especially oxidative phosphorylation, since a number of the respiratory chain complexes and several metabolite transporters require CL for optimal function (12;13). CL is found throughout nature in mitochondria of animals, plants, fungi and also is a component of bacterial
membranes (12). In eukaryotes, CL is synthesized from phosphatidylglycerol and cytidine-5'-diphosphate-1,2-diacyl-sn-glycero l in a reaction catalyzed by CL synthase (12;13). In animal tissues, especially in heart and muscle, CL contains almost exclusively C18 fatty acids, and 80% of this is typically linoleic acid, C18:2(n-6) (14). Because CL molecular species produced by CL synthase do not have the proper fatty acid side-chains, CL is remodeled to produce the required acyl-composition (7;13-15).

**Figure 2: Representative mass spectra of CL molecular species in cultured control and BTHS lymphoblasts (Same samples as shown in Figure 1). The same amount of cellular protein (2 mg) was extracted for both control and BTHS sample. The peak at m/z 619.5 corresponds to the (14:0)4-CL, the internal standard. In control lymphoblasts, 5 different CL clusters are present (A). BTHS lymphoblasts (B) clearly display CL deficiency, especially those with a high degree of unsaturation, e.g. the peaks within a CL cluster with low m/z (for example m/z 698.5, 711.5 and 724.5). A semi-quantitative representation of the levels of selected CLs, including their fatty acid composition, is depicted in (C). These values are the average ± SD of three different control and BTHS lymphoblast cell lines.
The proposed pathway for the remodeling of CL is a deacylation/reacylation mechanism, which involves the formation of the intermediate monolysocardiolipin (MLCL) by the action of phospholipase(s) and the reacylation of this compound by one or more acyltransferase(s) (16,17). It therefore has been suggested that the TAZ gene encodes an acyltransferase involved in the remodeling of cardiolipin. If so, one would expect that the intermediates of remodeling, i.e. MLCLs, would accumulate in BTHS. Recently, we and others have reported that a strain of Saccharomyces cerevisiae in which the yeast orthologue of the TAZ gene,
YPR140w, has been disrupted, not only displays CL deficiency but also accumulates MLCLs (18;19). We showed that the levels of both CL and MLCL normalized after transformation of the disruptant with human tafazzin (19), thereby confirming the direct involvement of TAZ in the remodeling of CL. Until now, however, MLCL accumulation has not been observed in BTHS. To investigate whether MLCLs also accumulate in BTHS we adapted our recently developed method for the measurement of CL (8) to measure CLs, MLCLs and other phospholipids in cells and tissues. We also investigated how TAZ deficiency affects other phospholipids in these tissues.

Our results show that in addition to CL deficiency in BTHS tissues/cells, there are also abnormalities in other phospholipid classes. Additionally, there is a clear accumulation of MLCLs in muscle, heart, lymphoblasts and lymphocytes but, strangely enough, not in fibroblasts and platelets. Since recent research indicates that MLCLs are pro-apoptotic lipids, we determined whether the MLCL accumulation leads to enhanced apoptosis in BTHS. Our investigations indicate that MLCLs do not lead to enhanced apoptosis in cultured BTHS cells.

Figure 4: Mass spectra of CL molecular species from control and BTHS heart tissue. The peak at m/z 619.5 corresponds to the (14:0)\(^4\)-CL, the internal standard. Like in lymphocytes, (18:2)\(^4\)-CL is the most abundant CL found in heart tissue of control sample (A). The levels of this CL are dramatically decreased in the BTHS heart (B). A semi-quantitative representation of the levels of selected CLs in two control and two BTHS hearts, including their fatty acid composition, is depicted in (C). This clearly shows that although there is a profound deficiency of (18:2)\(^4\)-CL (left graph), the less unsaturated CL molecular species are present at normal or even higher levels in BTHS heart (right graph).
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MATERIALS AND METHODS

All solutions were of analytical quality and were purchased from Merck (Darmstadt, Germany). The (14:0)₄-CL internal standard (I.S.) and MLCL were purchased from Avanti Polar Lipids (Alabaster, USA). Analytical HPLC LiChrospher Si 60 column (2 x 250 mm, 5 µm particle size) was purchased from Merck (Darmstadt, Germany).

