Cardiac myocyte-specific knock-out of calcium-independent phospholipase A2γ (iPLA2γ) decreases oxidized fatty acids during ischemia/reperfusion and reduces infarct size

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Cardiac Myocyte-specific Knock-out of Calcium-independent Phospholipase A$_2$$\gamma$ (iPLA$_2$$\gamma$) Decreases Oxidized Fatty Acids during Ischemia/Reperfusion and Reduces Infarct Size*

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Calcium-independent phospholipase A$_2$$\gamma$ (iPLA$_2$$\gamma$) is a mitochondrial enzyme that produces lipid second messengers that facilitate opening of the mitochondrial permeability transition pore (mPTP) and contribute to the production of oxidized fatty acids in myocardium. To specifically identify the roles of iPLA$_2$$\gamma$ in cardiac myocytes, we generated cardiac myocyte-specific iPLA$_2$$\gamma$ knock-out (CMiPLA$_2$$\gamma$KO) mice by removing the exon encoding the active site serine (Ser-477). Hearts of CMiPLA$_2$$\gamma$KO mice exhibited normal hemodynamic function, glicerophospholipid molecular species composition, and normal rates of mitochondrial respiration and ATP production. In contrast, CMiPLA$_2$$\gamma$KO mice demonstrated attenuated Ca$^{2+}$-induced mPTP opening that could be rapidly restored by the addition of palmitate and substantially reduced production of oxidized polyunsaturated fatty acids (PUFAs). Furthermore, myocardial ischemia/reperfusion (I/R) in CMiPLA$_2$$\gamma$KO mice (30 min of ischemia followed by 30 min of reperfusion in vivo) dramatically decreased oxidized fatty acid production in the ischemic border zones. Moreover, CMiPLA$_2$$\gamma$KO mice subjected to 30 min of ischemia followed by 24 h of reperfusion in vivo developed substantially less cardiac necrosis in the area-at-risk in comparison with their WT littermates. Furthermore, we found that membrane depolarization in murine heart mitochondria was sensitized to Ca$^{2+}$ by the presence of oxidized PUFAs. Because mitochondrial membrane depolarization and calcium are known to activate iPLA$_2$$\gamma$, these results are consistent with salvage of myocardium after I/R by iPLA$_2$$\gamma$ loss of function through decreasing mPTP opening, diminishing production of proinflammatory oxidized fatty acids, and attenuating the deleterious effects of abrupt increases in calcium ion on membrane potential during reperfusion.

The salvage of jeopardized regions of myocardium during ischemia/reperfusion (I/R)$^3$ has been a long-standing goal of heart research. Because mortality and morbidity are related to infarct size, a variety of hemodynamic, metabolic, and pharmacological approaches have been used to reduce the severity of myocardial infarction during ischemia (1–3). Recent studies have accumulated evidence that the irreversible opening of the mitochondrial permeability transition pore (mPTP) upon oxidative stress is a principal mechanism of apoptotic/necrotic cardiac cell death accounting for the majority of I/R injury (4–6). Although therapies for acute ischemia (e.g. reperfusion) have been extensively studied, at present there is no therapy for attenuating mPTP opening during reperfusion of ischemic zones in myocardium.

Although the precise chemical composition of the mPTP is incompletely understood (6), a variety of initiators and modulators of mPTP opening has been identified (7, 8). For example, during reperfusion, the reoxygeneration of ischemic tissue results in mitochondrial Ca$^{2+}$ overload and renormalization of intracellular and matrix pH, which are accompanied by the prodigious generation of reactive oxygen species that synergistically induce the opening of the mPTP. Furthermore, both fatty acids and their acyl-CoA derivatives increase dramatically during myocardial ischemia and each greatly facilitate mPTP opening (9–15). The extensive permeability of the inner mitochondrial membrane culminates in the release of proapoptotic factors and the efflux of toxic lipid metabolites into the cytosol that collectively precipitate irreversible myocardial necrosis and apoptosis (10, 16, 17).

Previously, we identified a novel calcium-independent phospholipase A$_2$$\gamma$ (iPLA$_2$$\gamma$; also known as PNPLA8) that was membrane-associated, present in multiple tissues, and possessed

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3 The abbreviations used are: I/R, ischemia/reperfusion; CL, cardiolipin; CMIPLA$_2$$\gamma$KO, cardiac myocyte-specific iPLA$_2$$\gamma$ knock-out; EET, epoxyeicosatrienoic acid; FLP, flippase; HDoHE, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; iPLA$_2$$\gamma$, calcium-independent phospholipase A$_2$$\gamma$; mPTP, mitochondrial permeability transition pore; oxoODE, oxo-octadecadienoic acid; PG, prostaglandin; L, internal standard; oxalam, oxidized linoleic acid metabolite; FCCP, trifluoromethoxy carbonylcyanide phenylnitrazonate; MRR, multiple reaction monitoring; AMPP, N-(4-aminomethylphenyl) pyridinium; TTC, triphenyltetrazolium chloride; D, diacyl; TPP$, tetraphenylphosphonium; LAD, left anterior descending; FRT, flippase recombinase target; MDMS-SL, multidimensional mass spectrometry-based shotgun lipidomics.
multiple discrete isoforms (18). Further studies demonstrated that iPLA\(_{2}\)\(\gamma\) transcription was tightly regulated through multiple complex mechanisms (19). Through immunohistochemistry and cardiac myocyte-specific expression, iPLA\(_{2}\)\(\gamma\) was shown to be localized to mitochondrial and peroxisomal compartments. Transgenic expression of iPLA\(_{2}\)\(\gamma\) resulted in the dramatic increase of 2-arachidonoyl lysophosphatidylcholine and 2-docosahexaenoyl lysophosphatidylcholine in cardiac myocytes (19, 20). Later studies also identified iPLA\(_{2}\)\(\gamma\) in the endoplasmic reticulum (21). To begin the mechanistic dissection of the roles of iPLA\(_{2}\)\(\gamma\) in biological function in health and disease, we generated a germ line knock-out of iPLA\(_{2}\)\(\gamma\) in mice (iPLA\(_{2}\)\(\gamma\) KO) (22–24). These studies revealed that iPLA\(_{2}\)\(\gamma\) loss of function dramatically reduced the opening of the mitochondrial permeability transition pore (mPTP) in liver mitochondria and that calcium channel of myocardial mitochondria obtained from the iPLA\(_{2}\)\(\gamma\) KO mouse markedly decreased the production of inflammatory eicosanoids in comparison with wild-type mice. However, germ line iPLA\(_{2}\)\(\gamma\) KO mice displayed multiple defects in virtually every organ system studied, thus rendering definitive mechanistic interpretation of responses to in vivo cardiac ischemia difficult. To traverse this difficulty, in this study we generated cardiac myocyte-specific iPLA\(_{2}\)\(\gamma\) knock-out mice (CMiPLA\(_{2}\)\(\gamma\)KO) by inserting flox sites proximal and distal to the active site serine of iPLA\(_{2}\)\(\gamma\) (Ser-477 in exon 5) and subsequently exciting the exon containing the active site by tamoxifen-activated cardiac myocyte-specific Cre recombinase. Utilizing this novel genetic mouse model, we have investigated the effects of cardiac myocyte-specific KO of iPLA\(_{2}\)\(\gamma\) on ischemia/reperfusion in vivo. The regiospecificity of iPLA\(_{2}\)\(\gamma\) toward phospholipid substrates is atypical among mammalian PLA2 enzymes in that the site of hydrolysis is dependent on the nature of the sn-2 aliphatic group (25). Specifically, if the sn-2 group is saturated or contains a single double bond, iPLA\(_{2}\)\(\gamma\) exhibits no preference for cleavage of the fatty acyl group at the sn-1 or sn-2 position. In sharp contrast, if the sn-2 substituent is polyunsaturated, iPLA\(_{2}\)\(\gamma\) serves predominantly as a PLA1, releasing the saturated fatty acid from the sn-1 position and generating 2-polyunsaturated fatty acyl lysolipids. Thus, the regiospecificity of hydrolysis is determined by the degree of unsaturation in the sn-2 phospholipid constituent. This unusual feature allows the enzyme to accomplish multiple regulatory functions in mitochondria, including the release of palmitate in the inner membrane, which opens the mPTP, the generation of polyunsaturated lysophospholipids, which are readily hydrolyzed by endogenous lipases to lead to the production of bioactive oxidized fatty acids (e.g. eicosanoids, docosanoids, etc.), and the provision of fatty acid substrates for use in mitochondrial energy generation.

