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_2γ (iPLA_2γ) Decreases Oxidized Fatty Acids during Ischemia/Reperfusion and Reduces Infarct Size*

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Calcium-independent phospholipase A_2γ (iPLA_2γ) 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_2γ in cardiac myocytes, we generated cardiac myocyte-specific iPLA_2γ knock-out (CMiPLA_2γKO) mice by removing the exon encoding the active site serine (Ser-477). Hearts of CMiPLA_2γKO mice exhibited normal hemodynamic function, glycerophospholipid molecular species composition, and normal rates of mitochondrial respiration and ATP production. In contrast, CMiPLA_2γKO 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_2γKO 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_2γKO 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_2γ, these results are consistent with salvage of myocardium after I/R by iPLA_2γ 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) 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 reoxygenation 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_2γ (iPLA_2γ; also known as PNPLA8) that was membrane-associated, present in multiple tissues, and possessed
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multiple discrete isoforms (18). Further studies demonstrated that iPLA$_2$γ transcription was tightly regulated through multiple complex mechanisms (19). Through immunohistochemistry and cardiac myocyte-specific expression, iPLA$_2$γ was shown to be localized to mitochondrial and peroxisomal compartments. Transgenic expression of iPLA$_2$γ resulted in the dramatic increase of 2-arachidonoyl lysophosphatidyicholine and 2-docosahexaenoyl lysophosphatidyicholine in cardiac myocytes (19, 20). Later studies also identified iPLA$_2$γ in the endoplasmic reticulum (21). To begin the mechanistic dissection of the roles of iPLA$_2$γ in biological function in health and disease, we generated a germ line knock-out of iPLA$_2$γ in mice (iPLA$_2$γ KO) (22–24). These studies revealed that iPLA$_2$γ loss of function dramatically reduced the opening of the mitochondrial permeability transition pore (mPTP) in liver mitochondria and that calcium challenge of myocardial mitochondria obtained from the iPLA$_2$γ KO mouse markedly decreased the production of inflammatory eicosanoids in comparison with wild-type mice. However, germ line iPLA$_2$γ 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$_2$γ knock-out mice (CMiPLA$_2$γKO) by inserting flox sites proximal and distal to the active site serine of iPLA$_2$γ (Ser-477 in exon 5) and subsequently excising 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$_2$γ on ischemia/reperfusion in vivo.

The regiospecificity of iPLA$_2$γ toward phospholipid substrates is atypical among mammalian PLA$_2$ 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$_2$γ 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$_2$γ serves predominantly as a PLA$_1$, 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$_2$γ 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$_2$γKO mice to investigate iPLA$_2$γ-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 demonstrated that myocardial loss of iPLA$_2$γ 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$_2$γ-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$_2$γ Knock-out Mice—To definitively identify the mechanistic importance of iPLA$_2$γ in cardiac myocytes, we engineered an inducible cardiac myocyte-specific knock-out of iPLA$_2$γ. Because of the presence of multiple transcriptional start sites in iPLA$_2$γ, 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$_2$γ allele in multiple tissues of the f/f mouse and PCR analyses for the identification of ablation of the PGK-neo cassette and iPLA$_2$γf/fCre$^+$ in the iPLA$_2$γ conditional KO mice are shown in Fig. 1. Northern and Western analyses demonstrated the specific ablation of iPLA$_2$γ in heart but not in other tissues in the CMiPLA$_2$γKO mouse (Fig. 1, E and F).

Demonstration That the Majority of iPLA$_2$γ Activity in Myocardium Is Present in Cardiac Myocytes and Discrete Tissue Distributions of iPLA$_2$γ 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$_2$γ activity and protein, the cell type of origin of iPLA$_2$γ is not known with certainty. Comparisons of WT Cre$^+$ with CMiPLA$_2$γKO mice definitively demonstrate that the overwhelming majority of iPLA$_2$γ 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$_2$γ isoforms (e.g. 88, 74, 63, and 52 kDa), which were previously identified by germ line knock-out and transgenic overexpression of iPLA$_2$γ (9, 19, 20). For example, note the predominance of the lower molecular mass iPLA$_2$γ isoforms (50–60 kDa) in liver in comparison with myocardium and brain. Collectively, these results demonstrate that iPLA$_2$γ in myocardium is predominantly located in cardiac myocytes and identify the tissue-specific distributions of different isoforms of iPLA$_2$γ.