CELL CULTURE AND TISSUES

Lymphoblasts were cultured on RPMI medium containing 10% FCS, 25 mM HEPES and 1% PEN/STREP. Cells were collected by centrifugation at 1500 rpm for 5 minutes and washed with PBS. Fibroblasts were cultured as described by Valianpour et al. (9). Pellets were used for the analysis either directly or were kept frozen at -80°C prior the analysis. All blood/tissue samples were collected according to institutional guidelines for blood/tissue sampling, including procurement of informed consent of the persons involved or their representatives.

LIPID EXTRACTION FROM CULTURED CELLS

Cell pellets were suspended in 300 µl of distilled water and sonicated on ice, 2x10s at 8 Watt. Of this cell suspension, 10 µL was used for protein measurement. Internal standard was added to the remaining homogenate (50 µL of a 7.8 µmol/L solution of (14:0)₄-CL in 2:1 chloroform:methanol (v/v)) followed by 3 ml of a 1:1 chloroform:methanol (v/v). This mixture was vigorously shaken, placed on ice for 15 min after which it was centrifuged at 1000 g for 5 min. The organic layer was transferred into another tube and the protein pellet was extracted with 3 ml 2:1 chloroform:methanol (v/v). The combined organic layers were evaporated under a stream of nitrogen at 50°C. The residue was dissolved in 150 µL 50:45:5 chloroform:methanol:water (v/v/v) and 5 µL of this solution was injected in to the HPLC-tandem MS system.

LIPID EXTRACTION FROM TISSUES

Approximately 2 mg of cardiac or muscle tissue was placed in a 1.5 ml Eppendorf vial and its exact wet weight was determined using a microbalance. The Eppendorf vial already contained the I.S., 150 µL of a 7.8 µmol/L solution of (14:0)₄-CL in 2:1 chloroform: methanol (v/v) (1170 pmol), which had been evaporated under a stream of nitrogen at 50°C. The tissue samples were freeze-dried overnight in a benchtop lyophilization system (LabConco, Kansas City, Missouri, USA). The lyophilized tissues were kept on ice and were grounded to powder using an eppendorf micropestle. 300 µL of 1:1 chloroform:methanol (v/v) was added to the tissue powder and this suspension was sonicated twice for 10 seconds at 2.5 Watt. Three ml of 1:1 chloroform:methanol (v/v) was added and this suspension was shaken vigorously, placed on ice for 15 min and centrifuged for 5 min at 1000 g. The organic layer was
transferred into another tube and the protein pellet was extracted with 3 ml 2:1 chloroform:methanol (v/v). The combined organic layers were evaporated under a stream of nitrogen at 50°C. The residue was dissolved in 250 μL 50:45:5 chloroform: methanol:water (v/v/v) and 5 μL of this solution was injected in to the HPLC-tandem MS system.

![Figure 5: Mass spectra of MLCL molecular species from control and BTHS heart tissue. The peak at m/z 619.5 corresponds to the (14:0)₄-CL, the internal standard. In control heart (A), only (18:2)₃-MLCL (m/z 592.5) was observed, however, in BTHS heart (B) there was a clear accumulation of more saturated MLCLs. A semiquantitative representation of the levels of selected MLCLs in two control and two BTHS hearts, including their fatty acid composition, is depicted in (C).](image-url)

The HPLC system consisted of an HP1100 series binary gradient pump, a vacuum degasser, and a column temperature controller (all from Hewlett Packard, Wilmington, DE, USA) and a Gilson 231 XL autosampler (Gilson, Villiers-le-Bel, France). 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 (Merck, Darmstadt, Germany). The phospholipids were separated from interfering compounds by a linear gradient between solution B (chloroform:
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methanol 98.2, v/v) and solution A (methanol:water 9:1, v/v). Both solutions contained 0.1 ml of 250 ml/l aqueous ammonia per liter of 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 8.1-16 min equilibration with 0% A. All gradient steps were linear; and the total analysis time, including the equilibration, was 16 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.

