Genetic Ablation of Calcium-independent Phospholipase A$_2$$\gamma$ Leads to Alterations in Mitochondrial Lipid Metabolism and Function Resulting in a Deficient Mitochondrial Bioenergetic Phenotype*

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Previously, we identified a novel calcium-independent phospholipase, designated calcium-independent phospholipase A$_2$$\gamma$ (iPLA$_2$$\gamma$), which possesses dual mitochondrial and peroxisomal subcellular localization signals. To identify the roles of iPLA$_2$$\gamma$ in cellular bioenergetics, we generated mice null for the iPLA$_2$$\gamma$ gene by eliminating the active site of the enzyme through homologous recombination. Mice null for iPLA$_2$$\gamma$ display multiple bioenergetic dysfunctional phenotypes, including 1) growth retardation, 2) cold intolerance, 3) reduced exercise endurance, 4) greatly increased mortality from cardiac stress after transverse aortic constriction, 5) abnormal mitochondrial function with a 65% decrease in ascorbate-induced Complex IV-mediated oxygen consumption, and 6) a reduction in myocardial cardiolipin content accompanied by an altered cardiolipin molecular species composition. We conclude that iPLA$_2$$\gamma$ is essential for maintaining efficient bioenergetic mitochondrial function through tailoring mitochondrial membrane lipid metabolism and composition.

Mitochondria transduce chemical energy from multiple dietary substrates into ATP and heat and are important participants in cellular lipid metabolism and signaling (1, 2). Through the precisely regulated partitioning of energy derived from substrates between ATP synthesis and heat production, a finely tuned dynamic balance is maintained to promote organism survival. In addition, excess energy derived from substrates, not immediately needed to fulfill chemical or thermodynamic demands, is directed into the production of lipid synthetic intermediates for energy storage (e.g. mitochondrial synthesis of lysophosphatidic acid, a precursor of triglycerides). Moreover, mitochondria generate diverse chemical signals that reflect the bioenergetic status of the cell (e.g. NO and cytochrome c) and thus integrate multiple energetic, metabolic, and signaling cascades (1–5).

Mitochondrial phospholipases are important participants in the regulation of mitochondrial function and signaling (6–11). Mitochondrial phospholipases catalyze the production of non-esterified fatty acids that regulate UCP function, release lipid second messengers, such as 2-arachidonoyl lysophosphatidylcholine (LPC), and modulate membrane molecular dynamics (12). Maladaptive activation of phospholipases has deleterious metabolic effects in many systems, and previous studies have implicated calcium-independent phospholipases A$_2$ (iPLA$_2$s) as the enzymic mediators of membrane dysfunction in diabetic cardiomyopathy (13–16). Metabolic flexibility is dependent on the efficient coordinated transitions of substrate flux that occur during alterations in energy sources (e.g. glucose versus fatty acid) or energy demand (e.g. exercise). Obesity and type II diabetes are thought to result from disruption of these transitions (17). Diabetic cardiomyopathy is characterized by altered lipid metabolism (i.e. greatly increased utilization of fatty acids) that results in changes in the chemical composition of cardiac membranes, modifying their biophysical properties and function (13, 18–20). Many of the metabolites used in mitochondrial energy metabolism possess dual roles in modulating signaling cascades (e.g. acyl-CoA, acylcarnitines, fatty acids, and lysophosphatic acid), thereby integrating lipid energy metabolism and cellular signaling functions (21–25). Ultimately, the disproportionate utilization of fatty acids in diabetic myocardium leads to a profound metabolic imbalance resulting in bioenergetic inefficiency, resulting, at least in part, from alterations of cardiolipin (CL) molecular species that occur in diabetic myocardium (18, 20, 26, 27).

Previously, we cloned and characterized iPLA$_2$$\gamma$, a major phospholipase activity in murine myocardium based on activity assays, mRNA content, and kinetic analyses using enantiomeric selective mechanism-based inhibitors (28). Subsequently,

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2 The abbreviations used are: LPC, Lysophosphatidylcholine; iPLA$_2$, calcium-independent phospholipase A$_2$; iPLA$_2$$\gamma$, calcium-independent phospholipase A$_2$$\gamma$ (AF263613); KO, knock-out; BEL, (6S)-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one; TAC, transverse aortic constriction; CL, cardiolipin; cPLA$_2$, cytosolic phospholipase A$_2$; WT, wild type; WAT, white adipose tissue; BAT, brown adipose tissue; UCP, uncoupling protein; nC, nucleotide; TMPD, tetramethyl p-phenylene diamine.
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### Experimental Procedures

**Materials**—Radiolabeled nucleotides and 1-palmitoyl-2-[1-$^{14}$C]arachidonoyl phosphatidylcholine were purchased from PerkinElmer Life Sciences. ECL reagents were purchased from GE Life Sciences. PCR reagents were purchased from Applied Biosystems (Foster City, CA) and used with an ABI GeneAmp PCR System 9700 thermocycler. Racemic BEL was purchased from Calbiochem. A rabbit antibody to a peptide derived from the human iPLA$_2$γ sequence was used in these studies and prepared by affinity chromatography as previously described in detail (28). Normal rabbit serum was affinity-purified in an identical manner for use as a control primary antibody. 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) and used as described previously (30). 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.

