Dramatic Accumulation of Triglycerides and Precipitation of Cardiac Hemodynamic Dysfunction during Brief Caloric Restriction in Transgenic Myocardium Expressing Human Calcium-independent Phospholipase A₂γ

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Previously, we identified calcium-independent phospholipase A₂γ (iPLA₂γ) with multiple translation initiation sites and dual mitochondrial and peroxisomal localization motifs. To determine the role of iPLA₂γ in integrating lipid and energy metabolism, we generated transgenic mice containing the α-muscle heavy chain promoter (αMHC) placed proximally to the human iPLA₂γ coding sequence that resulted in cardiac myocyte-restricted expression of iPLA₂γ (TGiPLA₂γ). TGiPLA₂γ mice possessed multiple phenotypes including: 1) a dramatic ~35% reduction in myocardial phospholipid mass in both the fed and mildly fasted states; 2) a marked accumulation of triglycerides during brief caloric restriction that represented 50% of total myocardial lipid mass; and 3) acute fasting-induced hemodynamic dysfunction. Biochemical characterization of the TGiPLA₂γ protein expressed in cardiac myocytes demonstrated over 25 distinct isoforms by two-dimensional SDS-PAGE Western analysis. Immunohistochemistry identified iPLA₂γ in the peroxisomal and mitochondrial compartments in both wild type and transgenic myocardium. Electron microscopy revealed the presence of loosely packed and disorganized mitochondrial cristae in TGiPLA₂γ mice that were accompanied by defects in mitochondrial function. Moreover, markedly elevated levels of 1-hydroxy-2-arachidonoyl-sn-glycero-3-phosphocholine and 1-hydroxy-2-docosahexaenoyl-sn-glycero-3-phosphocholine were prominent in the TGiPLA₂γ myocardium identifying the production of signaling metabolites by this enzyme in vivo. Collectively, these results identified the participation of iPLA₂γ in the remarkable lipid plasticity of myocardium, its role in generating signaling metabolites, and its prominent effects in modulating energy storage and utilization in myocardium in different metabolic contexts.

Maladaptive changes in lipid metabolism leading to the intracellular accumulation of triglycerides are increasingly recognized as the likely cause of the multiple end organ sequelae of the metabolic syndrome and diabetes (1–6). Many studies have demonstrated the correlation between the progression of obesity, the intracellular accumulation of triglycerides, and the resultant cellular dysfunction in multiple target organs (7–9). It is becoming increasingly evident that myocardial lipotoxicity results from the combined influences of an increased lipid burden in conjunction with inadequate utilization. In large part, this is precipitated by the dysfunctional uptake and inefficient oxidation of fatty acyl-CoAs in the mitochondrial and peroxisomal compartments. However, the chemical and enzymatic mechanisms that regulate the balance of lipid extraction, oxidation, and synthesis in myocardium are poorly understood (10). Recently, elegant studies using stable isotope techniques with mass spectrometry (11) have unambiguously demonstrated the sequential oxidation of long chain fatty acids first in the peroxisomes followed by their subsequent transport to mitochondria for further oxidation. In previous work, we identified an intracellular phospholipase, now termed iPLA₂γ (GenBank™ accession number AF263613), that contains dual subcellular localization sequences that can direct the enzyme into either the peroxisomal or mitochondrial compartments (12, 13). Accordingly, we postulated that the multiple isoforms of iPLA₂γ located in distinct subcellular compartments could participate in modulating the dynamic relationship between peroxisomal and mitochondrial energy production. This could serve to integrate myocardial chemical energy production and storage with heat generation to facilitate metabolic flexibility. In peroxisomes, fatty acyl-CoA oxidation is inefficient because of the exothermic nature of the initial reaction catalyzed by fatty acyl-CoA oxidase that reduces O₂ to generate H₂O₂ with-
out production of NADH. In addition, acetyl-CoA produced during peroxisomal β-oxidation cannot be efficiently utilized for energy production within the peroxisome. Thus, peroxisomes serve to dispose of the energy stored in fatty acids through the production of heat and the inefficient production of ATP. In contrast, in mitochondria, activation of fatty acyl-CoA is thermodynamically coupled to the production of NADH with near maximal conversion of the Gibbs free energy in fatty acids into ATP. It is now well established that mitochondrial bioenergetic efficiency is modulated by uncoupling proteins whose activity is dependent on the content of nonesterified fatty acids in the mitochondrial inner membrane. These fatty acids are likely generated either by mitochondrial phospholipases or fatty acyl-CoA thioesterases (14, 15). Accumulation of lipid in a cell is dependent on the metabolic clearance of fatty acid in comparison with its rate of extraction. In normal myocardium, the entry and oxidative metabolism of fatty acids are balanced to extract the necessary energy for physiologic function while avoiding lipid accumulation and its resultant toxic sequelae.

In recent work, we have demonstrated that iPLA$_2$γ can act as a signaling enzyme through the generation of 2-polyunsaturated acyl (20:4 or 22:6) lysophosphatidylcholines that serve as precursors of eicosanoid metabolites (by the action of lysophospholipases), cannabinoids (by the action of phospholipase C (16–19)), or through interactions with lysolipid receptors. In addition, Kudo and co-workers (20) have shown that cells expressing iPLA$_2$γ release increased amounts of arachidonic acid (relative to control cells), which is preferentially metabolized to PGE$_2$ by cyclooxygenase-1 relative to cyclooxygenase-2. Pfeiffer and co-workers (21) recently demonstrated that calcium-independent phospholipase A$_2$ plays an important regulatory role in mitochondrial function through the generation of fatty acids and modulation of the permeability pore transition. Despite these advances, the significance of iPLA$_2$γ in the regulation of cellular lipid metabolism, signaling, and bioenergetics is largely unknown.

