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Substantial Decrease in Plasmalogen in the Heart Associated with Tafazzin Deficiency

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Abstract

Tafazzin is the mitochondrial enzyme that catalyzes transacylation between a phospholipid and a lysophospholipid in remodeling. Mutations in tafazzin cause Barth syndrome, a potentially life-threatening disease with the major symptom of cardiomyopathy. In the tafazzin-deficient heart, cardiolipin (CL) acyl chains become abnormally heterogeneous unlike those in the normal heart with a single dominant linoleoyl species; tetralinoleoyl CL. In addition, the amount of CL decreases and monolysocardiolipin (MLCL) accumulates. Here we determined using high-resolution ³¹P NMR with cryoprobe technology the fundamental phospholipid composition, including the major but oxidation-labile plasmalogens, in the tafazzin-knockdown (TAZ-KD) mouse heart as a model of Barth syndrome. In addition to confirming less CL ($6.4 \pm 0.1 \rightarrow 2.0 \pm 0.4$ mol% of total phospholipid) and accumulated MLCL (not detected $\rightarrow 3.3 \pm 0.5$ mol%) in the TAZ-KD, we found a substantial reduction in plasmenylcholine ($30.8 \pm 2.8 \rightarrow 18.1 \pm 3.1$ mol%), the most abundant phospholipid in control wild type. Quantitative Western blot revealed that while the level of peroxisomes, where early steps of plasmalogen synthesis take place, was normal in the TAZ-KD, expression of Far1 as a rate-determining enzyme in plasmalogen synthesis was dramatically upregulated by $8.3 (\pm 1.6)$ -fold to accelerate the synthesis in response to the reduced plasmalogen. We confirmed lyso-plasmenylcholine (/plasmenylcholine) is a substrate of purified tafazzin for transacylation with CL (/MLCL). Our results suggest that plasmenylcholine, abundant in linoleoyl species, is important in remodeling CL in the heart. Tafazzin deficiency thus has the major impact on the cardiac plasmenylcholine level, and thereby its functions.

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Associated Content: Supporting Information

Appendices S1–4, Figures S1–9, Tables S1–3.

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Notes

The illustrated heart tissue images in the TOC graphic were made based on electron micrographs of the WT and TAZ-KD mouse heart in reference [44].

Graphical abstract

Introduction

Lipids play a tremendous variety of functional roles in biological systems that are performed according to the specific structure of the entire lipid molecule, including the hydrocarbon chains.¹ In recent years, lipid-protein interactions therefore are gaining attention together with our changes in fundamental understanding, with regard to both the specific binding and nonspecific regulation of the physical and chemical membrane environment.^{1–4} Barth syndrome is an X-linked potentially life-threatening recessive disease caused by mutations of a gene *G4.5* in distal Xq28,^{5–8} which encodes a transacylase named tafazzin;^{9–11} tafazzin catalyzes transfer of an acyl group between a phospholipid and a lysophospholipid in phospholipid remodeling.^{12–15} One of the major clinical symptoms due to the defect in the activity of tafazzin is cardiomyopathy.^{5, 6} The deficiency in tafazzin function leads to abnormal mitochondrial ultrastructure and impaired respiratory function^{5, 6}, where dramatic alterations have been elucidated in the state of cardiolipin (CL) as a key player with its cell-type specific distribution of acyl chain species¹⁶ in stabilizing the respiratory supercomplexes.¹⁷ These CL alterations are (i) a decreased level, (ii) diversification of acyl chain species contrary to the normal control, e.g. with one dominant species in the heart, and (iii), accumulation of monolysocardiolipin (MLCL).^{10, 11, 18} Therefore, CL has been the major focus among phospholipids in unveiling the molecular mechanism leading to Barth Syndrome.^{1, 19} In this work, we report high-resolution ³¹P NMR determination of the phospholipid composition of the heart of genetically engineered tafazzin-knockdown (TAZ-KD) mice.²⁰ We found a substantial reduction in the choline plasmalogen level ($30.8 \pm 2.8 \rightarrow 18.1 \pm 3.1$ mol% of the total phospholipid). The reduction was shown to be ~ 3-fold larger in magnitude than a reduction in CL ($6.4 \pm 0.1 \rightarrow 2.0 \pm 0.4$ mol%) that is known as the hallmark phenomenon of lipid alteration in Barth syndrome. The present finding was made by the simultaneous quantification of all the phospholipids along with adoption of the cutting-edge cryoprobe technology in ³¹P NMR to enhance the detection sensitivity. A particular precaution was made in the material processing to maintain the plasmalogen structure that is known to be labile to oxidation.

Plasmalogens constitute as much as 18% of the total phospholipid mass in humans,²¹ yet their physical, chemical, and biological roles are still largely unknown.^{21–23} The plasmalogen deficiency in relation to Barth syndrome has not been recognized previously. In general, situations that have been contributing to our poor understanding of plasmalogen

functions are due largely to (i) the nature of the structure that is not defined and characterized only by the headgroup, (ii) instability against oxidation, (iii) instability against acid during lipid extraction and chromatographic separation, and (iv) fragmentation complexity in the tandem mass spectrometry (MS). Plasmalogens are specifically characterized by the *sn*-1 vinyl ether linkage (Figure 1), in contrast to the counterpart ester linkage in the diacyl glycerophospholipids and the alkyl ether linkage in the plasm_{an}yl glycerophospholipids. There are only two major types of plasmalogens called plasm_{en}ylcholine and plasm_{en}ylethanolamine, having the choline or the ethanolamine headgroup, respectively. The heart is a rare organ which has prominently plasm_{en}ylcholine (26-41 mol% of the total choline glycerophospholipids in humans; the differences in the value come from different sources in literature) (cf. 1-3 mol% in the brain, 0-1 mol% in the liver), whereas plasmalogens in other organs are primarily ethanolamine glycerophospholipids.^{1, 24, 25} In the wild type (WT) mouse heart, plasm_{en}ylcholine was measured in this work to be 30.8 ± 2.8 mol%, and plasm_{en}ylethanolamine to be 8.2 ± 0.4 mol% of the total phospholipid. The numbers compare with the rest of choline glycerophospholipids (diacyl plus plasm_{an}yl) at 19.2 ± 1.9 mol% and ethanolamine glycerophospholipids (diacyl plus plasm_{an}yl) at 26.2 ± 1.2 mol%. Therefore, the drastic reduction of plasm_{en}ylcholine due to tafazzin deficiency is expected to have substantial effects on the integrity of the heart tissue; see Discussion.

MS methods have an advantage that they are more sensitive for signal detection than ³¹P NMR. MS is also capable of providing information about the hydrocarbon chain composition of various phospholipid classes and subclasses.²⁶ Quantification of phospholipid species in a biological material using MS is enabled by inclusion of a mixture of internal standard phospholipids of different headgroups and hydrocarbon species to correct for differences in their ionization efficiency, and/or by minimization of such differences in the ionization efficiency by taking a lower limit of lipid concentration for sample infusion to reduce formation of lipid aggregation that causes nonlinear responses in detection.^{27, 28} A phospholipid content of each class or subclass is determined by identification and summing up of contributions from various hydrocarbon chain species. The presence of differential fragmentation during collision-induced dissociation (CID) in tandem MS has to be taken into account. The tandem MS detection of plasmalogens that contain the vinyl ether linkage is known to be difficult and requires particular attention due to their unique behavior that is different from the typical neutral loss of the headgroup observed for diacyl and plasm_{an}yl glycerophospholipids.²⁹

³¹P NMR with inverse-gated ¹H decoupling has an advantage in accurately quantifying contents of phospholipid classes/subclasses, each of which gives a discrete signal as a sum of contributions from different hydrocarbon chain species. The signal separation is based on a unique chemical shift value of a phosphate group in each class/subclass that reflects its electronic structure influenced by specific types of nearby functional groups in the headgroup as well as the linkage with hydrocarbon chains. Plasmalogen ³¹P NMR signals can be distinctively observed for both the choline and ethanolamine classes.³⁰⁻³² In high resolution ³¹P NMR of phospholipid quantification, use of either (i) a solvent mixture, or (ii) detergent micelles in water are common to solubilize lipids to obtain sharp resonance lines.