### Table 1

| Primers | Sequence (5’–3’)
|---------|-----------------
| KO primers | AAAAAACTCGAGCCAAGAACATGCAGCACAGGCTTGAGGCCAT | P1
| | AAAAAACTCGAGCCAAGAACATGCAGCACAGGCTTGAGGCCAT | P2
| | AAAAAACTCGAGCCAAGAACATGCAGCACAGGCTTGAGGCCAT | P3
| | AAAAAACTCGAGCCAAGAACATGCAGCACAGGCTTGAGGCCAT | P4
| | AAAAAACTCGAGCCAAGAACATGCAGCACAGGCTTGAGGCCAT | P5
| | AAAAAACTCGAGCCAAGAACATGCAGCACAGGCTTGAGGCCAT | P6
| tail PCR primer set | AAAGGCCTACCCGCTTCCATTGCTTCA | NEO forward
| | GCTTCACAGAGGCTTGTTCTC | Common REV
| | KO band = 354 base pairs | iPLA$_2$γ FOR
| | WT band = 181 base pairs | Tail PCR primer set
| | 1 | CAAAAGCTCTTGATTACTAGGG
| | 2 | CAAAAGCTCTTGATTACTAGGG
| | 3 | GATTACATCTGTGGAGTAAGCCAGG
| | 4 | CCAAAGCTCTTGATTACTAGGG
| | 5 | ATGGACAATCTGGAGTAAGCCAGG
| | 6 | CCAAAGCTCTTGATTACTAGGG
| | GAPDH F | TGTTGCCATCAATGACCCCTTCA
| | GAPDH R | TGATGATCTTGAGGCTGTTGTC

iPLA$_2$γ was found to possess dual mitochondrial and peroxisomal localization signals, suggesting its importance in regulating myocardial bioenergetics and lipid metabolism. Next, we demonstrated that cardiac myocyte-specific overexpression of iPLA$_2$γ results in dramatic reductions of cardiac phospholipid mass and mitochondrial dysfunction (29). Remarkably, fasting of cardiac myocyte-restricted iPLA$_2$γ overexpressing mice resulted in large increases in myocardial triglyceride content that precipitated hemodynamic dysfunction (29).

To gain further insight into the role of iPLA$_2$γ in cardiac function, we inactivated the iPLA$_2$γ gene in mice by targeted deletion of exon 5 encoding the lipase active site (GVSTG). Genetic ablation of iPLA$_2$γ resulted in growth retardation, cold intolerance, and dramatically reduced exercise endurance. The importance of iPLA$_2$γ in the ability of myocardium to respond to stress was identified by the dramatically reduced survival of iPLA$_2$γ knock-out mice following transverse aortic constriction. Finally, mitochondrial function assays in mice null for iPLA$_2$γ demonstrated markedly decreased complex IV function that was associated with decreases in tetra-18:2 cardiolipin content. Collectively, these studies identify mitochondrial dysfunction resulting from alterations in mitochondrial lipid metabolism as the mechanistic basis for the phenotypic features of mice null for iPLA$_2$γ.
mouse genomic DNA sequence were utilized for detection of 7.4-kb WT and 5.9-kb KO iPLA₂γ bands by hybridization with a 459-nt genomic probe generated by PCR amplification with primers P5 and P6 (Table 1). Two clones of 238 screened by Southern analysis were positive for the homologous recombination. These clones were injected into C57BL/6 blastocysts, followed by implantation into pseudopregnant mice to generate the chimeric mouse founder line utilizing the Neurosciences Transgenic Core facility at Washington University. Chimeras were mated to C57BL6/j mice, and germ line transmission to heterozygous offspring was confirmed by Southern blotting of tail DNA. Interbreeding of heterozygous offspring was used to generate homozygous KO and WT littermates used in all studies. In addition to genomic Southern analysis, tail PCR was routinely performed for genotyping. “Neoforward” and “Common REV” primers (Table 1) were utilized for amplification of a 354-nt product from the knock-out allele, whereas paired “iPLA₂ FOR” and “Common REV” primers (Table 1) were utilized to amplify a 181-nt product for detection of the wild type allele. All experiments were performed using sex- and age-matched littermate controls as noted.