**MASS SPECTROMETRY**

A Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, UK), was used in the negative electrospray ionization 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 for MLCLs and CLs was 30 V. Mass spectra of CL and MLCL molecular species were obtained by continuous scanning from 400 to 1000 m/z with a scan rate of 200 Da/s in a time window of 2.5 minutes, during which both types of compounds elute from the column. Daughter fragments of CL and MLCL molecular species were obtained using optimal collision energy of 40 eV as described previously (8). Phosphatidyl-choline (PC) molecular species were measured using a parent ion scan (from m/z 400-1000) for m/z 184.1 in the positive ion mode. Phosphatidylethanolamine (PE) molecular species were measured in the positive ion mode using a neutral loss scan (from m/z 400-1000) of the mass 141.1.

In a separate analysis, using the same extracts, single-ion recording was used to perform a semi-quantitative determination of selected MLCLs and CLs. The area of each (ML)CL peak (A_{(ML)CL}) and that of the added internal standard (A_{IS}) was quantified using MassLynx 3.3 (Micromass, Manchester, United Kingdom). If appropriate, the peak areas were corrected for the contribution of naturally occurring isotopes. To compare individual samples the ratio (A_{(ML)CL}/A_{IS}) was also corrected for the protein content in case of cell homogenates, or wet tissue weight for tissue samples. Since the method is not validated for the different (ML)CL molecular species and because we did not determine whether the response of the internal standard was equal to that of the quantified (ML)CLs we have shown these values as arbitrary units, normalized per mg tissue or protein content.

**VALIDATION**

The linear response and detection limit for (18:2)₃-MLCL was established by injection of calibration mixtures with different concentrations of this analyte and a constant concentration of (14:0)₄-CL as the I.S. The extraction efficiencies for both the I.S. and analytes were obtained by comparing their peak intensities in the extracted calibration mixtures with those in the un-extracted calibration mixtures with the same final concentrations without extraction.
The intraassay (within-day) variation of the method was determined by measuring 10 times a non-enriched sample and a sample enriched with commercially available (18:2)_3_MLCL at low (2 nmol/mg of protein) and high (100 nmol/mg of protein) concentrations. The interassay (between-day) variation was determined by measuring blank samples and samples enriched with (18:2)_3_MLCL (10 and 100 nmol/mg of protein) over 3 separate weeks. The recovery of the method was established by measuring 10 different samples before and after enrichment with a known amount of (18:2)_3_MLCL.

We investigated ion suppression from interfering substances by comparison of the peak intensities of both (18:2)_4^CL and (14:0)_4^CL in the enriched samples with those of calibrator solutions with similar concentrations.

**SUBCELLULAR FRACTIONATION OF LYMPHOBLASTS AND ANALYSIS OF APOPTOSIS**

Control and BTHS lymphoblasts cells were harvested by centrifugation at 500 g and washed twice with phosphate buffered saline. Cells were resuspended in homogenization buffer (10 mM MOPS buffer containing, 250 mM sucrose, 1 mM EGTA, 2 mM KCl, pH 7.4) and homogenized by 5 passages through a cell-cracker (EMBL, Heidelberg, Germany). The postnuclear supernatant was obtained after centrifuging the homogenate for 10 min at 800 g at 4°C and protein concentration was determined using Bio-Rad Protein Reagent (Hercules, CA). The postnuclear supernatant was centrifuged for 60 min at 100,000 g to separate the organelles from the cytosol. The supernatant (cytosol) was collected and the organelle fraction was washed twice with homogenization buffer. Finally, the organelle pellet was resuspended in an equal volume as that of the cytosol.

Equal aliquots of the organelle and cytosolic fraction, corresponding to 75 µg of the post-nuclear supernatant protein, were separated by SDS-PAGE, blotted onto nitrocellulose membrane using a semi-dry electrophoretic apparatus and immunoblot analysis was performed as described previously (20). The a-Bid antibody (1:2000) and the monoclonal a-cytochrome c antibody (clone 7H8.2C12, 1:100) were from Cell Signaling Technology (Beverly, MA) and Pharmingen (San Diego, CA), respectively.