³³ Here we use SDS detergent micelles to measure the detailed phospholipid compositions of the TAZ-KD and WT mouse heart.

Accurate quantification of plasmalogens in a biological material requires precautions to avoid degradation of the structure due to oxidation during the sample preparation by including an antioxidant and a metal ion chelating reagent. The characteristic *sn*-1 vinyl ether linkage of plasmalogens is readily cleaved by reaction with reactive oxygen species (ROS), such as radicals and singlet oxygen.^{34–38} This vinyl ether has often been considered as the most probable target site for the oxidative reaction in phospholipids, including other susceptible sites like polyunsaturation in the acyl chain.³⁶ Such preferential oxidation at the vinyl ether is notable even in micelles³⁶ or in a membrane with large surface curvature like small unilamellar vesicles (SUVs),^{37, 38} where molecular motions of lipids are much less restricted and the degree of hydration is enhanced compared with that of a flat bilayer.³⁹ In fact, the high reactivity of the vinyl ether with ROS has suggested an antioxidant role of plasmalogens in the cell. Mutants of Chinese hamster ovary (CHO) cells that were deficient in plasmalogen biosynthesis were hypersensitive to photosensitized exposure to long wavelength UV light (>300 nm).^{35, 40} An inverse correlation between peroxide-producing potential of tissues and longevity of mammalian species is well-known.^{41, 42} Contrary to the inverse correlation for peroxide-producing potential as specified by the double-bond content in phospholipids, a positive correlation was observed with the maximum life span for the plasmalogen level, in support of the antioxidant function of plasmalogens.⁴³

Our ³¹P NMR observation of the drastic decrease in plasmalogen in the TAZ-KD mouse heart led us to investigate further the cause of the decrease as well as cellular responses to it based on quantitative Western blotting of relevant proteins.

Materials and Methods

Materials for the NMR experiments

Lipids: Most phospholipid standards used for signal assignment in the ³¹P NMR experiments, except sphingomyelin (SM) from Sigma (St. Louis, MO), were purchased from Avanti Polar Lipids (Alabaster, AL) as summarized in Appendix S1. **Detergents:** Sodium dodecyl sulfate (SDS) and sodium cholate hydrate used to dissolve lipids in water for ³¹P NMR measurements were from Bioshop Canada (Burlington, ON) and Sigma-Aldrich (St. Louis, MO), respectively. **Buffer:** 2-(N-morpholino) ethanesulfonic acid (MES) hydrate used to prepare the buffered SDS or cholate micellar solution was from Sigma-Aldrich (St. Louis, MO).

Ethylenediaminetetraacetic acid (EDTA), and cesium hydroxide hydrate, or cesium hydroxide solution used to prepare Cs-EDTA aqueous solution to homogenize the mouse hearts were from Sigma-Aldrich (St. Louis, MO). An antioxidant, butylated hydroxytoluene (BHT) was from Sigma-Aldrich (St. Louis, MO). **Solvent:** Methanol (HPLC grade) and chloroform (HPLC grade) were from Sigma-Aldrich (St. Louis, MO). **NMR solvent** deuterium oxide (99.9 atom%D) was from Cambridge Isotope Laboratories (Andover, MA).

Doxycycline-induced TAZ-KD in transgenic mice

All protocols were approved by the Institutional Animal Care and Use Committee of NYU School of Medicine and Langone Medical Center, and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH). The tafazzin-knockdown (TAZ-KD) transgenic mice (JAX stock #014648)⁴⁴ were housed in temperature-controlled conditions under a 12-hour light/dark cycle with free access to drinking water and food. The mice used in this study are the offspring of heterozygote (male)-wildtype (WT) (female) C57BL/6N crosses. To knockdown tafazzin expression, three-month-old transgenic mice were treated with doxycycline in drinking water, as well as their WT littermates, for the next 8 months. Briefly, drinking water containing 2 mg/mL doxycycline and 10% sucrose were prepared every 3-4 days. The use of 10% sucrose was necessary to improve palatability of the doxycycline solution. The phospholipid composition of the WT mouse heart showed no significant difference between with and without the doxycycline induction.

Preparation of the TAZ-KD and WT mouse heart phospholipid samples for ³¹P NMR

Cs-EDTA buffer was prepared by titration of free EDTA (at a final concentration of 0.2 M) in water with 50 w% CsOH aq. until the pH reached 6.0, followed by volume adjustment, addition of BHT at 50 μ M, pH readjustment to 6.0, and degassing by Argon-bubbling.³¹ Water used was Milli-Q purified water. The heart was taken from a freshly sacrificed TAZ-KD, or WT mouse, to be placed immediately in the cold Cs-EDTA buffer in a beaker. The buffer with the heart in it was swirled gently, excess blood removed with the buffer by decantation, and the buffer re-added (typically 1 mL for one heart). The heart tissue from two or three mice was used for one ³¹P NMR sample; for each sample, the hearts were minced in the cold buffer and homogenized in cold using a motor-driven Teflon pestle and a glass vessel. Here EDTA not only captures paramagnetic ions to prevent NMR line broadening, but also protects plasmalogens and polyunsaturated chains of phospholipids from ion-catalyzed oxidative degradation.^{34, 45, 46} Use of Cs cation is because of the absence of precipitate formation of cation-EDTA salt upon mixing with organic solvent.³¹ Lipids were further protected against oxidation by the inclusion of 50 μ M BHT. Lipid was then extracted from the homogenate by the Folch method⁴⁷ using a chloroform/methanol (2/1 (v/v)) solvent containing 250 μ M BHT. Solvent was evaporated under a stream of nitrogen gas to form a lipid film. The film was vacuum dried for 2 hours (note here high volatility of BHT), and dissolved in 600 μ L of degassed 10% (w/v) SDS aq. (pH 6.0, 50 mM MES, 50 μ M BHT, 10% (v/v) D₂O).

High-resolution ³¹P NMR

High-resolution ³¹P NMR spectra of the samples placed in 5-mm diameter NMR tubes were recorded with temperature control at 25 °C on a Bruker AVANCE-III 700 MHz spectrometer (³¹P frequency, 283.4 MHz) that was equipped with a QNP-cryoprobe. Spectra were acquired with an 80° excitation pulse on ³¹P nuclei and inverse-gated broadband ¹H-decoupling with the WALTZ-16 sequence at the decoupling power of 3.8 kHz. Free induction decays were acquired for 2048 scans over a 12 ppm (3.4 kHz) bandwidth with a 2.4-s acquisition time and a 1.0-s recycle delay. The chemical shift scale was referenced to

an external 85% (w/v) phosphoric acid standard set to 0 ppm. With the total 3.4-s pulse interval time on the 80° excitation pulse, ³¹P resonances of the measured phospholipids undergo full relaxation to ensure accurate quantification of their composition. This was confirmed by measurements of ³¹P spin-lattice relaxation times T_1 of the component phospholipids, as well as by direct comparison of the composition as a function of the excitation pulse angle varied in the range of 30-90° in a 10° increment. Parameters for the inversion-recovery experiments (d_I - 180° - t - 90° - acquire)_{*n*} were; a 5-s delay time d_I , a 12.9-μs 90° pulse on ³¹P nuclei, 256 scans *n* for each variable delay time *t* (0.10, 0.25, 0.50, 0.70, 1.25, 1.50, 1.75, 2.50, 3.00, 5.00 s), the WALTZ-16 ¹H decoupling at 1.6 kHz during the period (d_I - 180° - t) and at 3.8 kHz during the period (90° - acquire).