**General Animal Studies**—Animal protocols were in strict accordance with the National Institutes of Health guidelines for humane treatment of animals and were reviewed and approved by the Animal Care Committee of Washington University. Conscious animals fed *ad libitum* were weighed at various time points after weaning up to 10 months of age. Following euthanasia, tissues were dissected from male and female mice, weighed, and expressed as a fraction of total body weight. Rectal temperature was measured to the nearest 0.1 °C by inserting the probe of a thermocouple (Fisher) 20 mm into the rectum until a stable reading was obtained. For these temperature studies, mice were singly housed at ambient temperature with food and water provided *ad libitum*, and the mean of temperatures taken at 8 a.m. on three consecutive days was reported. For cold tolerance studies, male and female WT and KO mice were singly housed at 4 °C for up to 24 h with food and water provided *ad libitum*. For cold adaptation studies, core body temperatures were monitored utilizing a rectal probe at time 0 and hourly thereafter with constant monitoring of mice for signs of lethargy. Mice were removed from the study when their core temperature dropped below 28 °C. For food intake studies, *ad libitum* food intake of individually housed mice was measured at 1, 1.5, 4, and 9 months of age (*n* = 4) over a period of 3–5 consecutive days.

**RNA, DNA, and Protein Analyses**—For Northern blotting, total RNA was isolated utilizing an RNeasy tissue kit, and hybridizations were performed as previously described (8), utilizing labeled ~1-kb iPLA₂γ probe derived from IMAGE clone 6591346 (ATCC, Manassas, VA). For quantitative PCR analyses, total RNA was isolated from tissues using Trizol (Invitrogen) according to the manufacturer’s directions. RNA was quantified by spectrophotometry (NanoDrop, Wilmington, DE). cDNA was synthesized from RNA using Superscript III Reverse Transcriptase (Invitrogen). cDNA samples were amplified using Power SYBR Green PCR Master Mix (Applied Biosystems) on the ABI 7500 real time PCR system (Applied Biosystems). Acidic ribosomal protein 36B4 message was used for normalization, and results were analyzed using the comparative Ct method. Primers used for quantitative PCR were as follows (5’–3’): UCPI, TCTACAAGTTGAGGCCTCTA (forward) and TCGAGCTTCAACAGACCTCTGTA (reverse); UCIP2, TCACACAGCCTTACATCAAT (forward) and GACCATTACACATCTGAGGC (reverse); UCIP3, CAGAGGAGACATTGATGCTAC (forward) and AGGTTGAGATCCCAAACTTCT (reverse); 36B4, GCACGACACTGGGCTTCTTAAC (forward) and GTGCTCTTTGGGTGAACACGAAGCC (reverse). Results were analyzed by comparing the threshold crossing (Ct) of each sample after normalization to the control genes (∆∆Ct). The changes in the threshold crossing (∆Ct) were used to calculate the relative levels of each mRNA from the various samples using the formula 2–∆∆Ct.

Genomic Southern blot studies were performed by Psil digestion of 10 mg of genomic DNA, followed by electrophoreses on a 0.8% TAE gel and transfer to a Hybond-XL (GE Life Sciences) membrane for hybridization. Western blotting was performed as previously described (28) utilizing an ECL detection system (GE Life Sciences).

**Treadmill Studies**—Treadmill studies were performed utilizing the Mouse Cardiovascular Phenotyping Core at Washington University School of Medicine. WT and knock-out mice (ages 2–9 months) were run to exhaustion utilizing a motorized, speed-controlled, modular treadmill system (Columbus Instruments, Columbus, OH) equipped with an electric shock stimulus and an adjustable inclination angle as previously described (32). Runs were performed on a level inclination, and running velocity was initiated at 10 m/min and then incrementally increased at a rate of 3 m/min until exhaustion, defined as the inability to remain on the treadmill for 5 s.

**Transverse Aortic Constriction**—Transverse Aortic Constriction studies were performed by the Mouse Cardiovascular Phenotyping Core at the Washington University School of Medicine. The protocol for transverse aortic constriction (TAC) has been previously described (33, 34). In brief, surgery was performed on mice anesthetized with a xylazine (16 mg/kg) and ketamine (80 mg/kg) mixture, and a 7–0 silk suture was placed around the transverse aorta and tied around a 25-gauge blunt needle, which was subsequently removed. Following constriction, the gradient across the band was measured to ensure that adequate stenosis had been achieved. Subsequently, the thorax was closed, and mice were allowed to recover on a heating pad until responsive to stimuli. Surgeons were blinded to mouse genotypes. As controls, sham-operated animals underwent identical surgery but without aortic constriction.