Constitutional Characteristics of the CMiPLA$_2$γKO Mouse—In contrast to the global iPLA$_2$γ knock-out, which demonstrated a thin body habitus, decreased length, cognitive dysfunction, kyphosis, and decreased locomotor activity (22, 24), the CMiPLA$_2$γKO 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 CMIPLA\(_2\gamma\)KO 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 CMIPLA\(_2\gamma\)KO Mice—High resolution respirometry of myocardial mitochondria was performed to identify alterations in mitochondrial function and respiratory coupling efficiency in CMIPLA\(_2\gamma\)KO mice. To examine mitochondrial bioenergetic efficiency under different conditions, we utilized multiple substrates, including pyruvate/malate, palmitoyl-CoA/carnitine/malate, and pyruvate/glutamate/malate. Mitochondria from CMIPLA\(_2\gamma\)KO 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 O\(_2\) consumption during state 3 respiration, was not significantly different in WT versus CMIPLA\(_2\gamma\)KO mice (Fig. 2). These results demonstrate the ability of mitochondria from the CMIPLA\(_2\gamma\)KO to respire normally and efficiently synthesize ATP.

**FIGURE 1.** Cardiac myocyte-specific ablation of iPLA\(_2\gamma\) in mouse myocardium. A, a graphic representation of the iPLA\(_2\gamma\) conditional targeting strategy. Exons 4 and 5 (E4, E5) of the WT allele are depicted as open boxes, and the intronic sequence is represented as a solid line. PCR products generated for construction of the targeting vector with restriction sites used for cloning are as indicated. The targeting vector is shown with FLP sites (F) indicated as closed boxes flanking the PGK-neo cassette and IoxP sites indicated as triangle (L) flanking both the PGK-neo cassette (Neo) and E5. Below the targeting vector is a representation of the targeted allele in the conditional KO mouse. Breeding this mouse with an FLP recombinase mouse results in ablation of the PGK-neo cassette, generating the floxed allele. Finally, breeding with the Cre mouse results in ablation of E5, and the generation of the null allele is represented at the bottom.

B, a genomic Southern analysis of wild-type (WT) and Neo iPLA\(_2\γ\)\(^{+/+}\) mice shows the presence of only floxed iPLA\(_2\γ\) alleles in multiple tissues of the if/ if mouse. C, successful ablation of the PGK-neo cassette in the iPLA\(_2\γ\) conditional KO mouse. PCR analysis of tail DNA was utilized to identify mice from which the PGK-neo cassette had been ablated by crossing the conditional knock-out with a global FLP mouse. Lanes 1–4 identify heterozygous mice lacking the PGK-neo (Neo) cassette but have the floxed and WT alleles (iPLA\(_2\γ\)\(^{f/+}\)). Lane 5 identifies a heterozygous mouse having the PGK-neo allele (Neo). A mouse homozygous for the floxed allele was identified in lane 6 (iPLA\(_2\γ\)\(^{f/-}\)). D, PCR identification of heart-specific conditional KO Cre\(^\mathrm{−}\) mice. Tail PCR amplification of floxed (FL) and WT alleles along with Cre transgene (Cre) expression was used to identify iPLA\(_2\γ\)\(^{f/-}\) Cre\(^\mathrm{−}\) (lanes 2, 3, 5, and 9) and iPLA\(_2\γ\)\(^{f/+}\) Cre\(^\mathrm{−}\) (lanes 1, 7, and 8) mice. E, Northern analysis of RNA isolated from heart, kidney, and brain of WT and CMIPLA\(_2\γ\)KO (KO) mice. The results demonstrate the tissue-specific ablation of iPLA\(_2\γ\) in heart but not kidney or brain of the conditional knock-out. F, Western analysis of iPLA\(_2\γ\) expression in WT and CMIPLA\(_2\γ\)KO (KO) tissues. Lanes: heart, skeletal muscle (SkeM), liver, and brain.
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**TABLE 1**