Quantitative Western Blot

Quantitative Western blotting experiments were conducted on a V3 Western Workflow system (Bio-Rad) with some modifications in the procedure.

Sample preparation.⁴⁸⁻⁵¹—All the steps of sample preparation were carried out at 0-4°C. The homogenization medium (HM) (0.25 M sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH 7.4, protease inhibitor cocktail) as well as equipment used in the homogenization, centrifugation, and fractionation were pre-chilled. The heart immediately after being taken from a freshly sacrificed mouse was washed 3-4 times with HM. The heart was minced coarsely in 4 volumes of HM, and homogenized in a homogenizer with a pestle rotating at 500 rpm for 8 up-and-down strokes at a rate of ~10 sec/stroke. The homogenate obtained was centrifuged at 750g for 5 min. The supernatant was centrifuged one more time at 750g for 5 min. Thus obtained supernatant, i.e. postnuclear supernatant (PNS) was aliquoted and stored at -80°C until use. A part of PNS was fractionated into a pellet fraction containing peroxisomes and a supernatant fraction by ultracentrifugation at 100,000 g for 30 min. The pellet was re-suspended with an equal volume of HM to the supernatant volume. Protein concentration of PNS was determined by the Bradford assay (Bio-Rad). For Western blot experiments, the samples were heated at 95 °C for 5 min with SDS in the presence of a reducing agent (5% β-mercaptoethanol or 100 mM DTT) to denature proteins. The samples were briefly (~2 min) centrifuged at 6000 rpm, and supernatant was used for the Western blot experiment.

SDS-PAGE—Precision Plus Protein All Blue Prestained Protein Standards (Bio-Rad) was used as a molecular weight marker. Proteins were separated using 4-15% Mini-PROTEIN TGX Stain-Free Protein Gel (Bio-Rad) at 250V for 25 min. The amount of a sample to be loaded per lane for the quantitative Western blot analysis of the target proteins was determined by evaluating their respective linear dynamic range as a function of the total protein content (Figures S1 and S2). This evaluation of the linear dynamic range was done with a serial dilution of a mixture of the equal amount (per total protein) of the WT and TAZ-KD PNS fractions.

Introduction of fluorophore for quantification of the total protein content—UV-irradiation on the gel after the electrophoretic separation of proteins initiates reaction between trihalo compounds in the gel and tryptophan residues in proteins to introduce a

fluorophore. The UV-irradiation was conducted for 45 sec on a ChemiDoc Imaging System (Bio-Rad), after which ~10% of tryptophan residues in proteins was fluorophore-labeled. This ~10% labeling of tryptophan residues is sufficient to accurately quantify the total protein content, while having negligible effect on immunodetection of target proteins in the downstream. Fluorescence from the introduced fluorophore was quantified after transfer to the PVDF membrane to confirm the electrophoretic protein separation and to determine the lane-dependent transfer efficiency (Figure S2 legend).

Gel-membrane protein transfer and the efficiency—Proteins were transferred from the gel onto an Immun-Blot Low Fluorescence PVDF Membrane (Bio-Rad) at 2.5A and 25V for 7min using a Trans-Blot Turbo Blotting System (Bio-Rad). The transfer efficiency in each lane was determined by quantification of the total protein in the membrane on the ChemiDoc Imager System (Bio-Rad) (Figure S2 legend).

Incubation with antibodies—After blocking with 5% BSA in TBST, the membranes were incubated with the primary antibody at 4 °C overnight and then with the secondary antibody at room temperature for 1 hour. A wash was made after each incubation. Antibodies used are summarized in Table S1.

Quantification of target proteins—The membranes were incubated with ECL reagent, ⁵² and chemiluminescence was used to quantify the target proteins with a ChemiDoc Imaging System (Bio-Rad).

Results

High-resolution ³¹P NMR measurements of the phospholipid composition including oxidation-labile plasmalogens in the heart of WT and TAZ-KD mice

High-resolution ³¹P NMR in combination with the use of a solubilizing detergent is a powerful method to accurately determine a detailed phospholipid composition of a biological material in a single measurement, based on its ability to distinctively identify different phospholipid classes and subclasses according to their unique chemical shift values of the phosphate group. Sensitivity of the chemical shift to reflect the difference in the type of *sn*-1 linkage for plasmalogens is of particular importance in reliable quantification of those major but unique phospholipids.^{30–32} In ¹H and ¹³C NMR of phospholipids, a proton- or a carbon-signal from one of the *sn*-1 vinyl ether protons or carbons of plasmalogens, that is known to appear distinctively from all the other signals in the spectrum, may be used to quantify plasmalogens in a biological system.^{53–55} However, a procedure to identify relative contributions to the ¹H or ¹³C spectrum from various phospholipid classes/subclasses will be somewhat labor intensive also after assignment of heavily overlapping signals. Such quantification as well as cross-check for accuracy may be conducted by calculating a combination of a number of intensity-ratios for uniquely identifiable and well-separated signals from the individual phospholipid class/subclass. Calculation can be done using only minor fractions of the entire dynamic range of detection even for the major classes due to heavy signal overlap between classes/subclasses on their common structural moieties.

In some cases, use of a detergent with an extremely small aggregation number (n) like cholate ($n=4$) to dissolve lipids⁵⁶ for measurements in ^{31}P NMR not only results in resonance sharpening due to enhanced molecular motions but also causes specific detergent-lipid interactions that depend on hydrocarbon chain species and at least partially differentiate among them within a given class/subclass.³³ In the present work it turned out that use of SDS with the moderate aggregation number ($n=62$ -101)⁵⁶ is suitable to determine the phospholipid composition of a biological material, where each class and subclass gives a distinct single resonance as the sum of contributions to the intensity from various hydrocarbon chain species. This simplifies determination of the phospholipid composition without a need of identifying unevenly separated signals of diverse hydrocarbon chain species like in the case of using cholate, where some of them can overlap with, or appear in-between signals of chain species of another class that is nearby in the spectrum. Nevertheless we used cholate as well in this work to aid assignment of observed signals using SDS, according to a different profile of intensities vs. chemical shifts for the phospholipid classes and subclasses as will be presented later. Throughout the process for preparation of the NMR samples of the mouse heart phospholipids, particular caution was used, as described in Materials and Methods, to prevent plasmalogens from oxidation. Plasmalogens were confirmed to be fully protected and stable even in the micellar aqueous solution on the order of days according to ^{31}P NMR. Lipid extraction from the mouse heart homogenate was performed by the Folch method⁴⁷ using cold chloroform/methanol (2/1(v/v)) solvent containing 250 μM BHT (see Materials and Methods); the method is one of the most recommended in lipidomics studies.⁵⁷ We also confirmed that lysophospholipids of the heart tissue were efficiently extracted by the method (Figure S3).