**Mitochondrial Respiration Studies**—Mitochondria were prepared essentially as previously described (35, 36). Hearts were removed from WT and knock-out animals and immediately placed at 10% (w/v) into ice-cold pH 7 HEPES buffer containing 1 mM EDTA and 250 mM sucrose. Hearts were homogenized with a loose fitting dounce homogenizer. Next, the homogenate was centrifuged at 500 × g for 10 min to remove cellular debris, nuclei, and connective tissue. The supernatant solution was centrifuged at 9,000 × g for 35 min to isolate mitochondria, which were resuspended in homogenization buffer. Protein concentration was determined using the micro-BCA protein assay kit (Pierce). Oxygen consumption was measured polar-
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Graphically using a dual channel Instech dissolved oxygen measuring system. In the presence of malate and succinate, O$_2$ consumption was initiated with ADP to determine substrate-supported O$_2$ consumption and ability to generate ATP. Cytochrome oxidase activity, after the addition of rotenone, was determined by the addition of ascorbate and TMPD. The addition of azide completely abolished oxygen uptake in the rotenone-inhibited preparation.

Mass Spectrometric Analysis of CL—Enhanced shotgun lipidomics analyses of CL were performed on a QqQ mass spectrometer (Thermo-Fisher Scientific, San Jose, CA) equipped with an electrospray ion source as previously described (26). All electrospray ionization mass spectrometric analyses of lipids were conducted by direct infusion employing a Harvard syringe pump at a flow rate of 4 µl/min. Typically, a 1-min period of signal averaging was employed for each mass spectrum, and a 2-min period of signal averaging was employed for each tandem mass spectrometric spectrum. A mass resolution of 0.4 Thomson was employed for acquisition of mass spectra with a QqQ instrument.

Statistical Analysis—Data were analyzed using a two-tailed Student’s t test, χ test, one-way analysis of variance (for treadmill studies), and the log-rank test (for TAC studies). Differences were regarded as significant at p < 0.05. All data are reported as the mean ± S.E. unless otherwise noted.

RESULTS

Targeted Disruption of the iPLA$_2$γ Gene—Since iPLA$_2$γ has multiple promoters and splice variants (8, 28), we employed a knock-out strategy targeting the ablation of exon 5, which contains the active site. A neomycin-based gene-targeting vector was designed to undergo homologous recombination, leading to deletion of exon 5 of the murine iPLA$_2$γ gene. The strategy for the targeting event employed to effect homologous recombination and the multiple methods used for its identification are shown in Fig. 1A. First, a linearized vector was used to electroporate SCC#10 ES cells (derived from 129X1/SvJ mice) as described under “Experimental Procedures.” After neomycin selection, colonies were screened for targeted disruption of exon 5 by Southern blot analyses. Of the 238 ES clones identified possessing the neomycin resistance cassette, two independent clones (clones 7 and 48) exhibited evidence of the desired insertion/recombination event. These clones were separately injected into C57BL/6 blastocysts and implanted into pseudopregnant females as described under “Experimental Procedures.” Male chimeras (10 chimeras from clone 7 and 1 pseudopregnant female) were subsequently bred with C57BL/6 mice. Hetero-
mRNA purified from heart and other tissues in iPLA$_2^\gamma$ KO mice did not demonstrate any message corresponding to the altered transcript (Fig. 1D). Targeted disruption of the iPLA$_2^\gamma$ active site resulted in over a 60% reduction in the production of 2-[1-14C]arachidonoyl LPC from 1-palmitoyl-2-[1-14C]arachidonoyl phosphatidylcholine in murine mitochondria. This activity probably results from cPLA$_2^\gamma$ that is localized in the mitochondrial compartment and is both calcium-independent and not inhibitable by BEL (37). Taken together, these results support the conclusion that the gene-targeting event resulted in an iPLA$_2^\gamma$ null allele and that iPLA$_2^\gamma^{-/-}$ mice were viable.