For the analysis of cleavage of poly(ADP-ribose) polymerase (PARP), lymphoblasts were harvested by centrifugation, washed with phosphate buffered saline and lysed by sonication in homogenization buffer. The protein concentration of the homogenate was determined and 75 µg of protein was loaded on a 10% SDS-PAGE gel. Immunoblotting was performed using a mouse monoclonal a-PARP antibody (dilution 1:1000), which was purchased from Biomol (Plymouth Meeting, PA).
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**Figure 6**: Mass spectra of PE and PC molecular species in control and BTHS heart. PE molecular species found in control (A) and BTHS (B) are shown. The peak at 636.8 in both PE spectra corresponds to the (14:0) \_ PE, which was used as internal standard. 18:0/18:2-PE (m/z 744.8) clearly accumulates in both BTHS hearts (B and E). Compared to the control (C) the level of 34:2-PC (m/z 758.9, probably 16:0/18:2-PC) in BTHS (D and F) was elevated. In addition, the levels of arachidonic acid containing PE and PC (36:4 and 38:4) seemed to be decreased in BTHS (m/z values are indicated by arrows in figure B and C). 16:0-ethyl/18:2-acyl-PC (m/z 742.9), a plasmalogen, appears to be elevated in BTHS. A semiquantitative representation of the levels of 18:0/18:2-PE and 36:2-PC in two control and two BTHS hearts is depicted in (E) and (F), respectively.

**RESULTS**

Because MLCLs are present in the yeast model for BTHS (19) we set out to investigate whether these lysophospholipids also accumulate in cells and tissues of BTHS patients. We first adapted our recently developed method to measure CL to enable detection of MLCLs and validated this method. The main change in our method is the use of a one-phase extraction to increase the recovery of MLCLs. This change did not influence the recovery of CL and that of other phospholipids. Individual MLCL molecular species were measured by scanning
of negatively charged ions after separation by normal-phase HPLC. The response of the detected ions was linear to increasing concentrations of commercially available (18:2)₃-MLCL at least until 168 nmol/mg protein ($r^2 = 0.993$).

The recovery of the method was $68.2 \pm 4.9\%$ at low level and $78.2 \pm 2.2\%$ at high level of (18:2)₃-MLCL addition. The within-day coefficients of variation (CV %) at low and high levels of addition were 12.3 % and 2.8 %, respectively. The between-day CVs at low and high levels of addition were 17.3% and 4.2%, respectively. We used this validated method to determine the levels of both CLs and MLCLs in lymphoblasts, lymphocytes, fibroblasts, muscle and heart of controls and BTHS patients.

**MLCL AND CL ANALYSIS IN BTHS CELLS**

Figure 1 shows the MLCL molecular species isolated from cultured lymphoblasts of a control and a BTHS patient. This figure clearly shows that the levels of (18:1)₃-MLCL (m/z 595.5), (16:0)/(18:1)₂-MLCL (m/z 582.5), (16:1)/(18:1)₂-MLCL (m/z 581.5) and (16:0)₂/(18:1)-MLCL (m/z 568.5) are highly elevated in BTHS lymphoblasts (figure 1A) compared to the control cells (figure 1B). Semi-quantitative analysis showed that the levels of MLCL species in BTHS were 5-20 times higher than in controls (figure 1C). Figure 2 shows the CL molecular species in the same lymphoblast samples. Five clusters of doubly charged ions corresponding to different CL molecular species are present in the control sample (figure 2A). In each cluster, the most unsaturated CL is that with the lowest m/z value. The degree of saturation of the fatty acyl side-chains increases with higher m/z values within such a cluster.

The mass difference between each cluster corresponds to CLs with fatty acyl side-chains of different lengths. For example, 727.5 corresponds to a (18:1)₄-CL, whereas 713.5 corresponds to (16:1)/(18:1)₃-CL. In figure 2A and 2B, the CL clusters are numbered 1, 2, 3, 4, and 5. In controls, the most abundant CL molecular species are present in cluster 3 and 4, which contain 18:2, 18:1 and 16:1 as fatty acyl side-chains. Cluster 5 also contains unsaturated C20 fatty acids in addition to 18:2 and 18:1. Compared to the CL molecular species in control (figure 2A), the most unsaturated CLs in all clusters (e.g. the peaks with the lowest m/z of the cluster) are significantly decreased in BTHS patients (figure 2B) while the levels of less unsaturated CLs in each cluster such as (18:1)₄-CL (m/z 727.5) were present in normal amounts (figure 2C).