Plasmenylcholine drastically decreases in the TAZ-KD mouse heart—Figure 2A shows high-resolution ^{31}P NMR spectra of the TAZ-KD and the WT mouse heart phospholipids in SDS micellar solution. The phospholipid compositions were determined as summarized in Table 1 along with their changes from the WT to the TAZ-KD. Signal assignment was based on comparison with spectra of phospholipid standards as well as literature data.^{30–32} Measurements of the heart phospholipid ^{31}P NMR in cholate micellar solution gave another profile of intensities vs. chemical shifts for the component classes and subclasses, with which the assignment on the SDS micelle system was confirmed. Sensitive detection and accurate quantification of the phospholipids in the ^{31}P NMR experiments with inverse-gated ^1H decoupling were ensured by optimization of the experimental parameters according to the measured spin-lattice relaxation times T_1 (Table S2, Figure S4).

In addition to confirming the well-known changes in cardiolipin (CL) due to tafazzin deficiency, i.e. decrease in the amount of CL by -4.4 ± 0.4 mol% ($6.4 \pm 0.1 \rightarrow 2.0 \pm 0.4$ mol %) and accumulation of monolysocardiolipins (MLCLs) by $+3.3 \pm 0.5$ mol% (not detected $\rightarrow 3.3 \pm 0.5$ mol%) in the heart of the TAZ-KD mice (Table 1, Figure S5), we found a drastic decrease in the plasmenylcholine (at -0.163 ppm) by -12.7 ± 4.2 mol% ($30.8 \pm 2.8 \rightarrow 18.1 \pm 3.1$ mol%) (Figures 2A and B, Table 1), and a counterbalancing increase in the diacyl phosphatidylcholine (PC) (at -0.174 ppm) by $+7.8 \pm 3.5$ mol% ($19.2 \pm 1.9 \rightarrow 27.0 \pm 2.9$ mol%) (Figure 2A, Table 1) that are seen as clear inversion of spectral asymmetry with the partially overlapping signals. The mechanism of the counterbalance is separately

discussed in Appendix S2. The decrease in plasmalogen is drastic with regard to both (i) being the largest change in the content, and (ii) the most abundant phospholipid in the WT mouse heart (Table 1). Impact of such a drastic change in the most abundant phospholipid in the tissue is expected to be substantial not only as a part of lipid biochemistry of the cell but on membrane physical properties (see Discussion below). The magnitude of the decrease in terms of a change in the individual content was also estimated to be large as -41.3 ± 13.6 mol% (Table 1). This value is quite comparable to the decrease in CL by -68.7 ± 6.3 mol%. As a minor component, a plasmalogen signal overlaps with the diacyl PC signal (Figure S6), whereas contents of plasmalogen in the mammalian heart tissues are commonly less than 1/10 of that of diacyl PC^{25, 58} and essentially the same applies to the ratio between plasmalogenethanolamine and diacyl PE.^{25, 58} It is noted that in sharp contrast to the dramatic decrease in plasmalogen in the TAZ-KD heart, no significant change in the plasmalogenethanolamine content was detected (Figure 2A, Table 1).

Lysoplasmalogen as a counterpart of plasmalogen in transacylation by

tafazzin—The large decrease in plasmalogen due to deficiency in transacylation by tafazzin poses us a question if there is any change to the content of its lysophospholipid either as a precursor, or as a product of transacylation in the control WT. Although tafazzin has been shown to be a nonspecific transacylase for any phospholipid class examined,^{12–15} its reactivity for plasmalogen phospholipids has not been probed specifically to date. In our Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) MS experiments, lysoplasmalogens indeed were confirmed to be a substrate of tafazzin for both lyso-plasmalogen and lyso-plasmalogenethanolamine, using purified recombinant yeast tafazzin⁵⁹ (see Figure S7 about the reactivity). The reversible nature of the transacylation reaction by tafazzin, as has been reported in experiments using reversed pairs with regard to the donor/acceptor roles in each pair of different phospholipid classes^{14, 15}, indicates that lysoplasmalogens and plasmalogens are both substrates of tafazzin. The absence of the *sn*-1 unsaturation adjacent to the ether bond, corresponding to the structure of plasmalogen glycerophospholipids as minor components in mammalian organs including the heart,^{25, 58} showed in contrast, lack of reactivity with tafazzin.⁶⁰ The schematic analogy in reversibility of biochemical reactions to regulate a compositional equilibrium may be familiarly presented by transaminase to transfer an amino group between a donor and an acceptor in the bidirectional reaction.⁶¹ The net direction of the reaction mutually depends on concentrations of the four substrates, i.e. two substrates (a donor and an acceptor) on each side of the equation that correspond to the counterpart two products (an acceptor and a donor in the opposite reaction, respectively). While we chose here CL as a transacylation partner to directly probe the reaction for CL remodeling, in biological systems the partner can also be other lipid classes including the major PC and PE species as a part of the catalytic reaction cycle. Another important feature of the transacylation cycles by tafazzin is that only a trace amount of lysophospholipid is required and actually present in the membrane for progression of remodeling and attainment of the equilibrium in acyl-species distributions of the component phospholipids.¹⁵ Both choline and ethanolamine plasmalogens are major components of the heart mitochondria, and their fractions in this organelle are as abundant as they are in the whole heart tissue.⁶² Our preliminary data (not shown) in an ongoing subcellular fractionation study, using a single cell type system of lymphoblasts derived from

Barth syndrome patients that is ideal for studies of the mechanism, indicated that a decrease in plasmalogen (in this case plasmenylethanolamine as the dominant form) takes place almost exclusively in mitochondria.

The observed decrease in plasmenylcholine in the TAZ-KD mouse heart may suggest an increase in lyso-plasmenylcholine in the deficiency of tafazzin. Although the presence of only a trace amount of lysophospholipid is required to initiate the catalytic transacylation cycles by tafazzin according to orders of magnitude higher affinity for a lysophospholipid than for a parent phospholipid,¹⁵ it is conceivable that the maintenance of the plasmenylcholine level is associated with the lyso-plasmenylcholine level. In fact, a decrease in CL due to tafazzin deficiency is accompanied by an increase in MLCL.

In the ³¹P NMR spectra of both the WT and TAZ-KD mouse heart phospholipids (Figure 2A), we observed a signal at 0.208 ppm that can be unambiguously assigned to lyso PC as described below. Measurements on the standard lyso-plasmenylcholine and lyso PC in SDS micellar solution showed that the ³¹P NMR signal of lyso-plasmenylcholine appears slightly downfield of the signal of lyso PC whereas the separation between the two signals was about four-fold smaller than that observed for plasmenylcholine and diacyl PC (Figure 3A). Separation between those two lysophospholipids became more distinct in cholate micellar solution with an altered chemical shift profile for phospholipids, giving 10 times larger separation than in SDS micellar solution (Figure 3B). Measurements of the WT and TAZ-KD mouse heart phospholipids in cholate micellar solution confirmed the absence of a detectable level of lyso-plasmenylcholine in those samples (Figure 3B). The absence of detectable levels of lyso PE and lyso-plasmenylethanolamine in either the WT or TAZ-KD mouse heart was confirmed according to similar experiments comparing those different detergent systems (Figures 3C and D).