General Phenotypic Features of Mice Null for iPLA$_2^\gamma$—Subsequent large scale breeding of heterozygous mice resulted in normal litter sizes with no unexpected deaths of offspring. Genotypic analyses of 751 offspring from heterozygous crosses resulted in 201 WT, 395 heterozygous, and 155 homozygous KO offspring, indicating a small (17%) reduction in the expected Mendelian inheritance of the homozygous KO (0.89: 2.26:1.15 versus the expected 1:2:1 ratio (p < 0.02 by the $\chi^2$ test). Most of the reduction was due to the lower proportion of female KO mice (71 versus the expected 94), a 24% reduction in anticipated female offspring (p < 0.05). Although iPLA$_2^\gamma$ knockout mice appeared normal and healthy after birth and weaning by visual inspection, body weights of iPLA$_2^\gamma$ KO mice were consistently 9% lower compared with sex-matched WT littermates (8.78 ± 0.4 versus 10.95 ± 0.2 g respectively, at 28 days of age (p < 0.01)) and remained lower through approximately day 50 (Fig. 3A). In contrast to their WT littermates, which progressively increased in weight, mice null for iPLA$_2^\gamma$ did not gain significant weight after adolescence. By day 288, KO male animals had a mean weight of 22.2 ± 0.4 g versus 34.1 ± 3.1 g for WT males (i.e., 35% lower weight). Female KO animals weighed 21.4 ± 1.1 versus 31.2 ± 5.2 g for WT females at day 255 (31% lower weight) (Fig. 3B). Along with a reduction in body mass, by 9 months of age, KO male mice had a significantly shorter body length by ~8% (measured from the tip of the nose to the anus) (KO male mice were 9.3 ± 0.1 cm versus 10.0 ± 0.1 cm for WT littermates (p < 0.001, n = 11 WT and 13 KO). A slightly reduced food intake (9%) was identified for the 2–3-month-old iPLA$_2^\gamma$ KO animals, which was not statistically significant. In contrast, a 24% reduction in food intake was observed in 9-month-old male KO animals (3.7 g/day for KO versus 4.7 g/day for WT, p < 0.06, five separate measurements of n = 6 per group). This reduction was not significant after adjusting for differences in body weight. Both male and female KOs (between 3 and 12 months of age) had a significantly lower basal core body temperature by nearly 0.5 °C. The mean core body temperatures were 36.3 ± 0.1 °C (WT males) versus 35.7 ± 0.1 °C (KO males, p < 0.02) and 36.8 ± 0.1 °C (WT females) versus 36.4 ± 0.1 °C (KO females) based on n of ~19 per genotype and an average of 50 readings per genotype. The weights of brain, liver, kidney, skeletal muscle, brain, WAT, and BAT relative to total body weight were not significantly different compared with wild type littermates. Other measured parameters, including fasting blood glucose levels (116.5 ± 11.3 mg/dl), average blood pressure measured by five consecutive tail cuff readings (99.4 ± 8.16/86.2 ± 5.7 mm Hg for iPLA$_2^\gamma$ knockout mice versus 113.4 ± 1.97/96.9 ± 3.1 mm Hg for wild type littermates), and glucose tolerance (blood glucose measurements following 16 h of fasting and intraperitoneal glucose administration), were within the normal range and were not significantly different in 2–4-month-old KO and WT littermates. Echocardiographic analyses of iPLA$_2^\gamma$ mice at ages of...
2–3 months did not reveal any significant differences in chamber sizes or ventricular function compared with WT control mice fed ad libitum, after fasting, or after high fat feeding for 6 weeks. No differences were found between iPLA₂γ knock-out mice and iPLA₂γ controls in general ambulatory activity studies (data not shown).

Compromised Thermal Adaptation in iPLA₂γ KO Mice—To compare the ability of 4–6-month-old WT and iPLA₂γ KO animals to adapt to cold, decreases in core body temperature were examined as a function of time following exposure to 4 °C with ready access to food and water as described under “Experimental Procedures.” All WT littermates were able to maintain core body temperatures for 24 h at 4 °C. In sharp contrast, cold intolerance was manifest in both male and female mice null for iPLA₂γ (15.3 ± 2.7 h and 11.5 ± 3.4 h mean endurance times at 4 °C, respectively) (Fig. 4). Similarly, aged mice (9–11 months) null for iPLA₂γ also demonstrated cold intolerance, whereas...
their WT littermates did not. The mean endurance times for KO male and female animals were 8 ± 0.4 and 7.1 ± 0.6 h, respectively, whereas both WT male and female animals maintained their body temperatures throughout the entire experimental interval. Collectively, these results demonstrated that both young and old iPLA2γ knock-out mice had defects in cold adaptation.

Uncoupling of electron transport and conversion of the proton-motive force across the inner membrane into heat by uncoupling proteins (UCPs) is a well described mechanism for heat production. In particular, mitochondrial uncoupling in brown adipose tissue mediated by UCP1 has been previously shown to support body temperature after exposure to cold (reviewed in Ref. 38). Accordingly, we examined levels of UCP1 mRNA encoding UCP1 levels in brown adipose tissue by real-time PCR as described under “Experimental Procedures.” A, UCP1 message levels in brown adipose tissue were measured under ambient conditions (control) and during exposure to cold (4 °C). Animals were exposed to 4 °C until their core body temperature dropped to hypothermic levels (below ~28 °C). The mean cold tolerance time for KO animals was 8 h, and WT controls (still maintaining the core body temperatures) were sacrificed at the same mean time. B, no significant difference was observed for UCP2 expression levels in WT and KO hearts. C, a significant reduction in UCP3 expression was observed in the hearts of iPLA2γ KO mice (p < 0.05). n = 4 per group. Values represent arbitrary units (AU) after normalization to a ribosomal 36B4 internal standard. Data are presented as means ± S.E. Statistical significance was performed using the Student’s t test, and significant differences are indicated by brackets.