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

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

<table>
<thead>
<tr>
<th>Type</th>
<th>Body wt</th>
<th>HR</th>
<th>LVPWd</th>
<th>IVSd</th>
<th>LVIDd</th>
<th>LVPW</th>
<th>IVS</th>
<th>LVID</th>
<th>LVM</th>
<th>LVMI</th>
<th>RWT</th>
<th>FS</th>
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<td>WT</td>
<td>30.5 ± 1.7</td>
<td>638.7 ± 51.8</td>
<td>0.93 ± 0.04</td>
<td>3.59 ± 0.25</td>
<td>1.56 ± 0.20</td>
<td>1.67 ± 0.14</td>
<td>1.60 ± 0.20</td>
<td>124.7 ± 9.4</td>
<td>4.12 ± 0.28</td>
<td>0.54 ± 0.05</td>
<td>55.3 ± 5.0</td>
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<tr>
<td>KO</td>
<td>31.2 ± 2.9</td>
<td>651.0 ± 11.8</td>
<td>0.95 ± 0.06</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>
<td>0.51 ± 0.06</td>
<td>57.2 ± 4.9</td>
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**FIGURE 2.** High resolution respirometry of mitochondria from wild-type and cardiac myocyte-specific iPLA$_2$$\gamma$ knock-out mice. Heart mitochondria isolated from wild-type Cre$^+$ (WT) and cardiac myocyte-specific iPLA$_2$$\gamma$ knock-out (KO) mice were utilized to measure oxygen consumption and ATP production in the presence of the indicated substrates as described under “Experimental Procedures.” Oxygen consumption rates are expressed as nmol of O$_2$/min/mg protein in the presence of: A, pyruvate and malate (Pyr M); B, palmitoylcarnitine and malate (Pc M); C, pyruvate, glutamate, and malate (Pyr G M); D, ADP (1.25 mM), succinate (5 mM), rotenone (Rot, 0.5 $\mu$M), and oligomycin (O, 2.5 $\mu$M) were sequentially added. D, ATP to oxygen (P/O) ratios for WT and CMiPLA$_2$$\gamma$KO (KO) mice were determined by measurement of ATP production and O$_2$ consumption during state 3 respiration in the presence of ADP for 3 min. Data are presented as means ± S.E. (n = 3–4/group) from male mice 6 months of age. No significant differences in mitochondrial respiration and P/O ratios were found in WT versus CMiPLA$_2$$\gamma$KO mouse myocardium as determined by Student’s test.

**Lipidomic Analyses of Myocardium from WT and CMiPLA$_2$$\gamma$KO Mice**—To determine alterations in the myocardial lipidome of WT versus CMiPLA$_2$$\gamma$KO mice, we utilized multidimensional mass spectrometry-based shotgun lipidomics (MDMS-SL) (26). The major phospholipid classes in myocardium are choline and ethanolamine glycerophospholipids. Examination of choline glycerophospholipids demonstrated the presence of over 45 molecular species in murine myocardium that were largely composed of diacyl (D) phosphatidylcholine molecular species containing D16:0–22:6/D18:2–20:4, D18:0–22:6, D16:0–20:4/D18:2–18:2, D18:2–22:6, and D18:0–20:4/D18:2–20:2 in both the WT and the CMiPLA$_2$$\gamma$KO mice. Mirror plots of choline glycerophospholipids from averaged tandem mass spectra collected from six different mice demonstrated nearly identical profiles of individual molecular species (Fig. 3A). Similarly, MDMS-SL analysis of ethanolamine glycerophospholipids demonstrated over 30 diacyl phosphatidyethanolamine molecular species largely composed of D18:0–22:6, D16:0–22:6, D18:1–22:6, and D18:0–20:4 molecular species as well as 20 plasmalogen (P) ethanolamine phospholipid molecular species largely composed of P16:0–22:6, P18:1–20:4/P16:0–22:5, P18:0–22:6, and P18:1–22:6 molecular species. Mirror plots of ethanolamine glycerophospholipids from averaged mass spectra from six separate mice did not identify any significant differences between WT and CMiPLA$_2$$\gamma$KO mouse hearts (Fig. 3B). Triglyceride analysis by MDMS-SL demonstrated nearly identical total amounts of triglycerides and no differences in their molecular species composition in WT versus CMiPLA$_2$$\gamma$KO mice (Fig. 3C). Negative ion mass spectra did not reveal any significant differences in phosphatidylinositol, phosphatidylserine, 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 CMiPLAγKO myocardium, were nearly identical. Modest decreases in the levels of 18:2–18:2–18:2–22:6 CL and 18:2–18:2–18:2–18:2–22:6–22:6 CL (m/z 747.5 and m/z 771.5, respectively, in Fig. 4A) were present in CMiPLAγKO mice (Fig. 4B).