To gain insight into the role of iPLA$_2$γ in cardiac lipid metabolism, we generated mice that express human iPLA$_2$γ in a cardiac myocyte selective manner using the α-myosin heavy chain (αMHC) promoter. We now report that murine myocardium overexpressing iPLA$_2$γ can function normally with only 70% of its wild type endogenous phospholipid content. Remarkably, during 16 h of caloric restriction, triglyceride levels increase 7.5-fold in TGIpLA$_2$γ myocardium to account for ~50 mol% of total myocardial cellular lipid. Moreover, these acute changes in triglyceride levels precipitate hemodynamic compromise. Collectively, these results identify the major myocardial isoforms of iPLA$_2$γ, demonstrate both their peroxisomal and mitochondrial distribution in both wild type and transgenic myocardium, and underscore the role of iPLA$_2$γ in the adaptive regulation of lipid content and triglyceride metabolism in different metabolic contexts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radionucleotide 1-palmitoyl-2-[1-14C]arachidonyl phosphatidylcholine was purchased from PerkinElmer Life Sciences, and ECL reagents were purchased from Amer sham Biosciences. PCR reagents and the thermocycler were purchased from Applied Biosystems (Foster City, CA). Racemic BEL was purchased from Calbiochem. Mouse monoclonal anti-OxPhos complex IV antibody (COX) IgG$_{2a}$ was purchased from Molecular Probes (Eugene, OR). Mouse monoclonal anti-catalase IgG$_1$ was purchased from Sigma. IgG$_1$ and IgG$_{2a}$ isotype control antibodies were purchased from Sigma. An affinity-purified rabbit antibody to a peptide derived from the human iPLA$_2$γ sequence was utilized for these studies as described previously in detail (12). Normal rabbit serum was affinity-purified in an identical manner for use as a control primary antibody. Secondary antibodies were indocarbocyanine-conjugated goat anti-rabbit IgG (Cy3$^\text{TM}$) (Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 488$^\text{TM}$-conjugated goat anti-mouse IgG (Invitrogen). Synthetic phospholipids used as internal standards in mass spectrometric analyses were purchased from Avanti Polar Lipids (Alabaster, AL), Nu-Chek Prep, Inc. (Elysian, MN), and Cambridge Isotope Laboratories, Inc. (Cambridge, MA) as described previously (22). Solvents for sample preparation and for mass spectrometric analysis were purchased from Burdick and Jackson (Honeywell International Inc., Burdick and Jackson, Muskegon, MI). Most other reagents were obtained from Sigma.

**Generation of Mice Selectively Overexpressing iPLA$_2$γ in Cardiomyocytes**—Mice overexpressing human iPLA$_2$γ were prepared by exploiting the cardiomyocyte specificity of the αMHC promoter. Briefly, we utilized PCR to engineer Sall sites at the 5’ and 3’ ends of the full-length 2.3-kb coding sequence of human iPLA$_2$γ. The Sall-digested fragment was cloned into Sall-digested and alkaline phosphatase-treated α-MHC vector and sequenced in both directions. A NotI fragment containing the αMHC promoter in tandem with the iPLA$_2$γ sequence was utilized for microinjection of DNA directly into the pronuclei of mouse (B6CBAF1/J) zygotes, which resulted in integration of the transgene into the mouse germ line. Founder mice were identified by PCR analysis of mouse tail DNA and then bred with WT B6CBAF1/J mice (The Jackson Laboratories, Bar Harbor, ME) for at least three generations to establish the transgenic line. 3–4-Month-old hemizygous offspring mice were used in all studies. The degree of iPLA$_2$γ expression was determined by quantitative PCR, Western blotting, and fluorescent measurement of tissue sections.

**Electrospray Ionization Mass Spectrometry of Lipids**—Lipid extraction from mouse tissue and multidimensional ESI/MS analyses were performed as described previously utilizing a triple-quadrupole mass spectrometer (Thermo Electron TSQ Quantum, San Jose, CA) operating under Xcalibur software (22). Quantitative analysis and fingerprinting of TAG molecular species directly from lipid extracts were performed as described previously (23, 24). The first and third quadrupoles served as analyzers in tandem mass spectrometry, whereas the second quadrupole was used as the collision cell with collision gas pressure set at 1.0 millitorr, and collision energy varied with the classes of lipids as described previously (25). Under typical conditions, the profile mode utilized a 1-min period of signal averaging along with a 2-min period of signal averaging in each tandem MS spectrum. Multidimensional mass spectrometric...
data analyses were performed and authenticated as described previously (22, 25, 26).

**Electron Microscopy**—Mouse hearts were rinsed in PBS, placed in fixative solution containing glutaraldehyde (2%) and paraformaldehyde (1%) fixative in 0.8 M sodium cacodylate for at least 2 h, washed, placed in post-fixative (1% osmium tetroxide) for 1 h, dehydrated in graded alcohols, and then embedded in PolyBed 812 (Polysciences, Inc, Warrington, PA). Ninety-nanometer-thick sections were prepared and viewed with a JEOL model 1200EX electron microscope.

**Immunohistochemistry**—Paraffin-embedded sections were prepared essentially as described previously (27). Double labeling of affinity-purified iPLA$_2$γ and cytochrome c oxidase IV (COX) antibodies were performed using frozen ventricular sections as described previously (28). Primary antibody dilutions were 1:250 for the COX antibody, 1:100 for the iPLA$_2$γ antibody, and 1:100 for the catalase antibody. The secondary antibodies, indocarboxyanine-conjugated goat anti-rabbit IgG (Cy3$^{TM}$) (Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 488$^{®}$-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR), were added at 1:200 dilution in PBS for 2 h. Laser scanning confocal microscopy (model 2000, Amersham Biosciences) was performed utilizing an argon/krypton laser wavelength filter setting of 488/568 dual band pass with a laser attenuation of 10% using the 50 nm aperture, in combination with fluorescence microscopy using a ×40 oil immersion lens with numerical aperture of 1.0, lateral resolution of 0.23 μm, and depth resolution of 1.06 μm. The use of this combination resulted in a full width half-maximal focal plane with a thickness of ~1.0 μm. Identical dilutions of isotype-matched antibodies were utilized as controls for immunostaining with COX and catalase primary antibodies, and affinity-purified normal rabbit serum was used as a control for the iPLA$_2$γ primary antibody in these studies.