**Decreased Exercise Endurance of iPLA2γ KO Mice—**Considering the defects in cold adaptation in the iPLA2γ KO mice, we next focused on the ability of these mice to tolerate prolonged exercise on a treadmill in comparison with their WT counterparts. Interestingly, 53-day-old mice null for iPLA2γ exhibited a dramatic 50% decrease in exercise endurance (19.1 ± 1.3 min) compared with their WT littermates (39.3 ± 1.5 min, p < 0.001) when the animals were run to exhaustion on a treadmill (Fig. 6A). This exercise deficit was progressive, since the endurance time of 86-day-old KO mice relative to WT decreased to less than 25% relative to WT littermates (9.6 ± 0.7 versus 32 ± 3.2 min for the KO versus WT, respectively, p < 0.001) (Fig. 6B). By day 270, the KO treadmill time had further decreased to 5 min (5.3 ± 0.7 min) (Fig. 6B).

**Compromised Myocardial Functional Reserve in iPLA2γ KO Mice—**TAC with careful monitoring of pressure gradients has been a useful model in the evaluation of myocardial metabolic reserve in response to hemodynamic stress. Mice null for iPLA2γ were dramatically more susceptible to sudden death following transverse aortic constriction. Six of 8 knock-out mice (ages 2–4 months) died within 24 h following surgery.
(25% survival), whereas all of the sham operated mice or wild type littermates survived. No significant alterations in pressure gradients generated by banding between the two groups were present. These results demonstrate that iPLA$_2$$\gamma$ KO mice are unable to successfully compensate for the increased myocardial energy demands generated by hemodynamic-induced increases in ventricular afterload.

**Decreased Mitochondrial Function in iPLA$_2$$\gamma$ KO Mice Due to Inefficient Complex IV-mediated O$_2$ Reduction**—The markedly decreased survival of mice null for iPLA$_2$$\gamma$ in response to pressure overload suggested an iPLA$_2$$\gamma$-induced defect in cardiac mitochondrial function. To determine if this contributed to the increased TAC mortality rate and decreased exercise endurance observed in iPLA$_2$$\gamma$ knock-out mice, mitochondria were isolated from WT and iPLA$_2$$\gamma$ KO myocardium for measurement of respiratory function as described under “Experimental Procedures.” Initially, the ability of WT and iPLA$_2$$\gamma$ KO mitochondria to support cytochrome oxidase (Complex IV)-mediated reduction of molecular oxygen in the presence of a surrogate electron donor (ascorbate) was measured. Remarkably, the rate of O$_2$ consumption by iPLA$_2$$\gamma$ KO mitochondria was decreased by 65% compared with that of WT controls (Fig. 7A). To demonstrate the functional integrity of the isolated mitochondria, ADP, succinate, and malate were added in separate experiments to the mitochondria, which resulted in robust O$_2$ consumption in both WT and iPLA$_2$$\gamma$ KO mitochondria with only minimal differences in the rate of O$_2$ reduction (Fig. 7B). After correction for azide inhibition, a replot of ascorbate-TMPD oxygen consumption (relative site IV $V_{max}$) more dramatically demonstrated the reduced functional activity of complex IV in both young and old iPLA$_2$$\gamma$ KOs versus WT controls (Fig. 7C).

**Alterations in Cardiolipin Content and Molecular Species Distribution in iPLA$_2$$\gamma$ KO Mice**—To determine if genetic ablation of iPLA$_2$$\gamma$ resulted in changes in myocardial lipid composition, shotgun lipidomics was performed on heart tissue from WT and KO animals aged 4–6 months. No statistically different alterations in major lipid classes (e.g. phosphatidylcholine, phosphatidylethanolamine, phosphatidyglycerol, triacylglycerol, etc.) or molecular species examined were noted with the exception of a decrease in CL content and an altered CL molecular species distribution (Fig. 8). Specifically, the total CL content was reduced by 15 ± 0.2 mol % in iPLA$_2$$\gamma$ KO hearts in comparison with their wild type littermates (Fig. 9A). Moreover, a more marked decrease in the symmetric CL species tetra-18:2 CL was present in mice null for iPLA$_2$$\gamma$ in comparison with their WT littermates (33 ± 0.6 mol %, $p < 0.02$) (Fig. 9B). Similar results were obtained from analyses of hearts from animals 9–11 months of age (data not shown). The peak at $m/z$ 747.5 was composed of tri-18:2–22:6 CL and was not substantially changed in WT versus iPLA$_2$$\gamma$ KO mice. Collectively, these results demonstrate that iPLA$_2$$\gamma$ participates in mitochondrial cardiolipin metabolism, presumably by supplying the lysocardiolipin acceptor substrate for acylation/transacylation remodeling reactions. Thus, although iPLA$_2$$\gamma$ is not obligatory for the production of tetra-18:2 CL (i.e. tetra-18:2 CL is present in the iPLA$_2$$\gamma$ KO), the absence of iPLA$_2$$\gamma$ cannot be completely compensated for by other enzymes.