Plasmenylcholine vs. plasmenylethanolamine—Early steps of the biosynthesis of plasmalogens take place in peroxisomes, from which an intermediate precursor is transferred to the endoplasmic reticulum (ER) where further synthetic steps lead to the formation of plasmenylethanolamine (1-O-alk-1'-enyl-2-acyl-GPE) (Figure 4).^{21, 22, 63} Plasmenylcholine (1-O-alk-1'-enyl-2-acyl-GPC) is synthesized from plasmenylethanolamine via the cleavage in the headgroup either with or without steps including exchange of the acyl chain prior to the headgroup cleavage (Figure 4).^{21, 22, 63} The observed decrease in the plasmalogen level in the heart of TAZ-KD mice was exclusive for plasmenylcholine (Figure 2A, Table 1). Other important pieces of experimental evidence in on-going studies are that in other TAZ-KD mouse organs or human lymphoblast cells derived from Barth syndrome patients, there is a significant decrease in plasmenylethanolamine, the dominant form of plasmalogen in these cells/tissues (e.g., in the lymphoblast, $-3.5 \pm 2.2\%$ ($14.4 \pm 1.9\% \rightarrow 10.8 \pm 1.1\%$)) compared to control (unpublished results). A counterbalancing increase was observed as well, in overlapping signals corresponding to the other ethanolamine phospholipids (diacyl and plasmanyl) that most likely reflect an increase in diacyl PE (e.g., in the lymphoblast, $+7.5 \pm 1.8\%$ ($13.6 \pm 1.1\% \rightarrow 21.1 \pm 1.4\%$)).

Quantitative Western blot to monitor proteins for biochemical insights into the NMR observation of the plasmalogen decrease

In this section, we investigate levels of proteins in the mouse heart that may be considered to be relevant to the above observation of the substantial loss in plasmenylcholine due to the TAZ-KD. Monitoring of the proteins has been performed by quantitative Western blot. Quantification in a linear dynamic range of immunodetection was carefully assured for each target protein with regard to both the binding capacity to the polyvinylidene difluoride (PVDF) membrane, and integration of the chemiluminescence signal from the horseradish peroxidase (HRP) secondary antibody using a CCD camera (Figure S1). Lane dependent unevenness in the efficiency of transfer from the gel to the membrane was also taken into account and corrected by normalization to the total protein content that was quantified in each lane using Image Lab Software (Bio-Rad) (Figure S2 legend).

A loss of plasmalogen found in other disease like Zellweger syndrome is associated with a significant loss of peroxisomes,²⁴ where the early steps in biosynthesis of plasmalogen take place (Figure 4).^{21–23} In the present work it is noteworthy that the steady state level of plasmenylethanolamine that is a precursor for plasmenylcholine synthesis showed no significant change due to the TAZ-KD in contrast to the dramatic loss of plasmenylcholine (Figure 2A, Table 1). In light of the well-characterized cases of the other disease in which plasmalogen loss is associated with peroxisome deficiency, the clear difference observed for choline and ethanolamine plasmalogens in the TAZ-KD mouse heart was intriguing and lead us to evaluate the amount of peroxisomes. Among the series of peroxins that are responsible for functions of peroxisomes, Pex19p, Pex16p and Pex3p are known to be critical in *de novo* formation of the peroxisomal membrane structure.^{64–66} Pex19p is a water-soluble peroxin predominantly located in the cytosol, while Pex16p and Pex3p are membrane associated. Pex19p functions as a cargo protein as well as a chaperone to incorporate peroxisomal membrane proteins (PMPs) to the peroxisomal membrane. In this work, we quantified Pex19p as well as PMP70 that is a frequently used marker of the peroxisome level,^{67, 68} to investigate the amount of peroxisomes. Another reason to look into peroxisomes is related to a recent report that the *de novo* formation of peroxisomes is initiated by budding off vesicular pre-peroxisomal structures from mitochondria,⁶⁸ that are damaged in Barth syndrome, as well as from the ER. We also measured the level of catalase, which has been used as a histological marker of peroxisomes.⁶⁹ Catalase levels in the cytosol and in peroxisomes were monitored paying attention to the previously reported correlation of tafazzin deficiency with impaired respiratory function^{70–72} and relevant enhancement of ROS formation.^{73, 74}

In examining a direct response to a plasmalogen decrease in the cell, it is expected to be insightful to look into the expression level of fatty acyl-CoA reductase 1 (Far1). Far1 functions as a rate determining enzyme for plasmalogen synthesis,⁷⁵ converting fatty acyl-CoA to fatty alcohol (Figure 4).⁷⁶ Fatty alcohol is used to form a plasmalogen precursor 1-O-alkyl-1-dihydroxyacetone phosphate (alkyl-DHAP) in peroxisomes (acyl-DHAP → alkyl-DHAP) (Figure 4). The level of Far1 expression is known to be regulated by a feedback mechanism in response to the amount of plasmalogen in the cell;⁷⁵ in the case of a decrease

in plasmalogens, we would expect Far1 upregulation to increase the rate of plasmalogen synthesis.

In the mammalian heart, plasmenylcholine is degraded by consecutive enzymatic actions of (i) phospholipase A₂ to first cleave off the acyl chain at the *sn*-2 position and (ii) lysoplasmalogenase to next cleave off the fatty aldehyde at the *sn*-1 vinyl ether bond on lysoplasmalogen.⁷⁷ Note that plasmalogenase activity, that was observed in the heart to cleave the *sn*-1 vinyl linkage in plasmenylethanolamine,⁷⁸ has been found to be absent for plasmenylcholine in this organ.^{77, 79} In the present work we measured expression levels of calcium-independent phospholipase A₂ isoforms that are known to be plasmalogen selective; iPLA₂ β and γ .^{79–82}

The amount of peroxisomes is normal in the TAZ-KD mouse heart, while the catalase expression level is significantly increased—The ratio of expression of Pex19p in the TAZ-KD vs. WT mouse heart (TAZ-KD/WT) was determined to be 1.12 ± 0.10 , and that of PMP70 to be 0.97 ± 0.05 (Figure 5A, Table S3). The results indicate that the amount of peroxisomes is not reduced, and thus do not ascribe the NMR observation of the decrease in plasmenylcholine to a change in the amount of peroxisomes. Similar observations were made on the ratio for those proteins in human lymphoblast cells derived from Barth syndrome patients vs. healthy control (BTHS/control) with a somewhat higher ratio for Pex19p than in the case of the mouse heart; 1.29 ± 0.03 for Pex19p, 0.95 ± 0.01 for PMP70. The conclusion of no significant reduction in the amount of peroxisomes is in accord with the NMR observation that the plasmenylethanolamine content remains essentially unchanged (Figure 2A, Table 1). That is, the state of peroxisomes is normal in their ability to synthesize the precursors of plasmalogens.