Mass Spectrometric Analysis of Myocardial Eicosanoids, Docosanoids, and Oxidized Linoleic Acids—Previous studies have demonstrated the important roles of iPLAγ 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 CMiPLAγKO mice (Fig. 5). These include decreases in prostaglandins, 11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid (11-HETE), 12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), and 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE) as well as increased levels of 14(15)-epoxy-5Z,8Z,11Z-eicosatetraenoic acid (14,15-EET). Similarly, CMiPLAγKO mice had decreased levels of all observable oxidized linoleic acid metabolites (oxlams) except 9-oxo-10E,12Z-octadecadienoic acid (9-oxoODE) and had significant decreases in 22-carbon oxidized fatty acids, including 7,8R,17S-trihydoxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid (RVD-1), 19,20-di-hydroxy-4Z,7Z,10Z,13E,16Z-docosapentaenoic acid, and 7-hydroxy-4Z,8E,10Z,13Z,16Z,19Z-docosahexaenoic acid (7-HDoHE) (Fig. 5). These results identify iPLAγ as a prominent enzymic mediator for the generation of signaling oxidized fatty acids in myocardium.

Decreased Susceptibility of mPTP Opening in Myocardium from CMiPLAγKO Mice in Comparison with Wild-type Mice—Recent work in our laboratory led to the identification of iPLAγ as an important modulator of the Ca<sup>2+</sup>-induced opening of the mPTP in mitochondria isolated from liver (9). To determine the contribution of iPLAγ to the opening of the cardiac myocyte mPTP, we compared Ca<sup>2+</sup>-induced mitochondrial swelling in WT versus CMiPLAγKO 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 CMiPLAγKO mice (Fig. 6). Ca<sup>2+</sup>-induced swelling of mitochondria from both WT and CMiPLAγKO mice was demonstrated to be cyclophilin D (also known as peptidyl-prolyl cis-trans isomerase F)-dependent through nearly complete inhibition by 2 μM cyclosporin A. No observable differ-
**FIGURE 4.** Mass spectrometric analysis of cardiolipin molecular species in wild-type and cardiac myocyte-specific iPLA$_2$ 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 iPLA$_2$ 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 $M^+$/H$_{11001}$ 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 CMiPLA$_2$ KO (KO) mouse myocardium ($n=6$). **, $p<0.01$.

**FIGURE 5.** Cardiac oxidized fatty acids in wild-type and cardiac myocyte-specific iPLA$_2$ KO myocardial tissue. Myocardial tissue was isolated from wild-type (WT) and CMiPLA$_2$ KO (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; o xoODE, oxo-octadecadienoic acid; EpOME, epoxyoctadecaenoic acid; LT$_{BH_2}$, leukotriene B$_2$; TXB, thromboxane B; PG, prostaglandin; RVD, resolvin; DiHDHE, dihydroxydocosahexaenoic acid; DiDHPA, dihydroxydocosapentaenoic acid; and HDHE, hydroxydocosahexaenoic acid.
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Sanoids, 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 ($\Delta \Psi_{\text{mt}}$) 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 $\Delta \Psi_{\text{mt}}$ (approximately −160 mV) became less negative rapidly upon sequential calcium additions in the presence of either vehicle alone (control), 14,15-EET, PGE$_2$, or 9-oxoODE, 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, trifluoroethoxy carbonylcyanide phenylhydrazone (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.
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, 37, 38). 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 the mitochondrial matrix during ischemia (9, 39, 40). Prolonged Ca\(^{2+}\) overload, accumulation of inorganic phosphate, and induction of the calcium-induced opening of the mPTP are important regulator of the calcium-induced opening of the mPTP (9), myocardial mitochondria from myocardial ischemia (46).