Because of the presence of MLCLs in BTHS lymphoblasts we investigated the MLCL/CL profile of BTHS and control lymphocytes in order to determine whether these untransformed BTHS cells also display an accumulation of these aberrant cardiolipin species. Surprisingly, the CL spectra of control lymphoblasts and lymphocytes were very dissimilar.
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We found that (18:2)₄-CL is the most abundant CL in control lymphocytes (figure 3A), whereas lymphoblasts contain more saturated CL species and display a larger variety of acyl side-chain length (compare figures 2A and 3A). Like in BTHS lymphoblasts, we did observe a severe CL deficiency and a clear accumulation of MLCLs in BTHS lymphocytes (figure 3B and C). This is in contrast to platelets and fibroblasts, where no MLCL accumulation could be detected in BTHS cells (not shown).

MLCL and CL analysis in BTHS tissues

Since heart and muscle contain high amounts of CL and BTHS patients present with symptoms related to these tissues ((cardio)myopathy) we investigated the CLs and MLCLs in these tissues of control subjects and BTHS patients. In control heart, (18:2)₄-CL (m/z 723.5) is the most abundant CL (figure 4A and 4C left graph). This cardiolipin is severely deficient in BTHS heart while other CLs including (18:1)/(18:2)₃-CL (m/z 724.5), (18:1)₃/(18:2)₂-CL (m/z 725.5), (18:1)₃/(18:2)₁-CL (m/z 726.5) and (18:1)₄-CL (m/z 727.6) are still present
and their levels even appear to be slightly increased when compared to control heart (figure 4B and 4C right graph). The CL spectrum of skeletal muscle in both control and BTHS was very similar to that of heart (not shown). In control heart (figure 5A) as well as muscle (data not shown) we detected one MLCL with fatty acid composition \((18:2)_3\)-MLCL (m/z 592.5), which is present in low amounts when compared to \((18:2)_4\)-CL. In BTHS heart and muscle, however, there is a clear accumulation of different MLCLs, namely \((18:1)_3\)-MLCL (m/z 595.5), \((18:1)_2/(18:2)-\)MLCL (m/z 594.5), \((16:0)/(18:1)_2\)-MLCL (m/z 582.5) and \((16:1)/(18:1)_2\)-MLCL (m/z 581.5) (figure 5B).

None of these MLCL molecular species were detectable in control samples. The levels of these MLCL molecular species were 10 times higher in BTHS than in control samples (figure 5C) and were present in similar quantities as the remaining CLs. The levels of \((18:2)_3\)-MLCL in BTHS seemed to remain in the same range as in control (figure 5C).

**ANALYSIS OF OTHER PHOSPHOLIPIDS**

Using the same HPLC tandem MS system we investigated the composition and abundance of other phospholipids, including PE and PC, in BTHS and control tissues. Figure 6 shows clear abnormalities in PE molecular species isolated from heart tissue of a control (figure 6A) and BTHS heart sample (figure 6A). \((18:0/18:2)\)-PE level (m/z 744.8) was 5-6 times higher in BTHS heart when compared to control heart (figure 6B). The fatty acid composition of this PE, 18:0/18:2, was confirmed by tandem MS.

The same abnormalities were found in the PC fraction, but were less prominent than in PE. 34:2-PC levels (m/z 758.8, figure 7) appeared to be at least 2-3 times higher in BTHS samples when compared to the controls. This PC likely corresponds to 16:0/18:2 fatty acid composition, however, this was not checked experimentally. Additionally, m/z 742.9 in figure 6D, which likely represents a 16:0-enyl/C18:2 acyl-PC (a plasmalogen), appears to be elevated in BTHS heart. Both PE and PC molecular species containing arachidonic acid (m/z values indicated by arrows in figure 6B and 6D) seemed to be decreased in BTHS samples. We did not observe abnormalities in other investigated phospholipid classes, including phosphatidylglycerol, phosphatidylinositol and phosphatidylserine.