Accordingly, we hypothesized that loss of cardiac iPLA\(_{2}\)\(\gamma\) function would decrease I/R injury through a four-tiered synergistic mechanism involving the following: 1) attenuation of mPTP opening; 2) decreased inflammatory lipid second messengers; 3) preservation of mitochondrial membrane potential; and 4) attenuated release of toxic lipid metabolites (e.g. non-esterified saturated fatty acids, lysolipids, acyl-CoAs, and acylcarnitines) that accumulate during myocardial ischemia and are released during reperfusion.

In this study, we utilized CMiPLA\(_{2}\)\(\gamma\)KO mice to investigate iPLA\(_{2}\)\(\gamma\)-mediated mPTP opening upon calcium challenge, its role in the production of proinflammatory lipid metabolites (eicosanoids, docosanoids, and oxidized linoleic acid metabolites) in the border zone, and the development of cardiac necrosis after I/R in the absence of the confounding pathologies that were present in the germ line knock-out. Importantly, we demonstrate that myocardial loss of iPLA\(_{2}\)\(\gamma\) function substantially reduces infarct size after I/R in vivo and markedly decreases production of inflammatory oxidized fatty acids (oxylipins) in the ischemic border zone. Through ablation of iPLA\(_{2}\)\(\gamma\)-facilitated mPTP opening, generation of inflammatory lipid second messengers, and the release of toxic mitochondrial metabolites, a novel strategy to attenuate cardiac necrosis and inflammation during acute coronary syndromes has been identified.

**Results**

**Generation of Cardiac Myocyte-specific iPLA\(_{2}\)\(\gamma\) Knock-out Mice**—To definitively identify the mechanistic importance of iPLA\(_{2}\)\(\gamma\) in cardiac myocytes, we engineered an inducible cardiac myocyte-specific knock-out of iPLA\(_{2}\)\(\gamma\). Because of the presence of multiple transcriptional start sites in iPLA\(_{2}\)\(\gamma\), our strategy was to flox exon 5 containing the active site and remove it by tamoxifen induction of cardiac myocyte-specific Cre recombinase (Fig. 1). Southern analysis for the floxed iPLA\(_{2}\)\(\gamma\) allele in multiple tissues of the if/if mouse and PCR analyses for the identification of ablation of the PGK-neo cassette and iPLA\(_{2}\)\(\gamma\)\(\text{flox}\)/Cre\(\text{e}^+\) in the iPLA\(_{2}\)\(\gamma\) conditional KO mice are shown in Fig. 1. Northern and Western analyses demonstrated the specific ablation of iPLA\(_{2}\)\(\gamma\) in heart but not in other tissues in the CMiPLA\(_{2}\)\(\gamma\)KO mouse (Fig. 1, E and F).

**Demonstration That the Majority of iPLA\(_{2}\)\(\gamma\) Activity in Myocardium Is Present in Cardiac Myocytes and Discrete Tissue Distributions of iPLA\(_{2}\)\(\gamma\) Isoforms in Different Tissues**—Myocardium is composed of multiple cell types, including cardiac myocytes, endothelial cells, smooth muscle cells, fibroblasts, and macrophages. Although myocardium contains substantial amounts of iPLA\(_{2}\)\(\gamma\) activity and protein, the cell type of origin of iPLA\(_{2}\)\(\gamma\) is not known with certainty. Comparisons of WT Cre\(\text{e}^+\) with CMiPLA\(_{2}\)\(\gamma\)KO mice definitively demonstrate that the overwhelming majority of iPLA\(_{2}\)\(\gamma\) protein of murine myocardium is present in cardiac myocytes by tissue-specific knock-out mediated by the specificity of cardiac myocyte-specific expression of Cre recombinase. Moreover, the results of Fig. 1F demonstrate the diverse tissue-specific distribution of iPLA\(_{2}\)\(\gamma\) isoforms (e.g. 88, 74, 63, and 52 kDa), which were previously identified by germ line knock-out and transgenic overexpression of iPLA\(_{2}\)\(\gamma\) (9, 19, 20). For example, note the predominance of the lower molecular mass iPLA\(_{2}\)\(\gamma\) isoforms (50–60 kDa) in liver in comparison with myocardium and brain. Collectively, these results demonstrate that iPLA\(_{2}\)\(\gamma\) in myocardium is predominantly located in cardiac myocytes and identify the tissue-specific distributions of different isoforms of iPLA\(_{2}\)\(\gamma\).