Oxidized fatty acid metabolites from non-ischemic and ischemic border zones in non-ischemic and ischemic border zones were also demonstrated. This study demonstrates the unanticipated and dramatic increases in the production of multiple identified oxidized metabolites in ischemic border zones compared with non-ischemic zones. Furthermore, we demonstrated that submicromolar concentrations of free palmitic acid restored mPTP opening that was attenuated by loss of myocardial iPLA\(_2\). This is particularly relevant because iPLA\(_2\) has a marked position specificity for hydrolysis of diacyl phospholipids containing 18:2 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-lysophosphatidic acid, 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 important because iPLA\(_2\) has a marked position specificity for hydrolysis of diacyl phospholipids containing 18:2 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-lysophosphatidic acid, respectively, in the mitochondrial membrane (25).

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 iPLA\(_2\) 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 iPLA\(_2\) as an important regulator of the calcium-induced opening of the mPTP in liver mitochondria (9), myocardial mitochondria from the CMiPLA\(_2\)KO mouse demonstrate the regulatory role of iPLA\(_2\) 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 iPLA\(_2\). This is particularly relevant because iPLA\(_2\) has a marked position specificity for hydrolysis of diacyl phospholipids containing 18:2 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-lysophosphatidic acid, 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 important because iPLA\(_2\) has a marked position specificity for hydrolysis of diacyl phospholipids containing 18:2 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-lysophosphatidic acid, respectively, in the mitochondrial membrane (25).
diac myocyte iPLA$_2^\gamma$ in facilitating mPTP opening and the resultant increase in infarct size.

In addition to iPLA$_2^\gamma$-mediated release of saturated fatty acids from phospholipid pools, we previously reported marked iPLA$_2^\gamma$-dependent production of cardiac eicosanoids in the myocardium by utilizing cardiac myocyte-specific overexpression of iPLA$_2^\gamma$ and global iPLA$_2^\gamma$ knock-out mice (34). Our previous findings suggest that iPLA$_2^\gamma$-generated 2-polysaturated fatty acyl lysolipids and their downstream hydrolytic products (non-esterified polysaturated 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

FIGURE 8. Facilitation of Ca$^{2+}$-mediated mitochondrial membrane depolarization by oxidized polyunsaturated fatty acids. A and B, mitochondria were isolated from C57BL/6J mice (4–5 months of age) and 0.125 mg of protein/ml of mitochondria (mito) were placed into an OROBOROS Oxygraph 2K chamber containing a buffer solution of 0.23 M mannitol, 0.07 M sucrose, 3 mM HEPES, pH 7.4, 5 mM succinate, and 2 μM tetraphenylphosphonium chloride (TPP$^-$). The final concentrations of 0.1 mM KH$_2$PO$_4$ (KPi) and 1 mM oxidized fatty acids, including 12-HETE, 20-HETE, 14,15-EET, PGE$_2$, 9-oxoODE, and 8-HDoHE, or ethanol vehicle (control) were added to the chamber at the indicated times (arrows). CaCl$_2$ was sequentially added at 4-min intervals to the final concentrations of 10, 20, 40, 60, and 80 μM. Mitochondrial membrane potential (ΔΨ$_{mt}$) was determined by the concentration of extramitochondrial TPP$^+$ measured with an ion-selective electrode. Maximum depolarization of mitochondria was observed in the presence of 1.5 μM FCCP. *, p < 0.05, and **, p < 0.01 by Student’s test when compared with the controls (n = 3–4). C, representative potentiometric tracings are shown.
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 PGE\(_2\), 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\(_2\) 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 resulting 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\(_2\) 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\(_2\) 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\(_2\)'s 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\(_2\)-KO mice. Radiolabeled nucleotides ([\(\alpha\)-\(^32\)P]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\(_2\) was obtained from Sigma. Anti-iPLA\(_2\) 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. Mice
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'-TATAGAGATGCACAAACCAGTGAAGCGCG-3' and 5'-AGTTGGTAGTGTATGACTAGCACT-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 iPLA₂γ knockout generation