**DISCUSSION**

Through the use of targeted genetic ablation of iPLA$_2$$\gamma$, we have demonstrated the fundamental role of this enzyme in cellular bioenergetics that cannot be compensated for by alterations in other enzymic activities. Previously, we hypothesized that iPLA$_2$$\gamma$ had an important role in modulating cellular bioenergetics based on identification of its mitochondrial local-
Procedures." The levels of individual cardiolipin molecular species after identifi-
trometry analyses in the negative ion mode as described under "Experimental
fied Bligh and Dyer method and subjected to electrospray ionization/mass spec-
both total cardiolipin (WT and five KO animals between 3 and 6 months in age. Significant decreases in
FIGURE 9. Cardiolipin content of iPLA_{2γ} KO mice and their wild type littermates. Myocardial lipid extracts of wild type mice (A) and iPLA_{2γ} KO mice (B) were prepared by a modified Bligh and Dyer procedure. Negative ion electrospray ionization mass spectra were acquired using a QqQ mass spectrometer as described under "Experimental Procedures." Spectra have been nor-
malized to a CL internal standard (see insets). The asterisks indicate the doubly charged CL plus-one isotopologues whose ion peak intensities were utilized to quantify individual CL molecular species as described previously (26). The results indicate the paucity of tetra-18:2 CL molecular species in the iPLA_{2γ} KO in comparison with WT (compare peaks at m/z 723.5 in control versus iPLA_{2γ} KO). The insets on the right of each panel show the extended mass spectra that display additional internal standards and other major anionic phospholipids.

FIGURE 8. Expanded negative ion electrospray ionization mass spectra of myocardial lipid extracts from iPLA_{2γ}KO mice and their wild type littermates. Myocardial lipid extracts of wild type mice (A) and iPLA_{2γ} KO mice (B) were prepared by a modified Bligh and Dyer procedure. Negative ion electrospray ionization mass spectra were acquired using a QqQ mass spectrometer as described under "Experimental Procedures." Spectra have been nor-
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The absence of iPLA_{2γ} in murine myocardium probably results in a paucity of CL lysocardiolipin acceptor, which limits CL remodeling and the resultant synthesis of tetra-
18:2 CL (Fig. 9) (44). The deficiency of tetra-18:2 CL was anticipated based upon the known importance of phospho-
lipases in the remodeling of newly synthesized CL molecular species. This deficiency in symmetric CL molecular species probably results in inefficient Complex IV function, as dem-
onstrated by mitochondrial function assays in vitro. The inability of mitochondria to adapt effectively to a variety of stressors under pathophysiologic conditions underscores the importance of specific CL molecular species in mito-
chondrial bioenergetic efficiency and the important role of iPLA_{2γ} in generating the lysocardiolipin acceptor necessary for the generation of symmetric tetra-18:2 CL molecular species. We specifically point out that other alterations, in addi-
tion to the changes in CL content and molecular species composition, could contribute to the dysfunctional mito-
chondrial adaptations to stress that were demonstrated in this study, including cryptic changes in lipid-protein inter-
actions, alterations in membrane dynamics, and changes in membrane surface charge and distribution.

The iPLA_{2γ} KO phenotype is consistent with an extensive literature demonstrating the association between mitochon-
drial dysfunction and deficiencies in growth as well as cold and exercise tolerance (3, 45–48). Previously, it has been demonstrated in many models that mitochondrial integrity
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is essential for cold adaptation (38, 49–52). Thus, the ability to adapt to cold has previously been utilized to identify mitochondrial dysfunction in many different types of mice with genetic alterations in energy-producing pathways (e.g. MCAD (53), UCP1 (54), GATP1 (55), orphan nuclear receptor estrogen (56), and PGC-1β (57, 58)). Mutations in mitochondrial proteins encoded by both mitochondrial DNA and nuclear DNA have been implicated in a wide range of degenerative diseases, which include clinical features of myopathy, cardiomyopathy, growth retardation, movement disorders, and diabetes. Many animal models have confirmed that alterations in mitochondrial energy generation, ROS production, and apoptosis can all contribute to the pathophysiology of mitochondrial dysfunction (59–62).