**Constitutional Characteristics of the CMiPLA\(_{2}\)\(\gamma\)KO Mouse**—In contrast to the global iPLA\(_{2}\)\(\gamma\) knock-out, which demonstrated a thin body habitus, decreased length, cognitive dysfunction, kyphosis, and decreased locomotor activity (22, 24), the CMiPLA\(_{2}\)\(\gamma\)KO mice gained weight normally, possessed...
normal insulin sensitivity, did not develop kyphosis, and had no demonstrable sensory-motor abnormalities (data not shown).

Echocardiographic analyses of myocardial hemodynamic function in the CMiPLA₂γKO mice at 6 months of age (3 months after tamoxifen administration) revealed no significant alterations in left ventricular wall thickness, left ventricular mass index, or chamber diameters during end systole/diastole and displayed normal fractional shortening in comparison with WT littermates (Table 1).

**High Resolution Respirometry of Myocardial Mitochondria from WT and CMiPLA₂γKO Mice**—High resolution respirometry of myocardial mitochondria was performed to identify alterations in mitochondrial function and respiratory coupling efficiency in CMiPLA₂γKO mice. To examine mitochondrial bioenergetic efficiency under different conditions, we utilized multiple substrates, including pyruvate/malate, palmitoylcarnitine/malate, and pyruvate/glutamate/malate. Mitochondria from CMiPLA₂γKO mice demonstrated similar oxygen consumption rates in comparison with WT littermates during both state 2 and 3 respiration or after inhibition of complex I (rotenone) or complex V (oligomycin-induced state 4) (Fig. 2). The coupling of electron transport to oxidative phosphorylation (P/O ratio), which was determined by quantifying ATP production and O₂ consumption during state 3 respiration, was not significantly different in WT versus CMiPLA₂γKO mice (Fig. 2). These results demonstrate the ability of mitochondria from the CMiPLA₂γKO to respire normally and efficiently synthesize ATP.
iPLA$_2$γ Knock-out Decreases Eicosanoids during I/R

Echocardiographic comparisons of hemodynamic function in WT Cre$^+$ versus CMiPLA$_2$γKO mice at 6 months of age demonstrated no alterations in cardiac function after cardiac myocyte genetic ablation of iPLA$_2$γ. Parameters examined for each group were as follows: HR, heart rate (beats/min); LVPWd, left ventricular posterior wall thickness at end diastole (mm); IVSd, interventricular septal wall thickness at end diastole (mm); LVIDd, LV internal diameter at end systole (mm); LVM, left ventricular mass (mg); RWT, relative wall thickness; FS, fractional shortening (%). Data are presented as the mean ± S.D. utilizing six WT and six CMiPLA$_2$γKO male mice.

<table>
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<tr>
<th>Type</th>
<th>Body wt</th>
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<th>IVSd</th>
<th>LVIDd</th>
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<th>LVM</th>
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<td>0.96 ± 0.03</td>
<td>3.72 ± 0.25</td>
<td>1.63 ± 0.14</td>
<td>1.68 ± 0.12</td>
<td>1.59 ± 0.24</td>
<td>131.1 ± 7.6</td>
<td>4.22 ± 0.34</td>
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**TABLE 1**

Echocardiographic analysis of myocardial hemodynamic function in wild-type (WT) and cardiac myocyte-specific iPLA$_2$γ knock-out (KO) mice under light anesthesia

Lipidomic Analyses of Myocardium from WT and CMiPLA$_2$γKO Mice—To determine alterations in the myocardial lipoprotein of WT versus CMiPLA$_2$γKO mice, we utilized multidimensional mass spectrometry-based shotgun lipidomics (MDMS-SL) (26). The major phospholipid classes in myocardium are choline and ethanolamine glycerophospholipids. Examination of choline glycerophospholipids demonstrated the presence of over 45 molecular species in murine myocardium that were largely composed of diacyl (D) phosphatidylethanolamine molecular species largely composed of D18:0–22:6, D16:0–22:6, D18:1–22:6, and D18:0–20:4 molecular species as well as 20 plasmenyl (P) ethanolamine phospholipid molecular species largely composed of P16:0–22:6, P18:1–20:4/P16:0–22:5, P18:0–22:6, and P18:1–22:6 molecular species. Mirror plots of ethanolamine glycerophospholipids from averaged mass spectra from six separate mice did not identify any significant differences between WT and CMiPLA$_2$γKO mouse hearts (Fig. 3B). Triglyceride analysis by MDMS-SL demonstrated nearly identical total amounts of triglycerides and no differences in their molecular species composition in WT versus CMiPLA$_2$γKO mice (Fig. 3C). Negative ion mass spectra did not reveal any significant differences in phosphatidylinositol, phosphatidyleserine, or phosphatidylglycerol molecular species (Fig. 3D).
Next, because tetra-18:2 cardiolipin (CL) has been previously proposed to enhance mitochondrial efficiency by stabilizing the formation of mitochondrial supercomplexes (27–30), we determined the content and composition of myocardial CL using the M+1/2 isotopologue approach (Fig. 4) (31). The results demonstrated no significant differences in the total content of CL. The composition of most molecular species of CL, including symmetric tetra-18:2 CL (m/z 723.5 in Fig. 4A) in WT versus CMiPLA2 γKO myocardium, were nearly identical. Moderate decreases in the levels of 18:2–18:2–18:2–22:6 CL and 18:2–18:2–22:6–22:6 CL (m/z 747.5 and 771.5, respectively, in Fig. 4A) were present in CMiPLA2 γKO mice (Fig. 4B).