In skin fibroblasts of Barth syndrome patients, the level of catalase was observed to be upregulated.⁷⁴ Catalase is often used as a histological marker for peroxisomes by detecting peroxisomal catalase in comparison to the level of cytosolic catalase.⁶⁹ The presence of catalase in mammalian heart mitochondria was also indicated,⁸³ although its role in H₂O₂ removal in this organelle is likely to be minor compared to the role played by glutathione peroxidase.⁸⁴ In the present work, we observed an increase in catalase expression in the TAZ-KD mouse heart with the ratio TAZ-KD/WT = 1.47 ± 0.08 in measurements on the postnuclear supernatant (PNS) of the tissue (Figure 5B, Table S3). Distribution of catalase in the membrane pellet (P) and the supernatant (S) fractions of the PNS in the WT mouse heart was measured to be 45.4 ± 1.7 % and 54.6 ± 1.7 %, respectively (Figure 5B). The increased catalase level due to tafazzin deficiency was mostly attributed to the membrane fraction, i.e. peroxisomal catalase with the ratio of TAZ-KD/WT = 2.15 ± 0.03 , in contrast to the ratio of 1.10 ± 0.06 obtained for cytosolic catalase (Figure 5B). The increase in the former is in accord as well with the observations that there is no loss of the normal level of peroxisomes in the TAZ-KD according to quantification of Pex19p and PMP70 as above. The highly preferential increase in the peroxisomal catalase results in the catalase distribution in the P and S fractions in the TAZ-KD mouse heart to be 61.8 ± 0.2 % and 38.2 ± 0.2 %, respectively (Figure 5B). The conceivable mechanism leading to the catalase increase is described in Appendix S3. Our observation of upregulated catalase in the TAZ-KD mouse heart likely indicates enhanced defensive response to oxidation in the tissue. The more

prominent increase in peroxisomal catalase than in cytosolic catalase may reflect the critical role of catalase in detoxifying H_2O_2 in peroxisomes in contrast to its minor contribution compared to that of glutathione peroxidase in the cytosol.^{85, 86}

Rate of plasmalogen synthesis is enhanced in the TAZ-KD mouse heart by substantial upregulation of Far1 expression—Far1 is a rate determining enzyme of plasmalogen biosynthesis, the expression of which is regulated by a feedback mechanism in response to the amount of plasmalogen in the cell.⁷⁵ We compared Far1 expression levels of the TAZ-KD and WT mouse heart. The abundance of Far1 was found to be substantially enhanced by 8.3 ± 1.6 fold in the TAZ-KD compared to the control WT (Figure 5C, Table S3). The specificity of the immunodetection was further confirmed by use of a commercially available blocking peptide for Far1. The large upregulation of the Far1 level is consistent with a positive feedback response to the decreased steady state level of plasmenylcholine that is caused by the tafazzin deficiency. Yet the presence of the large upregulation of Far1 expression does not fully compensate for the decrease in plasmenylcholine. Another relevant observation is that there is no significant change in the content of plasmenylethanolamine (Figure 2A, Table 1). As plasmenylethanolamine is on the route to the production of plasmenylcholine (Figure 4), it is reasonable to consider that the observed lack of change in the steady state level of plasmenylethanolamine is merely a reflection of a balance between the accelerated plasmalogen synthesis caused by the Far1 upregulation, and a conceivably present decrease in plasmenylethanolamine by a mechanism analogous to that for the decrease in plasmenylcholine. In other words, the Far1 upregulation was sufficient to compensate for a decrease in plasmenylethanolamine due to the tafazzin deficiency but not for a decrease in plasmenylcholine.

Plasmalogen-selective iPLA₂ β is upregulated, contributing to the reduction of the plasmalogen level—The β isoform of calcium-independent phospholipase A₂, iPLA₂ β , is known to be plasmalogen-selective especially in releasing its *sn*-2 polyunsaturated fatty acid.^{79–81} It is therefore of interest to quantify the expression level of iPLA₂ β , when tafazzin is deficient compared with normal controls. We observed the ratio of expression TAZ-KD/WT to be 1.39 ± 0.14 (Figure 5D, Table S3). The significantly increased expression of iPLA₂ β in the absence of tafazzin likely contributes to the decrease in the plasmalogen level. No detectable amount of lyso-plasmenylcholine or lyso-plasmenylethanolamine in the NMR of the TAZ-KD (Figure 3) indicates that degradation of lysoplasmalogens is efficient to prevent their accumulation. The upregulation of iPLA₂ β in the tafazzin deficiency may be a part of spontaneous cellular responses to rescue the loss of transacylation, by elevated formation of a substrate for re-acylation by other acyl transferases in remodeling.^{19, 87} However, such responses can contribute to complication to increase phenotypic severity. In fact, knockout of iPLA₂ β in tafazzin deficiency is known to decrease the ratio of MLCL/CL as a molecular indicator of the inner mitochondrial membrane defect, and to alleviate phenotypic severity in disease models (Appendix S4). We also measured the iPLA₂ γ levels in the TAZ-KD and WT mouse heart. However, we detected only a faint band corresponding to iPLA₂ γ (Figure S8). There was no consistent change in the ratio of TAZ-KD/WT, ranging randomly between 0.5 and 3.1 in five sets of independent experiments (N=12) (Figure S8).

Discussion

Plasmalogen species in the mammalian heart, and potential influences of a reduction of plasmalogen in tafazzin-deficiency

Tafazzin function and CL remodeling in the context of the membrane

environment—CL species, in a biological system particularly with high energy turnover, are highly specific, typically containing one or two dominant unsaturated acyl chain species.¹⁶ The dominant and distribution of acyl chain species vary depending on the type of cell, tissue, organ, and organism.¹⁶ In the case of the mammalian heart, including the mouse heart investigated in this work,^{16, 88} the dominant CL species is tetralinoleoyl (18:2)₄. Tafazzin is centrally responsible for the regulation of the unique distribution of CL species via transacylation cycles in remodeling. Deficiency in tafazzin function results in a highly diverse and heterogeneous distribution of CL species.¹⁶ The molecular mechanism that regulates the system-dependent dominant CL species in remodeling is currently being actively investigated. Differences in dominant CL species in different systems are not explainable merely by confining our focus to an intrinsic specificity of tafazzin.^{1, 2, 19} Key observations in this regard are that expression of heterologous tafazzin yields formation of CL species characteristic to the expression system rather than CL species of the system, which the expressed tafazzin orthologue originally belongs to; e.g., human TAZ expressed in yeast yields yeast CL species,⁸⁹ human TAZ in *Drosophila* yields *Drosophila* CL species,⁹⁰ human or *Drosophila* TAZ in Sf9 insect cells yields CL species of Sf9 insect cells.¹⁵ Therefore, the physical and chemical membrane environment of a system including the composition of phospholipids and their acyl chain species must play a role in determining the distribution of CL species typically with one or two dominant species.

The observation of the substantially decreased steady-state level of plasmalogen, but not of other lipids except CL, in the TAZ-KD mouse heart (Figure 2A, Table 1) suggests that plasmalogen plays an important role in transacylation cycles between alternating lysophospholipid and phospholipid substrates of tafazzin in remodeling. The decrease in plasmalogen caused by the tafazzin deficiency may be associated with degradation of plasmalogen by the activity of upregulated plasmalogen-selective iPLA₂ β as shown above (Figure 5D, Table S3).