Recent investigations into the role of mitochondrial membrane dynamics have uncovered the critical role of mitochondrial membrane fusion and fission in facilitating mitochondrial function (63–65). Although the precise mechanisms that mediate fusion and fission are not known with certainty, it is clear that CL, which has a propensity for HII phase formation, plays a critical role in this process (66). The large difference in the volume occupied by the four highly unsaturated aliphatic chains of CL compared with that of the polar head groups probably promotes prominent curvature of the inner membrane (67). In addition, the doubly anionic charge present in CLs has profound effects on membrane surface charge and facilitates specific lipid-protein interactions. Although the structural features of polyunsaturated CLs have yet to be assigned specific mechanistic roles, the unique characteristics of this molecule and its highly symmetric configuration are probably critical to mitochondrial function on many levels. The production of symmetric CL species is facilitated by iPLA2γ and, as shown in the genetic knock-out, cannot be compensated for by the other phospholipases present in the mitochondrial compartment.

The iPLA2γ KO mice had significantly reduced cold tolerance, yet there were no large differences in expression of UCP1 under basal conditions or after cold induction. Considering the cold intolerance and lower basal core body temperature of the KO (probably due to mitochondrial inefficiency in heat generation), the small increase in KO UCP1 is not unexpected and may represent a compensatory mechanism for heat generation. Prior work has demonstrated the obligatory role of fatty acids in facilitating the function of uncoupling proteins (68–71). Thus, by providing fatty acids locally, mitochondrial phospholipases could function in the acute regulation of inner membrane UCP activity. The significance of the large down-regulation of UCP3 expression in the myocardium of iPLA2γ KO mice is unknown, as is the precise functional role of UCP3 itself.

Phospholipases could also play a key role in regulating the relative proportion of Gibbs free energy in substrate converted into either chemical energy (e.g. ATP) or heat in the mitochondria. In many systems previously studied, the role of phospholipases is to regulate signaling functions. In this regard, we point out that iPLA2γ selectively generates 2-arachidonyl (AA) (20:4) LPC, which serves as a node in lipid second messenger signaling pathways (12, 72, 73). Support for the role of iPLA2γ in the generation of 2-AA LPC as well as 2-docosahexaenoyl (2-DHA) (22:6)-LPC in vivo has come from transgenic mice overexpressing iPLA2γ in a cardiac restricted manner, where dramatic increases in 2-AA LPC and 2-DHA LPC have been demonstrated (29).

A fourth function of phospholipase activity in mitochondria may be to regulate the binding and dynamics of cytochrome c to the inner membrane, which is highly dependent on CL content, inner membrane surface charge, and membrane molecular dynamics (74). Thus, abnormal CL content in conjunction with alterations in the physical properties of the mitochondrial inner membrane could significantly change mitochondrial responses to physiologic and pathophysiologic perturbations. Some of the sequelae of altered CL content have been uncovered in the study of the genetic disease Barth syndrome, where a defect in tafazzin synthesis precludes the production of physiologic amounts of CL mass and molecular species (75–78). Notably, CL remodeling by tafazzin requires the generation of lysocardiolipin for subsequent transacylation/reacylation (44). Through the genetic knock-out of iPLA2γ, it appears that physiologic CL remodeling does not occur effectively, precipitating mitochondrial dysfunction. It is intriguing to note that the phenotype of Barth syndrome includes growth retardation and cardiomyopathy (79–81), two features recapitulated in the present study.

Genetic ablation of iPLA2γ results in many readily discernable phenotypic alterations. These stand in sharp contrast to the normal growth and development of cPLA2α and iPLA2β KO mice in which discernable alterations were either limited to the reproductive system or required careful manipulations of specialized cells to identify alterations (82–85). Recent studies have demonstrated the close structural similarity and evolutionary relationship between the active sites of the iPLA2 and cPLA2 families of enzymes (86). The results of this study clearly identify the role of iPLA2γ in mitochondrial bioenergetic function and underscore its importance in cellular energy metabolism and homeostasis. Based on the importance of mitochondrial efficiency in energy metabolism, it is likely that the alterations described in the present work represent only the major and most easily identifiable abnormalities apparent after genetic ablation. Future studies will probably uncover additional roles for iPLA2γ in mammalian biology as our understanding of the roles of phospholipases and their contribution to the regulation of mitochondrial function expands. The iPLA2γ KO mouse should provide a valuable model toward this goal.

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