Mass Spectrometric Analysis of Myocardial Eicosanoids, Docosanoids, and Oxidized Linoleic Acids—Previous studies have demonstrated the important roles of iPLA2 γ in releasing polyunsaturated fatty acids from mitochondria that are subsequently oxidized by a wide variety of downstream oxygenases (32–35). To gain access to the extremely low abundance regime necessary for accurate identification and quantification of oxidized fatty acids in myocardium, we used charge-switch derivatization with multiple reaction monitoring (MRM) in conjunction with high mass accuracy analysis of signature product ions from diagnostic transitions (36). Multiple differences in oxidized fatty acids containing 18-, 20-, and 22-carbons were observed in CMiPLA2 γKO mice (Fig. 5). These include decreases in prostaglandins, 11-hydroxy-5Z,8Z,12E,14Z-ecosatetraenoic acid (11-HETE), 12-hydroxy-5Z,8Z,10E,14Z-ecosatetraenoic acid (12-HETE), and 15-hydroxy-5Z,8Z,11Z,13E-ecosatetraenoic acid (15-HETE) as well as increased levels of 14(15)-epoxy-5Z,8Z,11Z-ecisatrienoic acid (14,15-EET). Similarly, CMiPLA2 γKO mice had decreased levels of all observable oxidized linoleic acid metabolites (oxlams) except 9-oxo-10E,12Z-octadecadienoic acid (9-oxODE) and had significant decreases in 22-carbon oxidized fatty acids, including 7,8,9R,10S,11E,12Z,13E-19Z-docosahexaenoic acid (RVD-1), 19,20-di-hydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid, and 7-hydroxy-4Z,8E,10Z,13Z,16Z,19Z-docosahexaenoic acid (7-HDoHE) (Fig. 5). These results identify iPLA2 γ as a prominent enzymic mediator for the generation of signaling oxidized fatty acids in myocardium.

Decreased Susceptibility of mPTP Opening in Myocardium from CMiPLA2 γKO Mice in Comparison with Wild-type Mice—Recent work in our laboratory led to the identification of iPLA2 γ as an important modulator of the Ca2⁺-induced opening of the mPTP in mitochondria isolated from liver (9). To determine the contribution of iPLA2 γ to the opening of the cardiac myocyte mPTP, we compared Ca2⁺-induced mitochondrial swelling in WT versus CMiPLA2 γKO mice. Incubation with calcium resulted in the anticipated swelling of WT myocardial mitochondria due to opening of the mPTP. In marked contrast, mitochondrial swelling was substantially attenuated in CMiPLA2 γKO mice (Fig. 6). Ca2⁺-induced swelling of mitochondria from both WT and CMiPLA2 γKO mice was demonstrated to be cyclophilin D (also known as peptidylprolyl cis-trans isomerase F)-dependent through nearly complete inhibition by 2 μM cyclosporin A. No observable differ-

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**FIGURE 3.** Mass spectrometric analysis of choline phospholipids, ethanolamine phospholipids, triglycerides, and anionic phospholipids by molecular ion spectra or tandem MS/MS spectra of lipid extracts of wild-type (WT) and cardiac myocyte-specific iPLA2 γ knock-out (KO) myocardium. A, averaged mass spectra of precursor ion scanning of m/z 184.1 (at collision energy 35 eV) of choline phospholipid (PC) molecular species in the positive ion mode from WT and CMiPLA2 γKO mouse myocardium. B, averaged molecular ion mass spectra of ethanolamine phospholipid (PE) molecular species in the negative ion mode from WT and CMiPLA2 γKO mouse myocardium. C, averaged molecular ion mass spectra of ammoniated triglyceride molecular species in the positive ion mode from WT and CMiPLA2 γKO mouse myocardium. D, averaged molecular ion mass spectra of negatively charged phospholipid molecular species in the negative ion mode from WT and CMiPLA2 γKO mouse myocardium. CL, cardiolipin; PG, phosphatidlyglycerol; PI, phosphatidylinositol; and PS, phosphatidylserine. All spectra were averaged from acquired individual mass spectra from four WT and six CMiPLA2 γKO mice (−6–7 months of age) after normalization to the peak intensity of internal standard (I.S.) in each panel (i.e. phosphatidythrenolamine I.S. in A; phosphatidythrenolamine I.S. in B; triacylglycerol I.S. in C; and PG I.S. in D).
iPLA₂γ Knock-out Decreases Eicosanoids during I/R

**FIGURE 4.** Mass spectrometric analysis of cardiolipin molecular species in wild-type and cardiac myocyte-specific iPLA₂γ KO myocardium. **A,** representative negative ion mode mass spectrum of anionic lipids for myocardium cardiolipin (CL) analysis from wild-type (WT) and cardiac myocyte-specific iPLA₂γ KO (KO) mice (6–7 months of age) after normalizing to tetra-14:0 CL internal standard (I.S., m/z 619.5). Cardiolipin molecular species were identified by the doubly charged peaks. The asterisks indicate examples of the M⁺/2 isotopologues of the doubly charged cardiolipin species (e.g. tetra-18:2 CL; 18:2–18:2–18:2–22:6 CL; and 18:2–18:2–22:6–22:6 CL) whose ion peak intensities were utilized to quantify individual cardiolipin molecular species. Tetra-18:2 CL is the predominant cardiolipin molecular species present at m/z 723.5. **B,** cardiolipin molecular species were identified in WT (n = 4) and CMiPLA₂γKO (KO) mouse myocardium (n = 6). ***, p < 0.01.

**FIGURE 5.** Cardiac oxidized fatty acids in wild-type and cardiac myocyte-specific iPLA₂γ KO myocardial tissue. Myocardial tissue was isolated from wild-type (WT) and CMiPLA₂γKO (KO) mice (~6–7 months of age) and flash-frozen in liquid nitrogen. Eicosanoids (A), oxlams (B), and docosanoids (C) were then purified by solid phase extraction and derivatized with AMPP. Quantitative analysis was performed by LC-MS/MS via MRM in the positive ion mode with accurate mass analysis of diagnostic product ions following separation of molecular species using a reverse phase column as described under “Experimental Procedures.” *, Values are the means ± S.E. of six preparations. *, p < 0.05 when compared with KO. HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; HODE, hydroxyoctadecadienoic acid; oxoODE, oxo-octadecadienoic acid; EpOME, epoxyoctadecenoic acid; LT₄, leukotriene B₄; TXB₂, thromboxane B₂; PG, prostaglandin; RVD, resolvin; DiHDoHE, dihydroxydocosahexaenoic acid; DiHDPA, dihydroxydocosapentaenoic acid; and HDoHE, hydroxydocosahexaenoic acid.
iPLA$_2^\gamma$ Knock-out Decreases Eicosanoids during I/R