Tafazzin has been shown to have reactivity with any phospholipid class examined,^{12–15} and choline phospholipids showed a high reactivity with CL in experiments using purified mitochondria as the source of tafazzin. In addition to the high reactivity at a given concentration, we note that the WT mouse heart contains plasmalogen at 30.8 ± 2.8 mol % as the single dominant phospholipid, followed by 19.2 ± 1.9 mol% of diacyl PC and a minor fraction of plasmalogen (Table 1). When quantification was made in a bilayer membrane on the transacylation reactivity by tafazzin between MLCL and a [¹⁴C]-labeled phospholipid in the 2-acyl chain in sonicated liposomes,¹⁴ > 3-fold faster reaction rates were observed for PC compared to PE having the corresponding acyl chain species, with marked preference in the reactivity observed for a linoleoyl (18:2) chain among others. Note here that tafazzin preferentially transfers an *sn*-2 acyl chain of a phospholipid^{91, 92} to either the *sn*-1 or *sn*-2 site of a lysophospholipid,^{15, 91} and the site of an acyl chain in a

lysophospholipid readily shuffles between the *sn*-1 and *sn*-2 positions via acyl chain migration. Lyso-plasmalogens are an exception whose hydrocarbon chain is vinyl ether bonded in the *sn*-1 position, therefore, does not migrate. An earlier work demonstrated the same trend in the reactivity preference (2-[¹⁴C]linoleoyl PC > 2-[¹⁴C]linoleoyl PE), although the difference was smaller.¹² On both PC and PE, the reactivity of their *sn*-2 oleoyl (18:1) or arachidonoyl (20:4) chain species was less than 10% of the reactivity of their *sn*-2 linoleoyl chain species.^{12, 14} Then the reactivity was compared between 2-linoleoyl PC with other lysophospholipid partners than MLCL, such as lyso PE and lyso PA.¹⁴ Lyso PE and lyso PA showed only ~15% and ~35% of the reactivity of MLCL, respectively. In a recent study, a similar preference in the reactivity of different PC species with MLCL was observed (PC(18:2-18:2) > PC(18:1-18:1), PC(16:1-16:1)) using purified recombinant yeast tafazzin reconstituted in large unilamellar vesicles,^{91, 92} while the effect of high concentrations of residual detergent that was used in the protein purification in the work was discussed in detail later.^{3, 59, 92} The discussion was made particularly because the tafazzin reactivity is known to be substantially accelerated in a highly disordered hydrocarbon chain environment, such as in micelles and inverted hexagonal phase membranes.² A similar acceleration of the reactivity toward lipid substrates in those disordered environments is being observed on another recombinant, human peripheral membrane protein, DGK ϵ , in our group⁹³ (Bozelli et al. Unpublished results). Those results suggest that membrane morphology including surface curvature (see Discussion) can have a large impact on the enzymatic reactivity and hence the phospholipid composition.

In the mammalian heart in general, the dominant acyl chain species of plasmenylcholine is a linoleoyl chain, being followed by an arachidonoyl or an oleoyl chain.^{58, 62, 94} The dominant *sn*-2 species of diacyl PC there is also a linoleoyl chain, being followed by an oleoyl chain. In the case of the mouse heart, the abundance of diacyl PC having an *sn*-2 linoleoyl chain is accompanied by the characteristic presence of diacyl PC having an *sn*-2 docosahexaenoyl or arachidonoyl chain.⁸⁸ In contrast, the dominant acyl chain species of plasmenylethanolamine in the mammalian heart are often comparable amounts of arachidonoyl and linoleoyl chains, while that in the *sn*-2 in diacyl PE is an arachidonoyl chain.^{58, 62, 94} In the mouse heart, docosahexaenoyl and arachidonoyl chains are the dominant species of plasmenylethanolamine and the *sn*-2 chain of diacyl PE.⁸⁸ The amounts (mol%) of those phospholipids in the WT mouse heart were determined in this work to be 30.8 ± 2.8 for plasmenylcholine, 19.2 ± 1.9 for diacyl PC plus a minor fraction of plasmanylcholine, 8.2 ± 0.4 for plasmenylethanolamine, and 26.2 ± 1.2 for diacyl PE plus a minor fraction of plasmanylethanolamine. The above mentioned high transacylation reactivity of choline phospholipid having an *sn*-2 linoleoyl chain with MLCL, along with the measured phospholipid composition in the mouse heart, suggest that plasmenylcholine and diacyl PC are the highly preferred reaction partners of MLCL in CL remodeling. This is in accord with the well-known fact that the single dominant CL species in the mammalian heart, including mouse, is tetralinoleoyl CL.^{16, 44, 88} The drastic reduction in plasmenylcholine but not in diacyl PC due to the tafazzin deficiency is suggestive of an important role of tafazzin to maintain the plasmalogen level. The importance can be related to the regulation of the dominant CL species, i.e. tetralinoleoyl CL in the case of the mammalian heart.

Plasmalogen and membrane morphology—The drastic loss of plasmalogen in the absence of functional tafazzin is likely to have major impact on the structure, function, and integrity of mitochondria. While there are reports about correlations between the lack of peroxisome biogenesis as the site of synthesis of plasmalogen precursors, and morphological/functional abnormalities of mitochondria especially in cristae,^{95, 96} little is known regarding physical and chemical roles of plasmalogens in mitochondria at the molecular level.

From a biophysical perspective, plasmalogen has a tendency to stabilize the inverse hexagonal membrane phase (H_{II}) above a transition temperature T_h between the liquid-crystalline (L_α) and the H_{II} phases.^{97, 98} This tendency is more notable than the one for diacyl PE and plasmalogen in the order of plasmalogen $\gg$ plasmalogen > diacyl PE. Thus, plasmalogen is highly capable of stabilizing and being stable in a negative surface curvature. The *sn*-2 acyl chain of diacyl PE and PC in a bilayer is known to take characteristic bent conformation near the ester bond, with which the uppermost carbon segments are almost parallel to the bilayer surface. In contrast, the presence of the *sn*-1 vinyl ether bond in plasmalogen and choline plasmalogens causes structural arrangement such that their *sn*-2 acyl chain takes preferential conformation with its primary axis, throughout the chain, perpendicular to the surface,^{98–100} and as a consequence plasmalogen molecules induce tighter lateral packing of lipids in a bilayer membrane compared to the diacyl counterparts.^{98, 101, 102} The tighter packing of the hydrocarbon chains brings the headgroups in closer contact, which favors H_{II} phase formation in the case of the plasmalogen membrane.

The mammalian heart mitochondria have at least comparable percentages of plasmalogens in the total lipid as the whole tissue does, according to a report of a study using guinea pigs.⁶² This applies to both plasmalogen and plasmalogen. The cristae membrane as the site for oxidative phosphorylation forms a tubular structure with a significant degree of curvature; an estimated outer diameter is ~35 nm.¹⁰³ Such curvature has been proposed to be stabilized by preferential localization of cone shaped lipids such as CL and PE in the inner leaflet of the membrane.^{103, 104} Indeed, PE deficiency in mammalian mitochondria impairs oxidative phosphorylation and alters mitochondrial morphology including the cristae.¹⁰⁵ CL deficiency impacts similarly; see, e.g. a review in the reference.¹⁹ Alterations of mitochondrial ultrastructures were also observed in the cells of Zellweger syndrome patients and mouse models, where plasmalogen as the dominant form of plasmalogen in the tissue was deficient due to deficiency in functional peroxisomes.^{95, 96} In the present work no significant change in the plasmalogen and diacyl PE contents was observed in the TAZ-KD mouse heart, while common reduction of plasmalogen was confirmed in cells and tissues where ethanolamine is the dominant form of plasmalogens, such as in lymphoblast cells derived from Barth syndrome patients as well as in other organs of the TAZ-KD mice (unpublished results). The reduction in the highly negative-curvature prone plasmalogen is considered to lower the stability of mitochondrial cristae membranes even in the presence of the counterbalancing increase in the less negative-curvature prone diacyl PE. Likewise, the reduction of plasmalogen is considered to affect the stability of the cristae membrane due to

reduction in the membrane packing ability despite the presence of the counterbalance by the diacyl PC. An indication that plasmalogen may play a critical role in maintaining the structure and function of mitochondrial cristae membranes is that loss of functional SLC25A46, a mitochondrial metabolite carrier that locates in the outer membrane and associates with ‘mitochondrial contact site and cristae organizing system’ (MICOS), results in prominent increase in the plasmalogen content along with abnormal mitochondrial architecture with markedly shortened cristae.¹⁰⁶

The dramatic plasmalogen reduction can be a useful diagnostic probe for Barth syndrome. A study to reverse the effects of the loss of tafazzin function associated with the plasmalogen level is in progress in our group using the tafazzin deficient human lymphoblast cell culture system. Findings in the study are expected to contribute to relief from the disease by recovering the reduced plasmalogen levels. Fundamental cellular mechanism of changes in lipid composition, not only in the mitochondria where tafazzin and CL are specifically located but also in other organelles, is being revealed as well in our group in view of the physical and chemical functions of those lipids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of plasmenylcholine and diacyl phosphatidylcholine (PC). Structures are drawn with the most abundant acyl chain species for those lipids in the mammalian heart.