**Isolation of Mitochondria**—Mice were euthanized by cervical dislocation, and their hearts were removed and placed in ice-cold myocardium isolation buffer (MIB: 0.21 M mannitol, 0.07 M sucrose, 3 mM HEPES, buffer solution of 0.23 M mannitol, 0.07 M sucrose, 3 mM HEPES, pH 7.2, 5 mM succinate, and 2 mM KH₂PO₄ and oxidized fatty acids (1 M) (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 (ΔΨ₀) measurement was performed using OROBOROS® Oxygraph 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.
For determination of mPTP opening, isolated mitochondria from wild-type and CmiPLA$_2$KO mouse hearts were placed in mitochondrial swelling buffer (3 mM HEPES, pH 7.0, containing 0.23 mM mannitol, 70 mM sucrose, 5 mM succinate, 1.25 mM rotenone, and 2 mM KH$_2$PO$_4$). 70 µg of mitochondria were placed in a 96-well plate with either ethanol vehicle alone (1%), 0.5 or 2 µM palmitic acid, and mitochondrial swelling was initiated by addition of 150 µM CaCl$_2$ (final) with comparisons with the addition of 10 µM 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).

Mitochondrial Swelling Assays—For determination of mPTP opening, isolated mitochondria from wild-type and CmiPLA$_2$KO mouse hearts were placed in mitochondrial swelling buffer (3 mM HEPES, pH 7.0, containing 0.23 mM mannitol, 70 mM sucrose, 5 mM succinate, 1.25 mM rotenone, and 2 mM KH$_2$PO$_4$). 70 µg of mitochondria were placed in a 96-well plate with either ethanol vehicle alone (1%), 0.5 or 2 µM palmitic acid, and mitochondrial swelling was initiated by addition of 150 µM CaCl$_2$ (final) with comparisons with the addition of 10 µM 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 prepped, and ventilated. After thoracotomy, the LAD artery was identified, and a 9-0 polypropylene suture was passed under the LAD artery. A knot was tied over a 1-mm section of PE-10 tubing placed directly over the vessel to create the occlusion. Ischemia was confirmed by an absence of blood flow and verified visually and by the presence of ST elevations on the electrocardiogram. The chest wall was approximated and covered with moistened gauze during the 30-min ischemia time. Reperfusion was induced by cutting the knot on top of the polyethylene tubing or simply removing the tubing piece. This allowed release of the occlusion, and resolution of ST segment elevations was observed. The chest was then closed, and mice were monitored closely for warmth and recovery until the end of the reperfusion time. After 24 h, the mice were given heparin (100 units, i.p.) and re-anesthetized with ketamine/xylazine, and the sternotomy was re-opened to expose the heart. The heart was excised andperfused retrograde through a catheter placed in the aorta. After slow perfusion of 1–2 ml of warmed phosphate-buffered saline (37 °C) to remove blood, the LAD was re-occluded with the 8-0 suture, and the heart was perfused with 5% Phthalo blue dye (Heucotech Ltd., Fairless Hill, PA) in saline to delineate the previously occluded and reperfused vascular bed (area-at-risk). The portion of the LV supplied by the occluded coronary was identified by the absence of blue dye. 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.

Statistics—Comparisons between the WT and CmiPLA$_2$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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References
iPLA$_2$γ Knock-out Decreases Eicosanoids during I/R


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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