FIGURE 6. Kinetics of calcium-induced swelling of mitochondria from wild-type and cardiac myocyte-specific iPLA$_2^\gamma$ knock-out mice. Myocardial mitochondria were isolated by differential centrifugation from wild-type (WT) and CMiPLA$_2^\gamma$KO (KO) mice (6 months old) and resuspended in swelling buffer containing 0.23 M manitol, 70 mM sucrose, 2 mM KH$_2$PO$_4$, 3 mM HEPES, pH 7.0, 5 mM succinate, and 1.25 mM rotenone. Mitochondria were placed in a 96-well plate with ethanol vehicle alone (1%), 2 μM cyclosporin A (CsA), 0.5 or 2 μM palmitate (PA). Following exposure to either 150 μM Ca$^{2+}$ or 10 μM EGTA (−Ca$^{2+}$), WT (A) and KO (B) mitochondrial swelling was monitored for decreases in absorbance at 540 nm at 15-s intervals at 23°C. Net changes in absorbance at 540 nm at 5, 10, and 20 min in WT and KO mitochondria were calculated in C where *, p < 0.05 and **, p < 0.001 when compared with the Ca$^{2+}$-induced absorbance decrease in WT mitochondria. Values are the average of four independent preparations ± S.E. Western blots against cyclophilin D (Cyp-D), adenine nucleotide translocase (ANT), and voltage-dependent anion channel (VDAC) in WT and CMiPLA$_2^\gamma$KO (KO) mouse hearts are shown in D (n = 4).

ences in cyclophilin D and adenine nucleotide translocase protein expression levels were present in WT versus CMiPLA$_2^\gamma$KO myocardium indicating that the attenuation of mPTP opening in CMiPLA$_2^\gamma$KO mouse hearts is not due to alterations in the expression of regulatory machinery of the mPTP by ablation of iPLA$_2^\gamma$ (Fig. 6D). Because iPLA$_2^\gamma$ selectively releases palmitate from the sn-1 position of polyunsaturated phospholipids, we investigated the role of low concentrations of palmitate on mPTP opening in WT and CMiPLA$_2^\gamma$KO mouse mitochondria. Addition of as little as 500 nm palmitate to mitochondria isolated from CMiPLA$_2^\gamma$KO mice completely recapitulated the calcium-induced swelling present in WT mice (Fig. 6).

Ischemia/Reperfusion Results in Dramatic Increases in Signaling Oxidized Fatty Acids That Are Attenuated in the CMiPLA$_2^\gamma$KO Mouse—Next, we determined whether iPLA$_2^\gamma$ loss of function results in alterations in lipid second messenger production during 30 min of ischemia followed by 30 min of reperfusion in vivo. High mass accuracy mass spectrometric analysis demonstrated 10–30-fold increases in multiple oxidized 18-, 20-, and 22-carbon fatty acids (i.e. oxlams, eicosanoids, and docosanoids, respectively) in the ischemic border zone versus non-ischemic regions of WT control hearts following I/R (Fig. 7). This dramatic increase was markedly attenuated in the border zone of ischemia in CMiPLA$_2^\gamma$KO mouse hearts. We specifically point out that the majority of signaling fatty acids induced by I/R result from lipoxygenase, cytochrome P450, and/or other oxidases acting on polyunsaturated fatty acids and do not originate from cyclooxygenase-mediated oxidation. These results are suggestive of fatty acid metabolic channeling from iPLA$_2^\gamma$ to downstream lipoxygenase, P450, and/or other as yet unidentified mitochondrial fatty acid oxidases.

Oxidized Fatty Acids, Including HETEs and 8-HDoHE, Facilitate Ca$^{2+}$-mediated Mitochondrial Membrane Depolarization—Because severe mitochondrial membrane depolarization is manifest upon calcium challenge, we investigated the effects of the oxidized fatty acid metabolites that dramatically increase during I/R on Ca$^{2+}$-mediated membrane depolarization of myocardial mitochondria. Mitochondrial membrane potential ($\Delta \Psi$) was determined by using a tetraphenylphosphonium (TPP$^+$) ion-selective electrode as described under “Experimental Procedures.” By measuring the extramitochondrial concentration of TPP$^+$, the changes in mitochondrial membrane potential were monitored following Ca$^{2+}$ titration in the presence of either vehicle (ethanol), 12-HETE, 20-HETE, 14,15-EET, PGE$_2$, 9-oxoODE, or 8-HDoHE, all of which were dramatically increased during I/R in vivo (see Fig. 7). The initial $\Delta \Psi$ (approximately −160 mV) became less negative rapidly upon sequential calcium additions in the presence of either vehicle alone (control), 14,15-EET, PGE$_2$, 9-oxoODE, or 8-HDoHE, but the membrane potential was partially restored within 4 min (Fig. 8). In contrast, 12-HETE, 20-HETE, or 8-HDoHE greatly facilitated mitochondrial depolarization at 60–80 μM calcium ion by dissipating the electric potential across the membrane resulting in no further depolarization upon addition of an uncoupling agent, trifluoromethoxy carbonylcyanide phenyl-hydrazone (FCCP) (Fig. 8).

Cardiac Myocyte-specific Ablation of iPLA$_2^\gamma$ Results in Dramatic Protection from Myocardial Ischemia/Reperfusion Injury in Vivo—Because mitochondria from CMiPLA$_2^\gamma$KO myocardium are resistant to mPTP opening and contained decreased amounts of inflammatory oxidized fatty acids that promote Ca$^{2+}$-mediated mitochondrial depolarization, we hypothesized that the CMiPLA$_2^\gamma$KO heart would be protected from I/R injury. Accordingly, we induced myocardial ischemia in vivo by ligation of the left anterior descending coronary artery for 30 min followed by 24 h of closed chest reperfusion, and we compared the infarct area to the area-at-risk in WT versus CMiPLA$_2^\gamma$KO mice. In WT mice, ischemia/reperfusion resulted in infarction of 40% of the area-at-risk (Fig. 9). Remarkably, in CMiPLA$_2^\gamma$KO mice, iPLA$_2^\gamma$ loss of function protected the heart from ischemia/reperfusion damage resulting in reduction of the infarct area to 16% of the area-at-risk (Fig. 9). Taken together, these results demonstrate that iPLA$_2^\gamma$ plays a prominent role in I/R-induced cardiac myocyte cell death illuminating iPLA$_2^\gamma$ inhibition as a novel multitiered therapeutic approach to significantly reduce infarct size during I/R.
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Discussion