**Substrate Utilization in an Isolated Working Mouse Heart Model**—Perfusion of isolated mouse working hearts was based on a procedure described previously (29). Adult mice (4–7 months old) were heparinized (100 units intraperitoneally) 10 min prior to anesthesia. Animals were then anesthetized with 5–10 mg of Na$^{+}$-pentobarbital (intraperitoneally). Hearts were excised and placed in an ice-cold Krebs-Henseleit bicarbonate (KHB) solution (118 mM NaCl, 25 mM NaHCO$_3$, 4.7 mM KCl, 0.4 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$, and 5.0 mM glucose, 70 micromoles/liter insulin, pH 7.4). Hearts were cannulated first via the aorta and perfused retrograde in Langendorff mode. The use of this combination resulted in a full width half-maximal focal plane with a thickness of ~1.0 μm. Identical dilutions of isotype-matched antibodies were utilized as controls for immunostaining with COX and catalase primary antibodies, and affinity-purified normal rabbit serum was used as a control for the iPLA$_2$γ primary antibody in these studies.

**Assay of DGAT Activity**—Heart tissue samples from fed and fasted WT and TG mice were homogenized in 50 mM Tris-HCl, pH 7.4, containing 50 mM KCl, and 0.25 M sucrose utilizing a tissue tearor homogenizer. The homogenates were centrifuged at 100,000 × g for 1 h, and the resultant pellets were then resuspended in homogenization buffer and sonicated (30 times with 1-s pulses at 20% power). The resuspended heart membrane fractions were assayed for DGAT activity in the presence of 50 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 1 mM EDTA, 100 mM MgCl$_2$, 0.5 mg/ml fatty acid-free bovine serum albumin, 100 μM [1-14C]oleoyl-CoA (80,000 dpm/reaction), and 200 μM 1,2-dioleoyl phosphatidylcholine (1% final concentration) as described previously (31) with minor modifications. Reactions were incubated for 5 min at 37 °C followed by extraction of the reactants and products into butanol. Radiolabeled triolein was resolved from unreacted [1-14C]oleoyl-CoA and [1-14C]oleic acid (due to endogenous acyl-CoA thioesterase activity) by thin layer chromatography (70:30:1 petroleum ether/diethyl ether/glacial acetic acid) on Partisil LK6D.
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silica gel plates (Whatman) and quantified by liquid scintillation counting.

**Miscellaneous Procedures**—Methods have been described previously for preparation of tissue homogenates (12), SDS-PAGE (32), Western analysis (12), and echocardiographic analyses (15). Calcium-independent PLA\(_2\) activity of cytosolic and membrane fractions was measured by quantifying the release of radiolabeled fatty acid from 1-palmitoyl-2-[\(^{1-14}\)C]arachidonoyl oleic acid from radiolabeled substrate (L-1-palmitoyl-2-[\(^{1-14}\)C]oleoylphosphatidylcholine, \(5\) \(\mu\)M final concentration) following incubation at 37 °C for 2 min in 100 mM Tris acetate, pH 8.0. Reactions were terminated by extraction of remaining substrate and products into butanol, separation by TLC, and quantification of released fatty acid by scintillation spectrometry as described under “Experimental Procedures.” Data presented are the averages ± S.E. of six separate determinations from tissue samples from three WT and three TGiPLA\(_2\gamma\) mouse hearts.

**RESULTS**

Cardiac Myocyte-restricted Expression of Calcium-independent Phospholipase A\(_2\)\(\gamma\) Identifies a Complex Pattern of Isoform Expression—Previous work by our group and others has demonstrated the robust expression of iPLA\(_2\gamma\) mRNA in myocardium in comparison with other tissues (12, 35) and has identified the presence of peroxisomal and mitochondrial localization signals (13, 36). However, the principal functions of iPLA\(_2\gamma\) in myocardial phospholipid metabolism, lipid signaling, and energy homeostasis, are currently unknown. We reasoned that cardiac myocyte-specific overexpression of iPLA\(_2\gamma\) would provide important insights into these and other cellular processes. Accordingly, transgenic mice selectively expressing iPLA\(_2\gamma\) in cardiac myocytes were generated through placing an αMHC promoter upstream of the iPLA\(_2\gamma\) coding sequence. SDS-PAGE and Western blot analysis of myocardial membrane proteins identified the presence of three major bands at 70, 63, and 50 kDa using an antibody directed toward a 15 amino acid epitope ~50 residues from the C terminus of iPLA\(_2\gamma\) (as described under “Experimental Procedures”) (Fig. 1A). Whereas the 70- and 63-kDa isoforms corresponded approximately to the predicted masses resulting from utilization of downstream AUG start sites, the 50-kDa protein product likely is a product of in vivo proteolysis because no translation initiation codon is present that would produce a polypeptide of the corresponding molecular weight. Two-dimensional electrophoresis and Western blotting of the human iPLA\(_2\gamma\) isoforms demonstrated a remarkable diversity in the post-translational modifications of TGiPLA\(_2\gamma\) revealing over 25 discrete isoforms (Fig. 1B). Membrane fractions from transgenic myocardium possessed robust phospholipase activity that was not present in other