**MLCL AND CL-DEFICIENCY AND APOPTOSIS**

Recently, it was shown that CL and MLCL bind to Bid, a proapoptotic Bcl-2 family member, which is involved in death-receptor-mediated apoptosis in many cell systems (21). Upon an apoptotic stimulus, cytosolic Bid is somehow recruited to the mitochondrial membrane where it, in concert with other Bcl-2 family members, initiates the release of apoptogenic factors like cytochrome c, thereby promoting apoptosome-mediated apoptosis. In this process, full length Bid (f1. Bid) is cleaved by caspase 8 resulting in a truncated protein termed


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tBid, which is considered as the active form. Espositi and coworkers have shown that although both CL and MLCL interact in vitro with (t)Bid, MLCL binds with higher affinity and also is capable of stimulating the cytochrome c releasing capacity of tBid. The observation that, in vivo, MLCL levels increase upon Fas-induced apoptosis, is suggestive that MLCL production in mitochondria could be involved in the initiation of apoptosis by the recruitment of (t)Bid to mitochondria.

We therefore hypothesized that the accumulation of MLCLs in BTHS cells and tissues could result in enhanced apoptosis, which could lead to pathological findings observed in BTHS patients. To test this hypothesis we used BTHS lymphoblasts, which were found to accumulate MLCLs, and determined several relevant apoptotic parameters.

First, we investigated a late-stage apoptotic marker, namely the cleavage of poly(ADP-ribose) polymerase (PARP). After induction of apoptosis, PARP is cleaved by caspase 3, which prevents PARP from repairing apoptosis-induced DNA damage. Figure 7A shows that, like in control cells, there is no cleavage of PARP in BTHS lymphoblasts and that most, if not all, of the protein is full length. Second, we determined the subcellular distribution of (t)Bid and cytochrome c in three BTHS and three control lymphoblasts cell lines. Figure 7B and C show that there is no difference between the distribution of Bid and cytochrome c between mitochondria and cytosol in BTHS and control lymphoblasts. Bid is present as full Bid and only detected in the cytosol. In contrast, cytochrome c is only found in mitochondria and is not present in the cytosol. The distribution of the mitochondrial enzyme glutamate dehydrogenase confirms that the mitochondria were intact and well separated from the cytosol (figure 7C).

DISCUSSION Since we and others recently discovered that the yeast disruptant of the TAZ orthologue in Saccharomyces cerevisiae not only displays CL deficiency but also accumulates MLCLs ([18;19]) we set out to investigate whether MLCL accumulation also occurs in BTHS. Therefore, we successfully adapted our method to enable the measurement of MLCLs in addition to CL and other phospholipids. As observed in the yeast model for BTHS, our data show an accumulation of MLCL molecular species in some BTHS cell lines and tissues. We found that MLCLs accumulate in BTHS heart, muscle, (cultured EBV-transformed) lymphoblasts and lymphocytes. In contrast, no MLCL accumulation was observed in cultured primary fibroblasts and platelets of BTHS patients.

The accumulation of a supposed interme-diate of CL remodeling in BTHS further supports the earlier suggestion that the decreased levels of CL in BTHS are due to a defect in the remodeling process.
The fact that some cell types show accumulation of MLCLs while other do not is surprising and suggests that remodeling of CLs may be different in different cell types or tissues. It has been known for some time that CL is remodeled via the deacylation/reacylation mechanism (16), with MLCL as an obligate intermediate. The enzyme responsible for the acyl-CoA-dependent reacylation of MLCL, MLCL acyltransferase, has been purified from pig liver mitochondria by Hatch and coworkers (17), but the corresponding gene has not been identified. Recently, Xu et al. reported that in rat liver, both CL and MLCL can also be remodeled via transacylation, using PC and PE as acyl donors (22). They showed that the acyl transfer activity in rat liver mitochondria was specific for linoleate-containing phospholipids, which differed from lymphoblasts, where PE containing oleate could also be used as acyl donor. The mere fact that MLCLs accumulate in certain tissues, however, does not allow discrimination between the involvement of either deacylation/reacylation and transacylation mechanisms, since MLCL formation theoretically occurs in both processes. Still, analysis of the fatty acid composition of the accumulating MLCL molecular species could contribute to our understanding of the CL remodeling process. When considering this, there appears to be a direct relation between the fatty acid composition of the MLCLs and that of the CLs of a certain tissue or cell type.