Previous studies have emphasized the central roles of the mPTP in mediating cardiac damage during ischemia/reperfusion through opening of the channel precipitated by calcium overload, accumulation of inorganic phosphate, and induction of oxidative stress that is amplified by the production of saturated fatty acids and oxidized lipid metabolites (4, 7, 8). The large amounts of acyl-CoA and acylcarnitine that accumulate in the mitochondrial matrix during ischemia accelerate mPTP opening and are directly released into the cytosol along with cytochrome c after mPTP opening (9, 39, 40). Prolonged Ca²⁺/H₉₂₅₃ causes irreversible dissipation of the mitochondrial membrane potential and loss of membrane integrity leading to extensive mitochondrial damage (41). The resultant mitochondrial depolarization exacerbates mitochondrial dysfunction by autoamplification of membrane potential-sensitive iPLA₂γ activity (42). Furthermore, mPTP opening results in the release of apoptogenic factors (e.g. cytochrome c and apoptosis-inducing factor) from the intermembrane space that triggers cell death programs rather than homeostatic clearance of metabolically inefficient mitochondria (e.g. mitophagy). This study demonstrates the unanticipated and dramatic accumulation of oxidized fatty acids, including large amounts of oxidized linoleic acid metabolites, which likely originate from cardiolipin, the major pool of esterified linoleic acid in the mitochondrial compartment as well as a plethora of eicosanoid metabolites known to have adverse effects on cardiac myocyte membrane proteins, inflammation, and bioenergetics (43–45). The benefits of iPLA₂γ loss of function investigated in this study include the attenuation of many of the molecular mechanisms known to predispose to myocardial tissue damage during pathological processes, including cardiac ischemia/reperfusion (46).

Consistent with our prior work identifying iPLA₂γ as an important regulator of the calcium-induced opening of the mPTP in liver mitochondria (9), myocardial mitochondria from the CMiPLA₂γKO mouse demonstrate the regulatory role of cardiac iPLA₂γ on the mitochondrial permeability transition. Furthermore, we demonstrated that submicromolar concentrations of free palmitic acid restored mPTP opening that was attenuated by loss of myocardial iPLA₂γ. This is particularly relevant because iPLA₂γ has a marked sn-1 regiospecificity for hydrolysis of diacyl phospholipids containing sn-2 arachidonic acid or docosahexaenoic acid leading to the release of saturated fatty acids from the sn-1 position concomitant with the generation of 2-arachidonoyl- and 2-docosahexaenoyl-lysophosphatidylcholine, respectively, in the mitochondrial membrane (25). The rapid lateral diffusion of the released saturated fatty acid in the plane of the inner membrane allows it to directly interact with the mPTP without sequestration by cytosolic fatty acid-binding proteins. The regulatory effects of palmitate on the mPTP are further aggravated by its ability to induce ER Ca²⁺ depletion and reactive oxygen species generation (47, 48) and by acting as an endogenous ionophore (49). Supporting these mechanisms, deletion of mitochondrial membrane-associated iPLA₂γ led to the remarkable and robust salvage of damaged regions of myocardium after I/R, which emphasizes a prominent role of car-

![Graph A](http://example.com/GraphA.png)

![Graph B](http://example.com/GraphB.png)

![Graph C](http://example.com/GraphC.png)

![Graph D](http://example.com/GraphD.png)
In addition to iPLA$_{2}$$y$-mediated release of saturated fatty acids from phospholipid pools, we previously reported marked iPLA$_{2}$$y$-dependent production of cardiac eicosanoids in the myocardium by utilizing cardiac myocyte-specific overexpression of iPLA$_{2}$$y$ and global iPLA$_{2}$$y$ knock-out mice (34). Our previous findings suggest that iPLA$_{2}$$y$-generated 2-polyunsaturated fatty acyl lysolipids and their downstream hydrolytic products (non-esterified polyunsaturated fatty acids) are further channeled to multiple metabolic pathways to produce numerous oxidative metabolites (34, 50). A variety of oxidized polyunsaturated lipids generated by multiple oxygenases (e.g. cyclooxygenases, lipoxygenases, and P450 hydroxylases) have been identified as pro-inflammatory mediators in diverse tissues and cell types (45, 51). The deleterious sequelae of pro-inflammatory oxylipins in myocardial I/R injury are also well known, although the precise complement and functions of individual signaling oxylipin molecular species are poorly understood (52–54). To determine the types and changes in extremely low abundance signaling oxidized fatty acids released during pathological processes, we utilized a mass spectrometric “charge-switch” high mass accuracy product ion approach that resulted in a marked increase in sensitivity and...
successful exclusion of false-positive identification through high mass accuracy analysis of informative product ions (36). Although the myocardial lipidome of CMiPLA_2γKO (KO) mouse hearts at similar levels demonstrate excellent definition of the areas of infarction (IA bordered by yellow dashed line, arrows), area-at-risk (AAR, red dashed line), and the left ventricle (LV, black dashed line) after a 30-min occlusion and 24 h of reperfusion of the left anterior descending artery (LAD). At the end of the reperfusion interval, the heart was excised and perfused with Phthalo blue dye with the LAD reoccluded (to define the previous area-at-risk) followed by TTC staining to define the infarct size. B, dramatic decreases in IA/left ventricle and IA/area-at-risk were quantified and subjected to statistical analysis. Data are presented as means ± S.E. utilizing 9 WT and 12 KO male mice (~6–7 months of age).

Because oxidized fatty acids have a multitude of effects on transmembrane proteins, including ion channels and receptors (55, 56), we monitored the changes in mitochondrial membrane potential (ΔΨ_m) in the presence of multiple oxidized lipid metabolites to determine their effects on Ca^{2+}-mediated potential dissipation. During sequential calcium challenges, mitochondria in the absence of extramitochondrial oxidized fatty acids partially recovered their membrane potential from multiple rapid initial losses of transmembrane potential induced by additions of Ca^{2+}. In contrast, hydroxylated polyunsaturated fatty acids (e.g. 12-HETE, 20-HETE, and 8-HDoHE), but not 14,15-EET, 9-oxoODE, or PGE2, sensitize mitochondria to the calcium-induced loss of membrane potential. These findings are supported by previous studies that showed arachidonic acid- and 12-HETE-facilitated Ca^{2+} overload resulting in abnormal oxidative stress and mitochondrial dysfunction (44, 49). Therefore, the results of this study suggest that iPLA_2γ facilitates production of oxidized lipid metabolites by providing PUFAs and/or polyunsaturated fatty acyl lysolipids, which can be further hydrolyzed to non-esterified PUFAs by lysophospholipases and subsequent oxidation by downstream oxygenases. The resultant oxidized fatty acids likely regulate ion channels through selective binding to transmembrane domains of ion channels and ion transporters, direct disruption of interactive membrane domains, and/or the formation of pores in the membrane bilayer. Collectively, it seems likely that the enzymic activity of iPLA_2γ integrates metabolic information from multiple pathways to regulate myocardial networks that control cell fate decisions, electrophysiological function, and receptor-mediated alterations in cardiac myocyte metabolism.