**FIGURE 1. Expression and phospholipase A\(_2\) activity of TGiPLA\(_2\gamma\).**  A, immunoblot analysis of myocardial membranes from WT and TGiPLA\(_2\gamma\)-overexpressing mice. Myocardial membrane proteins (100 μg/lane) from WT and TGiPLA\(_2\gamma\) (iPLA\(_2\gamma\)) were resolved by SDS-PAGE (10% gel), transferred to a polyvinylidine difluoride membrane, and incubated with immunoaffinity-purified anti-iPLA\(_2\gamma\) antibody (12), and immunoreactive bands were visualized by enhanced chemiluminescence as described under “Experimental Procedures.” Molecular weight markers are indicated on the left, and the predicted sizes of the iPLA\(_2\gamma\) isoforms are indicated on the right. Results are representative of separate Western analyses from three different sets of mice.  B, detection of multiple iPLA\(_2\gamma\) isoforms expressed in TGiPLA\(_2\gamma\) mouse heart by two-dimensional electrophoresis and immunoblot analysis. Protein extracts were separated by two-dimensional gel electrophoresis by electrofocusing (pI) in the first dimension and by size in the second dimension (MW) prior to immunoblot analysis as described above. C, distribution of iPLA\(_2\gamma\) activity in WT and TGiPLA\(_2\gamma\) mouse tissues. Phospholipase A\(_2\) activity of membrane fractions of the indicated tissue homogenates was quantitated by measuring release of \(^{[1-14}\)C]-radiolabeled fatty acid from 1-palmitoyl-2-[\(^{1-14}\)C]arachidonoyl oleic acid from radiolabeled substrate (L-1-palmitoyl-2-[\(^{1-14}\)C]oleoylphosphatidylcholine, 5 \(\mu\)M final concentration) following incubation at 37 °C for 2 min in 100 mM Tris acetate, pH 8.0. Reactions were terminated by extraction of remaining substrate and products into butanol, separation by TLC, and quantification of released radiolabeled fatty acid by scintillation spectrometry as described under “Experimental Procedures.” Data presented are the averages ± S.E. of six separate determinations from tissue samples from three WT and three TGiPLA\(_2\gamma\) each performed in duplicate. D, differential selective inhibition of TGiPLA\(_2\gamma\) by (R)- and (S)-BEL. TGiPLA\(_2\gamma\) membrane fractions were preincubated with the indicated concentrations (R)-BEL, (S)-BEL, or ethanol vehicle alone for 3 min followed by assay of phospholipase A\(_2\) activity as described above. Calcium-independent PLA\(_2\) activity was plotted as a percent of the control reaction (ethanol vehicle alone), and (R)- and (S)-BEL inhibitable activity was significantly different at 1 \(\mu\)M and higher concentrations (\(p < 0.05\), indicated by the asterisk). Data were averaged from analyses of duplicate determinations from three separate TGiPLA\(_2\gamma\) and three separate WT mouse hearts.
tissues examined (Fig. 1C) and were ~10-fold more sensitive to inhibition by (R)-BEL in comparison with (S)-BEL (IC$_{50}$ for (R)-BEL ~ 2 μM) (Fig. 1D). Thus, the in vivo expression of mature iPLA$_2$γ protein in myocardium is complex and utilizes multiple different translation initiation sites in conjunction with proteolytic processing.

**Calcium-independent Phospholipase A$_2$γ Is Present in Both Mitochondrial and Peroxisomal Compartments in Wild Type and Transgenic Murine Myocardium**—To identify the subcellular distribution of iPLA$_2$γ in both WT and TG mice, immunohistochemical analyses of myocardial tissue sections were performed. Immunohistochemical staining of wild type (WT) and TGiPLA$_2$γ heart tissue with antibodies directed toward either cytochrome c oxidase (COX) or catalase demonstrated different staining patterns consistent with their known localization in mitochondria or peroxisomes, respectively (Fig. 2). No substantial differences in the individual staining patterns of catalase or cytochrome c oxidase were observed in TGiPLA$_2$γ myocardium relative to those of WT controls. Similar analyses of TGiPLA$_2$γ myocardium utilizing antibody directed against iPLA$_2$γ clearly identified a staining pattern that was distinct from that of the mitochondrial or peroxisomal markers alone.

**FIGURE 2. Dual colocalization of iPLA$_2$γ to mitochondrial and peroxisomal compartments in mouse myocardium.** Paraffin sections from TGiPLA$_2$γ (TG) (A and B) and WT mouse heart (C and D) were dual labeled with iPLA$_2$γ antibody (column 2) and with either antibody against cytochrome c oxidase 1V (COX) for mitochondrial localization or antibody against catalase for peroxisomal localization. Image overlays of the respective immunohistochemical staining patterns in columns 1 and 2 are presented in column 3 (overlay). The green punctate pattern observed for iPLA$_2$γ (column 2) was nearly identical with the red COX mitochondrial marker (column 1) in myocardial cells from both TGiPLA$_2$γ (A) and WT (C) mouse heart resulting in a yellow punctate pattern in regions of overlap (column 3). Nonmitochondrial iPLA$_2$γ was localized to the peroxisomal compartment by dual staining with iPLA$_2$γ and catalase antibody. Comparison of catalase-stained hearts for TGiPLA$_2$γ (B) and WT (D) with iPLA$_2$γ staining (column 2) suggested similarities in staining and overlay of the column 1 and 2 images resulted in a punctate yellow pattern (column 3). Bleed through was minimized in all experiments by adjustment of red and green windows utilizing single transfections. Parallel studies utilizing isotype-specific control antibodies and affinity-purified rabbit serum showed no staining. Data are representative of that obtained from analyses of heart tissue from three separate animals.