In control heart and muscle samples, we only found one MLCL molecular species, namely (18:2)3-MLCL, the lysodervative of (18:2)4-CL. In general, MLCL molecular species in BTHS tissues and cells were predominantly composed of mono unsaturated and even saturated fatty acids, which also reflected the composition of the remaining CLs. This was also true for the accumulating MLCLs in lymphoblasts and lymphocytes, which again reflect the CL fatty acid composition. Xu et al observed that the transacylase activity for oleate-containing PE was reduced in lymphoblasts from patients with BTHS, which led them to suggest that cardiolipin is remodeled by acyl specific phospholipid transacylation, which involves tafazzin. If this is indeed true, MLCL acyltransferase is encoded by a different gene.

The observation that not all cell types display accumulation of MLCLs could reflect the expression pattern of this enzyme, rendering tissues that have low or no MLCL acyltransferase expression less- or incapable of reacylation MLCLs, which then accumulate. Obviously, another factor could be the activities of the different phospholipases, which are needed for the generation of MLCLs. Since the fatty acid composition of the MLCLs parallels that of the CLs it is likely that the formation of MLCLs is not a specific process. This in turn is in agreement with the observed substrate specificity of MLCL acyltransferase, which is capable of using linoleoyl-, oleoyl- and even palmitoyl-CoA, albeit less efficiently (17). This less specific reacylation of MLCL could be followed by a more linoleoyl-specific transacylation.
Monolysocardiolipins accumulate in Barth syndrome but do not lead to enhanced apoptosis

reaction involving tafazzin, resulting in the formation of the predominant tetralinoleoylcardiolipin. The existence of such a mechanism is supported by the fact that PC and PE molecular species containing linoleic acid, which are reported to function as acyl donors in the transacylation of cardiolipin accumulate in BTHS (this study and (22)).

The results of Xu et al. demonstrated the transacylation reaction has a high specificity for linoleoylContaining PC or PE in rat liver. In fact, palmitoyl- and oleoyl-containing PC or PE were totally ineffective donors in the transacylation reaction. It is noteworthy, however, that although in liver the transacylation reaction was very specific for linoleoyl-containing donors, in lymphoblasts this transacylation could also be performed with oleoyl-containing PE (22). This suggests that their may be a tissue-dependent substrate specificity for the transacylation reaction. Since MLCLs recently have been suggested to promote apoptosis by the binding of the proapoptotic protein Bid and its concomitant recruitment to the mitochondrial outer membrane (21), we investigated whether BTHS lymphoblasts displayed a higher level of apoptosis using several parameters, including cleavage of PARP and the subcellular distribution of (t)Bid and cytochrome c. None of these investigations indicated that BTHS lymphoblasts displayed a higher level of apoptosis when compared to control lymphoblasts. Although these results are in agreement with the findings of Kuijpers and coworkers, which showed that BTHS neutrophils are not apoptotic even though they are Annexin-V positive (23), MLCL could still have different effects in other cell types. Also, one should keep in mind that the lymphoblasts used for these studies are EBV-transformed cells, which might not be the best model system to study apoptosis. Further investigations in freshly isolated BTHS blood cells are currently underway.

The observation that MLCLs accumulate in lymphocytes also has impact on the way BTHS is diagnosed. Previously, we published a method to diagnose BTHS patients by analyzing CL levels in platelets using HPLC-MS (8). Although this method has been and still is very useful, it is based on a decrease of CL levels, which has less diagnostic power than the accumulation of (what appears to be) a disease specific compound. We are now investigating whether determination of both MLCL and CL levels (and possibly a ratio of these two) in lymphocytes is a better diagnostic parameter for BTHS.

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