Taken together, this study identifies a critical role of cardiac myocyte iPLA_2γ in the Ca^{2+}-induced opening of the mPTP and the generation of inflammatory signaling oxidized fatty acids that each contribute to cardiac damage during I/R, which can be largely ablated by iPLA_2γ loss of function. Thus, inhibition of a single enzyme has multiple salutary effects during I/R providing a novel synergistic approach for the pharmacological treatment of acute coronary syndromes and multiple myocardial diseases.

**Experimental Procedures**

**Materials**—PCR reagents were purchased from Applied Biosystems (Foster City, CA) for genotyping of WT and CMiPLA_2γKO mice. Radiolabeled nucleotides ([α-32P]dCTP) were purchased from PerkinElmer Life Sciences. Synthetic phospholipids used as internal standards in mass spectrometric analyses were purchased from either Avanti Polar Lipids (Alabaster, AL) or Nu-Chek Prep, Inc. (Elysian, MN). Oxylinps, including deuterated stable isotopes used as internal standards, and FCCP were obtained from Cayman Chemical (Ann Arbor, MI). Tamoxifen utilized for heart-specific conditional ablation of iPLA_2γ was obtained from Sigma. Anti-iPLA_2γ antibody was generated in our laboratory as described previously (9).

Cyclosporin A was obtained from EMD Millipore (Billerica, MA). Antibodies for cyclophilin D, voltage-dependent anion channel, and adenine nucleotide translocase were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Most other supplies and reagents were obtained from Sigma or Fisher.

**General Animal Studies**—Animal protocols were in strict accordance with guidelines of the National Institutes of Health Office of Laboratory Animal Welfare and were approved by the Animal Studies Committee at Washington University.
were fed a standard diet (PicoLab Rodent 20 from LabDiet (St. Louis, MO) containing 5% total fat (13% of total calories) and 0.94% saturated fat) ad libitum unless otherwise indicated. Echocardiographic analyses were performed under light anesthesia as described previously (57, 58). Following euthanasia by cervical dislocation, heart tissues were dissected from male mice, weighed, and either flash-frozen in liquid N2 or the fresh tissue was used immediately.

**Generation of Cardiac Myocyte-specific iPLA2γ Knock-out Mice**—To elucidate the specific roles of iPLA2γ in myocardium, we engineered a conditional iPLA2γ targeting construct containing 7208 bases of the mouse iPLA2γ gene (mouse BAC clone bMQ-391E22, Geneservice Ltd., Cambridge, UK) with an inserted loxP-flippase (FLP) recombinase target (FRT)-neomycin-FRT resistance cassette and a loxP site encompassing exon 5 of the iPLA2γ gene (Fig. 1). Deletion of exon 5 has been previously shown to result in a genotype null for iPLA2γ and complete ablation of iPLA2γ protein expression in multiple tissues (22). The sequence of the targeting vector was verified prior to electroporation into EDJ22 ES cells at the Mouse Genetic Core, Washington University. PCR analyses using iPLA2γ-specific primers 5′-TATAGAGATGCACAACCCAGTGAAAGCGG-3′ and 5′-AGTGGGTAGTGATGACTAGCAGCT-3′ identified three targeted ES clones; however, Southern blot analyses revealed that two of the clones also contained an additional random incorporation event. Therefore, only the ES cell clone containing the single targeted event was expanded and used for injection of blastocysts and implantation into pseudo pregnant female C57BL/6 mice. Chimeric mice were identified by PCR injection of blastocysts and implantation into pseudo pregnant mice obtained from mating to C57BL/6 mice were similarly genotyped and revealed germ line transmission of the floxed mice. PCR analyses using iPLA2γ-inducible Cre recombinase in myocardium. The progeny were genotyped by duplex PCR using the above-mentioned primers 5′-CGGTGGAATGATACTAGCAGGC-3′ and 5′-AGTGGTAGTGATGACTAGCAGCT-3′ (Fig. 1). Deletion of the PGK-neo cassette by Southern blot analyses of tail gDNA as described for the ES cell clones. F1 female C57BL/6 mice. Chimeric mice were identified by PCR in myocardium, the progeny were genotyped by duplex PCR using the above-mentioned primers 5′-CGGTGGAATGATACTAGCAGGC-3′ and 5′-AGTGGTAGTGATGACTAGCAGCT-3′ (Fig. 1). Deletion of the PGK-neo cassette by Southern blot analyses of tail gDNA as described for the ES cell clones. F1 female C57BL/6 mice. Chimeric mice were identified by PCR

Our floxed iPLA2γ allele (abbreviated g/g′) mice were next crossed with an αMHC-MerCreMer mouse line (stock no. 005657, The Jackson Laboratory), which produces a tamoxifen-inducible Cre recombinase in myocardium. The progeny were genotyped by duplex PCR using the above-mentioned iPLA2γ-specific primers combined with Cre-specific primers 5′-CGGTGGAATGATACTAGCAGGC-3′ and 5′-AGTGGTAGTGATGACTAGCAGCT-3′ (Fig. 1). Transgenic αMHC-MerCreMer and g/g′ mice were backcrossed onto a C57BL/6 background for at least four generations prior to generating double transgenic αMHC-MerCreMer/g/g′ mice. Myocardial iPLA2γ gene ablation was induced in 1.5–3-month-old αMHC-MerCreMer/g/g′ mice by intraperitoneal tamoxifen injections (30 μg/gm body weight) twice daily for 2 consecutive days. Initially, two control groups of 3-month-old mice (one group possessing only the functional floxed iPLA2γ alleles (g/g′) and a second group bearing the inducible Cre transgene but no loxP sites) were identically treated with tamoxifen. This dosage of tamoxifen in 3-month-old mice induced a level of Cre-recombinase that produces no observable pathology in controls but was sufficient to attain a null iPLA2γ gene.

**Mass Spectrometric Analyses of Eicosanoids, Docosanoids, and Oxidized Metabolites of Linoleic Acid**—Mass spectrometric analyses of signaling eicosanoids, docosanoids, and oxlams were performed using a charge-switch strategy by derivatization with N-(4-aminomethylphenyl) pyridinium (AMPP) and subsequent LC-MS/MS with MRM and accurate mass determination of diagnostic product ions as described previously (36).