**FIGURE 3. Mass level alterations of choline, glycerophospholipids, ethanolamine glycerophospholipids, and triacylglycerides in wild type (WT) and transgenic (TGiPLA$_2$γ) myocardium.** Mouse heart tissues were obtained from 3- to 4-month-old wild type or TGiPLA$_2$γ mice fed ad libitum or fasted overnight for 16 h. Mouse myocardial lipids were extracted in the presence of 50 mM LiCl in an aqueous phase and subjected to ESI/MS analyses in the positive ion mode as described under “Experimental Procedures.” Bar graphs A and B show alterations in major lipid classes fed ad libitum or after 16 h of fasting for wild type (A) and TGiPLA$_2$γ (B) hearts. n = 4 per group. Bar graph C shows alterations in 16:0–22:6 and 18:0–20:4 PtdCho species fed ad libitum or after 16 h of fasting conditions for wild type and TGiPLA$_2$γ hearts. n = 4 per group. Note the substantial decrease in PtdCho and PtdEtn mass levels in TGiPLA$_2$γ relative to WT heart tissue and the dramatic increase in myocardial TAG that occurs upon caloric deprivation of TGiPLA$_2$γ myocardium (A and B) as well as the decrease in TGiPLA$_2$γ 16:0–22:6 PtdCho and increase in TGiPLA$_2$γ 18:0–20:4 PtdCho species during nonfasted conditions (C). * indicates p < 0.05; **, p < 0.001.
Merging of the images obtained from iPLA$_2$ staining with those obtained from cytochrome c oxidase clearly demonstrated their colocalization in the mitochondrial compartment in in vivo myocardium consistent with its N-terminal mitochondrial localization sequence (Fig. 2A). A strikingly similar immunostaining pattern for both iPLA$_2$ and cytochrome c oxidase was also seen in WT myocardium identifying the presence of endogenous iPLA$_2$ in mitochondria (Fig. 2C). These results demonstrate that the observed colocalization was not an artifact of the expression system utilized because iPLA$_2$ was identified in the mitochondrion of both TGiPLA$_2$ and WT mice (Fig. 2C). Additionally, no staining was detected in control studies utilizing isotype-specific control antibodies and affinity-purified rabbit serum, further demonstrating the specificity of the immunofluorescence patterns obtained (results not shown). Comparison of the immunohistochemical staining patterns of catalase and iPLA$_2$ in TGiPLA$_2$ myocardium revealed the presence of iPLA$_2$ in peroxisomes as predicted from its C-terminal SKL peroxisomal localization motif (Fig. 2, B and D). Importantly, immunostaining of wild type myocardium also established the colocalization of endogenous iPLA$_2$ with catalase in peroxisomes (Fig. 2D). Collectively, these results demonstrate the predicted dual localization of iPLA$_2$ in the peroxisomal and mitochondrial compartments and substantiate the similar subcellular distribution of both endogenous and recombinant iPLA$_2$ in intact wild type and transgenic myocardium.

A Dramatic Decrease in Lipid Content Accompanied by Molecular Species Alterations in Choline and Ethanolamine Glycerophospholipids Is Present in TGiPLA$_2$ Hearts—To begin to assess the potential biochemical roles of iPLA$_2$ in myocardium, alterations in the cellular lipid content of TGiPLA$_2$ hearts relative to WT controls were initially examined by shotgun lipidomics as described previously (22). Remarkably, both choline (PtdCho) and ethanolamine (PtdEtn) glycerophospholipids were dramatically decreased in TGiPLA$_2$ myocardium relative to WT mice fed ad libitum (Fig. 3, A and B). The contents of the PtdCho and PtdEtn classes were decreased by 30 and 40 mol %, respectively, in TGiPLA$_2$ myocardium (i.e. 53.2 ± 5.1 nmol of PtdCho and 40.4 ± 3.9 nmol of PtdEtn per mg of protein) in comparison with WT mice (i.e. 76.3 ± 2.7 nmol of PtdCho and 67.3 ± 2.8 nmol of PtdEtn per mg of protein) (p < 0.01). Mass spectrometry demonstrated that iPLA$_2$ overexpression selectively decreased PtdCho molecular species containing docosahexaenoate (22:6) (i.e. 16:0–22:6 PtdCho (m/z = 812.7) was 26.9 ± 1.5 and 11.6 ± 0.9 nmol/mg protein in WT and TGiPLA$_2$, respectively, p < 0.02) (Fig. 3C). The corresponding sn-2 22:6 lysolipid was increased. In contrast to the reduction in 22:6-containing PtdCho molecular species, a remarkable 45% increase in 18:0–20:4 PtdCho content was present (4.2 ± 0.3 versus 6.7 ± 0.5 nmol/mg protein in WT and TGiPLA$_2$, respectively, p < 0.05) (Fig. 3C) demonstrating an individual molecular species selectivity for hydrolysis in in vivo myocardium.

Considering the substantial decrease in PtdCho and PtdEtn in TGiPLA$_2$ myocardium in mice fed ad libitum, we were next interested to determine the lipid metabolic response of the TGiPLA$_2$ mice to caloric restriction. Shotgun lipidomic analyses of phospholipids following 16 h of fasting in WT mice demonstrated a substantial decrease in total PtdCho mass in comparison with mice fed ad libitum (Fig. 3A). As anticipated from previous results (25), fasting resulted in a substantial decrease in PtdCho molecular species containing long chain unsaturated acyl groups with a corresponding increase of shorter aliphatic chains. Additional decreases in the low basal levels of choline phospholipid occurred during fasting (53.2 ± 5.1 versus 33.0 ± 2.9 nmol of lipid/mg of protein under fed and fasted conditions, p < 0.05) in TGiPLA$_2$ mice (Fig. 3B).

Fasting Results in the Dramatic Accumulation of Triglycerides in TGiPLA$_2$ Myocardium That Is Accompanied by Alterations in TAG Molecular Species Profiles—Caloric restriction for 16 h in WT animals resulted in a 1.6-fold increase in the content of triacylglycerols (4.4 ± 0.5 nmol of TAG/mg of protein in fed versus 7.2 ± 1.4 nmol of TAG/mg of protein in fasted mice, p < 0.05). In contrast, a dramatic 7.5-fold increase in TAG levels was observed upon fasting TGiPLA$_2$ mice (10.1 ± 1.5 nmol/mg of protein in fed versus 75.6 ± 4.4 nmol/mg of protein in fasted mice,
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$p < 0.001$). This accumulation of triglyceride mass represented $\sim 50$ mol % of the total lipid content in 16 h fasted transgenic myocardium (Fig. 3). Two-dimensional mass spectrometric profiles of lipids extracted from calorically restricted TGiPLA$_2^\gamma$ myocardium demonstrated remarkable differences in the acyl chain composition of individual TAG molecular species relative to those from TG mice fed ad libitum (Fig. 4). Examination of TAG molecular species containing 16:0 fatty acid in fed (Fig. 4A) and fasted (Fig. 4B) TGiPLA$_2^\gamma$ transgenic myocardium (NL 256.2 mass spectra in Fig. 4) revealed a shift in the relative intensity of the ion peaks at $m/z$ 837.7–839.7 and from $m/z$ 863.7 to 865.7. These alterations indicate a remodeling of TAG, increasing the content of saturated acyl chains (i.e., decreasing the relative unsaturated acyl chain content) after 16 h of caloric deprivation. Furthermore, the NL 256.2 mass spectra also demonstrated the fasting-dependent depletion of an abundant ion peak cluster at $m/z$ 911.7 (corresponding to 16:0–18:1–22:6 TAG). Similar results were also obtained for TAG molecular species containing 18:2 and 18:1 fatty acids (i.e., NL 280.2 and NL 282.2 mass spectra in Fig. 4, respectively). Collectively, these results indicate that multiple TAG molecular species containing unsaturated fatty acids are selectively catabolized and remodeled during caloric restriction.