Figure 2.

(A) ^{31}P NMR spectra of phospholipids in the heart of the wild type (WT, blue trace) and tafazzin-knockdown (TAZ-KD, red trace) mice. A magnified region on the right shows inversion of the spectral asymmetry, indicating a drastic decrease in plasmalogen choline (-0.163 ppm) and a counterbalancing increase in diacyl PC (-0.174 ppm) in due to the TAZ-KD. The signal from the minor component plasmalogen choline (see the main text) overlaps with the signal from diacyl PC (Figure S6). A magnified region on the upper left shows a large decrease in CL (0.745 ppm) along with increases in 2-MLCL (1.137 ppm) and 1-MLCL (1.009 ppm). An increase in PG (0.845 ppm) is also seen. *A part of the CL decrease is masked by contributions from the resonance of a phosphate group in the diacyl half of MLCLs (Figure S5). (B) A spectrum of the standard plasmalogen choline (green trace) overlaid with the WT mouse heart phospholipid spectrum (blue trace).

Figure 3.

^{31}P NMR identification of lysophospholipids in the mouse heart, using different solubilizing detergent systems of SDS and cholate. (A) ^{31}P NMR of lyso PC and lyso-plasmenylcholine (lyso-plsCho) in SDS micelles yields similar chemical shift values. (B) Use of cholate micelles results in distinct signal separation for lyso PC and lyso-plsCho, thus unambiguously identifying the presence of lyso PC and the absence of a detectable amount of lyso-plsCho in the WT and TAZ-KD mouse heart. (C) The absence of a detectable amount of lyso PE in the WT and TAZ-KD mouse heart is evident from the measurements in SDS micelles. Lyso-plasmenylethanolamine (lyso-plsEtn) shows the chemical shift close to that of CL and the diacyl half of MLCLs at 0.745 ppm in SDS micelles. (D) Use of cholate micelles enabled to identify the absence of a detectable amount of lyso-plsEtn in the WT and TAZ-KD mouse heart.

Figure 4. The biosynthesis pathway for plasmalogens (1-O-alk-1'-enyl-2-acyl-GPE (plasmenylethanolamine) and 1-O-alk-1'-enyl-2-acyl-GPC (plasmenylcholine)) in the cell.

Figure 5.

Quantitative Western blot of proteins in the mouse heart that are indicative of (i) the amount of peroxisomes (Pex19p, PMP70, catalase), (ii) a degree of oxidative stress (catalase), (iii) a degree of feedback regulation of the plasmalogen level (Far1), and (iv) a degree of plasmalogen-selective lipid degradation (iPLA₂β). (A) The ratio of expression levels (TAZ-KD/WT) in the TAZ-KD and WT mouse heart on cytosolic Pex19p (N=3) and peroxisomal integral membrane protein PMP70 (N=3). Measurements to determine the ratio were conducted on the postnuclear supernatant (PNS), while fractionation by ultracentrifugation (see Materials and Methods) results in detection of Pex19p exclusively in the supernatant fraction (S), and PMP70 exclusively in the membrane pellet fraction (P) that includes peroxisomes. (B) The TAZ-KD/WT ratio on catalase determined for the PNS (N=9). Furthermore, fractionation was performed to determine the TAZ-KD/WT ratio in each of the S (N=3) and P fractions (N=3). The Western blotting image shows quantities of catalase in the PNS, S and P fractions of the WT and TAZ-KD mouse heart homogenate. Distributions of cytosolic (in S) and peroxisomal (in P) catalase in the WT and TAZ-KD samples are illustrated as pie charts. (C) The Western blotting image showing greatly enhanced expression of Far1 in the TAZ-KD mouse heart, where the large decrease in plasmalogen was observed by ³¹P NMR. The fractionation experiment resulted in detection of Far1 dominantly in the supernatant. The TAZ-KD/WT ratio on the PNS is shown (N=10). (D) The Western blotting image showing quantities of iPLA₂β. The fractionation experiment resulted in detection of the protein in the supernatant fraction. The TAZ-KD/WT ratio determined from the PNS is shown (N=10).

Table 1
Contents and Changes (mol%)¹ of the Heart Phospholipids of the WT vs. TAZ-KD Mice

Phospholipid	Chemical shift ² /ppm	Content		Change in contribution to the total phospholipid TAZ-KD-WT	Change in terms of the individual content (TAZ-KD-WT)/WT×100
		WT	TAZ-KD		
diacyl PC (+ plasmanylcholine) ³	- 0.174	19.2 ± 1.9	27.0 ± 2.9	+7.8 ± 3.5	+40.4 ± 18.1
plasmenylcholine	- 0.163	30.8 ± 2.8	18.1 ± 3.1	- 12.7 ± 4.2	- 41.3 ± 13.6
PI	0.039	3.6 ± 0.1	4.1 ± 0.3	+0.5 ± 0.3	+14.3 ± 9.1
PS	0.128	1.8 ± 0.2	2.1 ± 0.4	+0.3 ± 0.5	+15.9 ± 26.8
lyso PC	0.208	0.5 ± 0.1	0.6 ± 0.1	+0.1 ± 0.1	+10.9 ± 23.3
diacyl PE (+ plasmanylethanolamine) ⁴	0.295	26.2 ± 1.2	30.0 ± 1.1	+3.8 ± 1.6	+14.6 ± 6.2
plasmenylethanolamine	0.336	8.2 ± 0.4	8.3 ± 0.4	+0.2 ± 0.5	+2.2 ± 6.5
SM	0.409	2.5 ± 0.2	3.1 ± 0.5	+0.7 ± 0.5	+26.9 ± 21.1
CL	0.745	6.4 ± 0.1	2.0 ± 0.4	- 4.4 ± 0.4	- 68.7 ± 6.3
PG	0.845	0.9 ± 0.2	1.4 ± 0.1	+0.6 ± 0.2	+65.7 ± 20.0
1-MLCL	1.009	N.D. ⁵	0.9 ± 0.4	+0.9 ± 0.4	N.A. ⁶
2-MLCL	1.137	N.D. ⁵	2.4 ± 0.2	+2.4 ± 0.2	N.A. ⁶

¹ Average, and error shown as the standard deviation, are obtained from three independent biological samples (N = 3) for each of the WT and TAZ-KD mice.

² Values are from observation at 25°C in 10%(w/v) SDS micellar solution at pH=6 (50 mM MES, 50 μM BHT, 10% (v/v) D₂O), in reference to an external standard 85%(w/v) phosphoric acid in water set to 0 ppm.

^{3,4} Signal of plasmanyl glycerophospholipid overlaps with signal of the diacyl counterpart (Figure S6 shows the case of plasmanylcholine and diacyl PC).

⁵ Not detected.

⁶ Not applicable.