**MDMS-SL Analyses**—Lipidomic analyses of WT and CMIpla2γKO mouse myocardium were performed as described previously (22, 23). Lipid extracts were reconstituted with 1:1 (v/v) CHCl3/CH3OH, flushed with nitrogen and stored at −20 °C prior to electrospray ionization-MS using a TSQ Quantum Ultra Plus triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an automated nanospray apparatus (Advion Biosciences Ltd., Ithaca, NY) and customized sequence subroutine operated under Xcalibur software. Enhanced MDMS-SL analysis of cardiolipins was performed with a mass resolution setting of 0.3 Thomson using the M +1/2 isotopologue approach as we described previously (31, 59).

**Isolation of Mitochondria**—Mice were euthanized by cervical dislocation, and their hearts were removed and placed in ice-cold mitochondria isolation buffer (MIB: 0.21 M mannitol, 0.07 M sucrose, 0.1 M K-EDTA, 10 mM Tris-HCl, 1 mM EGTA, 0.5% BSA, pH 7.4) in a Petri dish on ice. Heart tissue was immediately diced into small pieces with a razor blade and transferred to a 10-ml Potter-Elvehjem tissue grinder with 5 ml of MIB. The tissue was homogenized using a rotorized homogenizer with a Teflon pestle set at 120 rpm. The homogenate was then diluted to 10 ml with MIB and centrifuged for 7 min at 850 × g. The supernatant was carefully collected and centrifuged at 10,000 × g for 10 min. The final pellet was resuspended in MIB with no BSA.

**High Resolution Mitochondrial Respirometry**—High resolution respirometry was performed using an OROBOROS® Oxgraph 2K (Innsbruck, Austria) as described previously (23). Respiration was started by the addition of palmitoylcarnitine (20 μM)/malate (5 mM), pyruvate (5 mM)/malate, or pyruvate/glutamate (10 mM)/malate (state 2) followed by sequential addition of ADP (1.25 mM) (state 3), succinate (5 mM) (state 3 Max), rotenone (0.5 μM), oligomycin (2.5 μM) (state 4), and antimycin A (3.75 μM). For measurement of ATP production, a 10-μl aliquot was collected from the respirometry chamber during state 3 respiration for 3 min following addition of ADP, mixed with an equal volume of DMSO, and stored at −80 °C for subsequent measurement of ATP synthesis using an ATP determination kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Finally, the ATP/O (P/O) ratio was determined by ATP production and O2 consumption during state 3 respiration.

**Mitochondrial Membrane Potentiometry**—Mitochondrial membrane potential (ΔΨm) measurement was performed using OROBOROS® Oxgraph 2K equipped with a TTP ion-selective electrode. Mitochondria isolated from C57BL/6J mice (4–5 months of age) were placed into a chamber containing a buffer solution of 0.23 mM mannitol, 0.07 mM sucrose, 3 mM HEPES, pH 7.2, 5 mM succinate, and 2 μM TPP-Cl at 30 °C. 0.1 mM KH2PO4 and oxidized fatty acids (1 μM 12-HETE, 20-HETE, 15-19 HETEs, 4-15 DHTEs, and 0-4 20-HETEs) were added to the chamber at a concentration of 2 μM. Mitochondria were added to the chamber at a concentration of 2 μM and incubated for 15 min before the ΔΨm measurement.
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14.15-EET, PGE$_2$, 9-oxoODE, or 8-HDoHE) or ethanol vehicle for control were added to the chamber. CaCl$_2$ was sequentially injected at 4-min intervals to 10, 20, 40, 60, and 80 $\mu$m final concentration. Mitochondrial membrane potential was calculated by following the instructions provided by the manufacturer (OROBOROS INSTRUMENTS Corp.).

Mitochondrial Swelling Assays—For determination of mPTP opening, isolated mitochondria from wild-type and CmiPLA$_2$$\gamma$KO mouse hearts were placed in mitochondrial swelling buffer (3 mM HEPES, pH 7.0, containing 0.23 M mannitol, 70 mM sucrose, 5 mM succinate, 1.25 mM rotenone, and 2 mM KH$_2$PO$_4$). 70 $\mu$g of mitochondria were placed in a 96-well plate with either ethanol vehicle alone (1%), 0.5 or 2 $\mu$m palmitic acid, and mitochondrial swelling was initiated by addition of 150 $\mu$m CaCl$_2$ (final) with comparisons with the addition of 10 $\mu$m EGTA as control. Decreases in absorbance (540 nm) are indicative of swelling of the mitochondria by opening of the mPTP and were monitored every 15 s using a SpectraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA) (9).

Myocardial Ischemia Reperfusion Studies—The methods of Weinheimer et al. (60) were used. Mice were subjected to reversible left anterior descending (LAD) coronary artery occlusion to induce ischemia for 30 min, followed by 24 h of reperfusion. Briefly, mice were anesthetized with a mixture of ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg), surgically prepared, and ventilated. After thoracotomy, the LAD artery was identified, and a 9-0 polypropylene suture was placed under the LAD artery. The heart was then wrapped in Saran wrap and placed in a 20 °C freezer for 10 min. The ventricles were then cut with a scalpel in 1–2-mm transverse sections, and the slices were photographed on both sides to identify the perfused myocardium. The slices were stained by immersion with 1% triphenyltetrazolium chloride (TTC) (in phosphate buffer, pH 7.4, 37 °C), which forms an insoluble red formazan product in the presence of active dehydrogenase enzymes. The slices were weighed and re-photographed at low magnification on both sides. The images from dye perfusion (area-at-risk) and TTC staining were digitized to permit computerized videoplanimetry of TTC stained and unstained tissue as well as the area perfused and non-perfused with Phthalo dye on the surface of each slice. The percentage of the surface area-at-risk that was infarcted was averaged for each group of mice, and the degree of infarction was calculated as a percentage of the area-at-risk.

Miscellaneous Procedures—Standard methods were used for SDS-PAGE and Western analyses. Protein concentration was measured by a Bradford assay (Bio-Rad) or bicinchoninic acid assay (Thermo Scientific) utilizing bovine serum albumin as standard. Northern and Southern analyses were performed as described previously (22).

Statistics—Comparisons between the WT and CmiPLA$_2$$\gamma$KO groups studied were made using a two-tailed Student’s $t$ test. A value of $p < 0.05$ was considered significant. All data are reported as the means ± S.E. unless otherwise noted.

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Cardiac Myocyte-specific Knock-out of Calcium-independent Phospholipase A2\(^\gamma\) (iPLA\(^\gamma\)) Decreases Oxidized Fatty Acids during Ischemia/Reperfusion and Reduces Infarct Size

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