Alterations in Lysophosphatidylcholine Molecular Species—Selective increases in multiple lysophosphatidylcholine species were observed in TGiPLA$_2^\gamma$ myocardium relative to those in WT heart tissue (representative spectra are shown in Fig. 5). A 2.5-fold increase in total lysophosphatidylcholine content was present in TGiPLA$_2^\gamma$ myocardium (3.52 ± 0.20 versus 1.42 ± 0.06 nmol/mg of protein in TGiPLA$_2^\gamma$ and WT myocardium, $p < 0.02$) (Fig. 5B). TGiPLA$_2^\gamma$ 20:4 lyso-PtdCho ($m/z$ 566.48) content increased 11-fold (from 0.04 ± 0.01 to 0.45 ± 0.04 nmol/mg of protein in WT versus TGiPLA$_2^\gamma$ myocardium, respectively, $p < 0.02$), whereas a 4-fold increase in TGiPLA$_2^\gamma$ 22:6 lyso-PtdCho ($m/z$ 590.5) content was observed (from 0.24 ± 0.01 to 0.99 ± 0.13 nmol/mg protein, in WT versus TGiPLA$_2^\gamma$ myocardium, respectively, $p < 0.02$) (Fig. 5B). Previously, we identified the selective production of sn-2 20:4 lyso-PtdCho from 16:0 to 20:4 PtdCho utilizing purified iPLA$_2^\gamma$ in an in vitro system (16). Collectively, these results confirm the substrate selectivity of iPLA$_2^\gamma$ previously demonstrated in vitro and support the notion that iPLA$_2^\gamma$ participates in the trafficking of arachidonic acid and docosahexaenoic acid containing lipids in intact myocardium.

Cardiac Myocyte-restricted Expression of iPLA$_2^\gamma$ Results in a Dysfunctional Mitochondrial Phenotype—Considering the prevalence of the mitochondrial localization of iPLA$_2^\gamma$ (Fig. 2), we were next interested to determine whether elevated expression of the enzyme altered mitochondrial morphology, respiratory function, and/or substrate utilization. Electron micrographs of myocardial tissue from WT and TGiPLA$_2^\gamma$ mice revealed that mitochondria from TGiPLA$_2^\gamma$ mice were remarkable for their loosely packed and disorganized cristae (Fig. 6B) relative to WT controls (Fig. 6A). In myocardium, the overwhelming majority of phospholipid mass resides in the mitochondrial compartment (37). Thus, the observed disorganization of the cristae and reduced surface area of the inner mitochondria membrane are consistent with the decreased phospholipid mass present in TGiPLA$_2^\gamma$ myocardium (Fig. 3).

Next, to address the effects of iPLA$_2^\gamma$ on mitochondrial function, the respiration rates of isolated WT and TGiPLA$_2^\gamma$ heart mitochondria were determined utilizing palmitoylcarnitine, pyruvate, and succinate as substrates. Analysis of the basal respiration rate (state 2) revealed a minor deficiency in $O_2$ utilization by TGiPLA$_2^\gamma$ heart mitochondria with pyruvate as substrate (Fig. 7A, middle panel), although $O_2$ consumption was normal with palmitoylcarnitine and succinate (Fig. 7A, upper and lower panels). In contrast, maximal ADP-stimulated (state 3) respiration was significantly decreased in the TGiPLA$_2^\gamma$ mitochondria compared with their WT counterparts, with a $>50\%$ reduction in $O_2$ utilization using either palmitoyl-l-carnitine or pyruvate as substrate. Similar iPLA$_2^\gamma$-dependent defects in mitochondrial respiration occurred following uncoupling of

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**FIGURE 5.** Electrospray ionization mass spectra of lysophosphatidylcholine (LPC) molecular species in fasted WT and TGiPLA$_2^\gamma$ myocardium. Mouse heart lipid extracts were obtained and analyzed by ESI/MS for lysophosphatidylcholine molecular species as described under “Experimental Procedures.” A, top spectrum demonstrates total lysophosphatidylcholine molecular species from fasted WT mouse heart, and the lower spectrum represents the lysophosphatidylcholine molecular species from fasted TGiPLA$_2^\gamma$ mouse heart. Peaks were normalized to the internal standard (17:0 LPC, IS) at $m/z$ 532.49. Selective increases in multiple lysophosphatidylcholine molecular species were observed in TGiPLA$_2^\gamma$ myocardium relative to those in WT myocardium. B, bar graphs of lysophosphatidylcholine species from WT and TGiPLA$_2^\gamma$ hearts. Asterisks indicate statistically significant differences ($p < 0.02$) between WT and TGiPLA$_2^\gamma$ mice. Error bars indicate the means ± S.E.
the electron transport chain with oligomycin (state 4) (Fig. 7A). The respiratory control quotients (state 3/state 4) of TGiPLA2γ mitochondria were compromised relative to WT. These trends were statistically significant in the case of succinate as substrate (p < 0.05) (Fig. 7). These deficiencies in mitochondrial function observed in vitro were substantiated by a 2-fold increase in acylcarnitine in TGiPLA2γ hearts indicating an inability to process fatty acid derivatized to acylcarnitine destined for mitochondrial oxidation (Fig. 7B). To gain insight into the ability of intact TGiPLA2γ hearts to utilize glucose and fatty acid substrates, working hearts were isolated, perfused with either [1H]palmitate or [U-13C]glucose, and the amounts of 3H2O and 14CO2 produced in each case were analyzed and compared with WT controls. Metabolic analyses of substrate utilization revealed significant alterations in glucose as well as palmitate metabolism. Comparisons of metabolic profiles from TGiPLA2γ myocardium with WT control hearts demonstrated that glucose oxidation was ~20% lower in TGiPLA2γ myocardium, whereas palmitate oxidation increased to levels ~30% higher than WT animals demonstrating a metabolic shift from glucose to fatty acid utilization in the transgenic heart (Fig. 8). Notably, an increased reliance on fatty acid substrate has been shown previously to be a feature of myocardial lipotoxicity associated with obesity and diabetes (38–40). Significantly, these alterations in substrate utilization were consistent with a mitochondrial respiratory defect substantiating the mitochondrial dysfunction present in TGiPLA2γ hearts (Fig. 7).

**Northern Blot Analyses of mRNA Identify Increased DGAT-1 Message during Fasting**—The dramatic accumulation of triglycerides in TGiPLA2γ heart following fasting could potentially result from either a decrease in intracellular TAG hydrolysis or an increase in TAG synthesis. DGAT catalyzes the rate-
were similar in TGiPLA2 pyruvate dehydrogenase, porin, and cytochrome WT and TGiPLA2 as described under “Experimental Procedures.”

Diabetic Restriction Induces a Lipotoxic Phenotype Precipitating Acute Myocardial Hemodynamic Dysfunction in TGiPLA2 hearts. Although TGiPLA2 mice displayed normal exercise tolerance when fed ad libitum, TGiPLA2 mice developed acute and profound ventricular dysfunction following a 16-h fast. Echocardiographic measurements revealed a statistically significant decrease in TGiPLA2 heart rate with fasting (679.8 ± 21.1 bpm in the prefasted condition versus 601.9 ± 13.7 bpm in the postfasted condition, p < 0.02). Comparing WT and TGiPLA2 after fasting, TGiPLA2 shows a dramatic increase in left ventricular diastolic function.

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A. Palmitate Oxidation  
B. Glucose Oxidation  

FIGURE 8. Glucose and palmitate oxidation in WT and TGiPLA2γ isolated working mouse hearts. An isolated working mouse heart perfusion system was utilized to measure glucose and palmitate oxidation rates in wild type controls and iPLA2γ transgenic hearts (TGiPLA2γ) isolated from adult mice (4–7 months old). A, palmitate oxidation rates in WT and TGiPLA2γ isolated hearts were determined based upon the release of 14CO2 from 14C-palmitate as described under “Experimental Procedures.” B, glucose oxidation rates in WT and TGiPLA2γ isolated hearts were measured as the production of 13CO2 from [U-13C]glucose as described under “Experimental Procedures.” A significant decrease in glucose oxidation and a significant increase in palmitate oxidation (*, p < 0.05) were observed (n = 5) for each group examined.

A.  
B.  
C.  

FIGURE 9. Expression of mitochondrial markers in WT and TGiPLA2γ hearts. A, Northern analysis of WT and TGiPLA2γ hearts. Total RNA was extracted from fresh heart tissues and utilized in Northern analysis as described under “Experimental Procedures.” The relative expression of message in nontransgenic control (WT) and TGiPLA2γ (TG) hearts under conditions of feeding ad libitum (fed) and 16 h of fasting (fast) were determined for DGAT-1, DGAT-2, MCAD, ATP synthase, iPLA2γ, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S ribosomal RNA. B, Western analysis of mitochondrial markers from WT and TGIPLA2γ hearts. Mitochondrial proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, probed with antibodies against pyruvate dehydrogenase (PDH) or porin and visualized by ECL staining as described under “Experimental Procedures.” W, wild type littermate control; T, TGIPLA2γ. Bands are shown corresponding to pyruvate dehydrogenase (PDH) and porin. C, in additional experiments, cytochrome c oxidase IV (COX) was compared in nontransgenic control (W) and TGIPLA2γ (T) and shown to be present in equivalent amounts. Data are representative of three independent determinations.

Caloric Restriction Induces a Lipotoxic Phenotype Precipitating Acute Myocardial Hemodynamic Dysfunction in TGIPLA2γ Hearts. Although TGIPLA2γ mice displayed normal exercise tolerance when fed ad libitum, TGIPLA2γ mice developed acute and profound ventricular dysfunction following a 16-h fast. Echocardiographic measurements revealed a statistically significant decrease in TGIPLA2γ heart rate with fasting (679.8 ± 21.1 bpm in the prefasted condition versus 601.9 ± 13.7 bpm in the postfasted condition, p < 0.02). Comparing WT and TGIPLA2γ after fasting, TGIPLA2γ shows a dramatic increase in left ventricular diastolic function.
During feeding, the intracellular triglyceride content of cardiac myocytes from transgenic mice expressing iPLA_2γ was significantly increased compared to wild-type controls. This increase in triglycerides was not accompanied by increases in other lipid classes. Thus, although not apparent under basal conditions, the cryptic and intrinsic dysfunction of TGIPLA_2γ mitochondria is unmasked during the metabolic stress of fasting, which presents myocardium with a significantly increased fatty acid burden. In this compromised state, TGIPLA_2γ mitochondria become impaired in their ability to oxidize fatty acids, which results in a substantial accumulation of triglyceride molecular species that precipitates acute hemodynamic dysfunction.

DISCUSSION

In this study, transgenic mice selectively expressing iPLA_2γ in cardiac myocytes were generated, and their resultant phenotypes were characterized demonstrating the following: 1) the profound depletion of specific phospholipid classes and individual molecular species; 2) gross morphologic abnormalities in mitochondrial cristae; 3) multiple defects in mitochondrial function, including changes in state 3 and state 4 respiration; and 4) marked augmentation of fasting-induced accumulation of triglycerides that was accompanied by the acute precipitation of cardiac hemodynamic dysfunction. Despite these radical distortions, myocardium from TGIPLA_2γ mice was surprisingly functional as assessed by echocardiography and exercise capacity when mice were fed ad libitum. However, the physiologic sequelae of iPLA_2γ overexpression were unmasked during mild caloric restriction, resulting in increased intracellular TAG levels and alterations in normal heart function.

During fasting, myocardium actively extracts fatty acids from serum resulting in increases in the intracellular triglyceride content of cardiac myocytes from \( \approx 2-3 \text{ mol} \% \) in the fed state to \( \approx 5-6 \text{ mol} \% \) in the fasted state. This increase in triglycerides serves not only as an energy reservoir but also as a gauge of the recent metabolic history of the myocardium. Notably, this additional triglyceride pool is preserved for at least an additional 24 h after feeding presumably to compensate for future potential energy deficits (22). During feeding ad libitum, TGIPLA_2γ mice had near normal levels of triglyceride mass present, indicating their ability to balance fatty acid uptake and metabolism to maintain lipid homeostasis. However, during modest caloric restriction, TGIPLA_2γ mice accumulated dramatic amounts of triglycerides that were not accompanied by increases in other lipid classes.

To further elucidate the impact of intramyocardial lipid accumulation, myocardial fatty acid burden was increased iPLA_2γ expression, leading to the metabolic dysfunction in diabetic myocardium (5), the possibility that the high risk of sudden death in diabetic patients could result from accelerated hormone and lipid-induced mitochondrial stress meriting consideration. Caloric restriction is accompanied by de novo CD36 synthesis to facilitate increased fatty acid import (43). The biochemical mechanisms that are responsible for the trafficking of fatty acids from the sarcolemma to intracellular loci are not well defined. Modulation of the trafficking of fatty acids to either the peroxisomal or the mitochondrial compartment clearly is the key process that contributes to the adaptive metabolic responses of myocardium and other tissues. Recent stable isotope studies have demonstrated that a substantial portion of extracted fatty acids undergoes initial processing in peroxisomes prior to subsequent mitochondrial β-oxidation (11). Thus, the coordinated regulation of peroxisomal and mitochondrial fatty acid processing in maintaining myocardial energy homeostasis is evident. Through the transgenic expression of iPLA_2γ, normal myocardial lipid metabolism has been perturbed resulting in a dramatic decrease in total mitochondrial phospholipids. Therefore, these alterations limit the metabolic flexibility of myocardium and identify a novel pathway leading to the metabolic dysfunction in diabetic myocardium.

At least 80% of the total lipid mass of myocardium is contained within the mitochondrial compartment (14, 37), and most of this lipid mass is located in the inner mitochondrial membrane. The dramatic loss of phospholipid in the TGIPLA_2γ heart reflects loss of mitochondrial membrane potential (14, 37). The discordant expression patterns of CD36 and iPLA_2γ suggest that CD36, an auxiliary fatty acid transporter, is regulating the trafficking of fatty acids to either the peroxisomal or the mitochondrial compartment in diabetic myocardium (5). However, the physiologic sequelae of iPLA_2γ overexpression were unmasked during mild caloric restriction, resulting in increased intracellular TAG levels and alterations in normal heart function.
Transgenic Mice Expressing iPLA$_2^\gamma$

of at least a portion of the cristae phospholipid pool that can contribute to both energy production and compensation during acute caloric restriction or during myocardial ischemia (22). Recently, we have demonstrated that myocardial phospholipases are activated during fasting. These phospholipases result in alterations of myocardial membrane composition that presumably are an adaptive attempt to meet required energy needs during a caloric deficit (22). Thus, iPLA$_2^\gamma$ could participate in the generation of mitochondrial free fatty acids from cellular phospholipids leading to uncoupling of the electron transport chain and exacerbation of mitochondrial dysfunction in the fasted state. The resultant endogenously hydrolyzed fatty acids likely contribute to the observed accumulation of intracellular triglycerides following conversion to their acyl-CoA derivatives. The deleterious consequences of elevated iPLA$_2^\gamma$ underscore the importance of endogenous fatty acids derived from phospholipids in these pathologic alterations in myocardial lipid homeostasis during caloric deprivation (22).

In previous work, we demonstrated that iPLA$_2^\gamma$ possesses a highly selective phospholipase A$_2$ catalytic activity for sn-2 polyunsaturated aliphatic chains resulting in the production of 2-arachidonoyl lyso-PtdCho in vitro (16). This study illustrates the in vivo importance of those initial observations as evidenced by the accumulation of 2-arachidonoyl lyso-PtdCho and 2-docosahexaenoyl lyso-PtdCho. These results identify the presence of a novel myocardial signaling pathway in vivo that is initiated by the actions of iPLA$_2^\gamma$ to generate moieties (sn-2 polyunsaturated lysolipids) that can potentially serve as precursors for a wide variety of signaling molecules. For example, lysophospholipase acting upon 2-arachidonoyl lyso-PtdCho generates arachidonic acid for eicosanoid production, whereas its action on 2-docosahexaenoyl lyso-PtdCho would release docosahexaenoic acid that possesses signaling functions in several systems (44–47). Similarly, the actions of nucleotide pyrophosphatase/phosphodiesterase 6 (NPP6) or related phosphodiesterases would generate cannabionoids, thereby representing secondary branches of dual signaling pathways sharing common lipid precursors.

In conclusion, these results demonstrate that transgenic myocardial expression of iPLA$_2^\gamma$ results in a marked loss of cardiac phospholipids from mitochondria that is accompanied by mitochondrial dysfunction. These results clearly demonstrate the importance of mitochondrial membrane lipid composition and phospholipases in the function of this organelle. Finally, the data indicate that cryptic mitochondrial dysfunction can be unmasked by fasting where metabolic flexibility is required to adapt to an increased fatty acid burden in myocardium. Collectively, these studies underscore the key roles of phospholipases in coordinate coordinating peroxisomal and mitochondrial membrane composition and their function to facilitate appropriate adaptive responses to metabolic and nutritional stresses.

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