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

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Calcium-independent phospholipase A$_2$γ (iPLA$_2$γ) is a mitochondrial enzyme that produces lipid second messengers in cardiac myocytes, we generated cardiac myocyte-specific iPLA$_2$γ knock-out (CMiPLA$_2$γKO) mice by removing the exon encoding the active site serine (Ser-477). Hearts of CMiPLA$_2$γKO mice exhibited normal hemodynamic function, glycerophospholipid molecular species composition, and normal rates of mitochondrial respiration and ATP production. In contrast, CMiPLA$_2$γKO 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$_2$γKO 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$_2$γKO 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$_2$γ, these results are consistent with salvage of myocardium after I/R by iPLA$_2$γ 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$_2$γ (iPLA$_2$γ; 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 iPLA₂γ transcription was tightly regulated through multiple complex mechanisms (19). Through immunohistochemistry and cardiac myocyte-specific expression, iPLA₂γ was shown to be localized to mitochondrial and peroxisomal compartments. Transgenic expression of iPLA₂γ resulted in the dramatic increase of 2-arachidonoyl lysophosphatidylcholine and 2-docosahexaenoyl lysophosphatidylcholine in cardiac myocytes (19, 20). Later studies also identified iPLA₂γ in the endoplasmic reticulum (21). To begin the mechanistic dissection of the roles of iPLA₂γ in biological function in health and disease, we generated a germ line knock-out of iPLA₂γ in mice (iPLA₂γKO) (22–24). These studies revealed that iPLA₂γ 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 iPLA₂γKO mouse markedly decreased the production of inflammatory eicosanoids in comparison with wild-type mice. However, germ line iPLA₂γ 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 iPLA₂γ knock-out mice (CMiPLA₂γKO) by inserting flox sites proximal and distal to the active site serine of iPLA₂γ (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 iPLA₂γ on ischemia/reperfusion in vivo.

The regiospecificity of iPLA₂γ 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, iPLA₂γ 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 polunsaturated, iPLA₂γ serves predominantly as a PLA1 releasing the saturated fatty acid from the sn-1 position and generating 2-polyunsaturated fatty acyl lysophospholipids. 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 polunsaturated 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 iPLA₂γ 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, lysophospholipids, acyl-CoAs, and acylcarnitines) that accumulate during myocardial ischemia and are released during reperfusion.

In this study, we utilized CMiPLA₂γKO mice to investigate iPLA₂γ-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 iPLA₂γ 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 iPLA₂γ-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 iPLA₂γ Knock-out Mice—To definitively identify the mechanistic importance of iPLA₂γ in cardiac myocytes, we engineered an inducible cardiac myocyte-specific knock-out of iPLA₂γ. Because of the presence of multiple transcriptional start sites in iPLA₂γ, 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 iPLA₂γ allele in multiple tissues of the f/f mouse and PCR analyses for the identification of ablation of the PGK-neo cassette and iPLA₂γfl/Cre+ in the iPLA₂γ conditional KO mice are shown in Fig. 1. Northern and Western analyses demonstrated the specific ablation of iPLA₂γ in heart but not in other tissues in the CMiPLA₂γKO mouse (Fig. 1, E and F).

Demonstration That the Majority of iPLA₂γ Activity in Myocardium Is Present in Cardiac Myocytes and Discrete Tissue Distributions of iPLA₂γ 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 iPLA₂γ activity and protein, the cell type of origin of iPLA₂γ is not known with certainty. Comparisons of WT Cre+ with CMiPLA₂γKO mice definitively demonstrate that the overwhelming majority of iPLA₂γ 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 iPLA₂γ isoforms (e.g. 88, 74, 63, and 52 kDa), which were previously identified by germ line knock-out and transgenic overexpression of iPLA₂γ (9, 19, 20). For example, note the predominance of the lower molecular mass iPLA₂γ isoforms (50–60 kDa) in liver in comparison with myocardium and brain. Collectively, these results demonstrate that iPLA₂γ in myocardium is predominantly located in cardiac myocytes and identify the tissue-specific distributions of different isoforms of iPLA₂γ.

Constitutional Characteristics of the CMiPLA₂γKO Mouse—In contrast to the global iPLA₂γ knock-out, which demonstrated a thin body habitus, decreased length, cognitive dysfunction, kyphosis, and decreased locomotor activity (22, 24), the CMiPLA₂γKO 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 CMiPLA2 γKO 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 CMiPLA2 γKO Mice—High resolution respirometry of myocardial mitochondria was performed to identify alterations in mitochondrial function and respiratory coupling efficiency in CMiPLA2 γKO mice. To examine mitochondrial bioenergetic efficiency under different conditions, we utilized multiple substrates, including pyruvate/malate, palmitoyl carnitine/malate, and pyruvate/glutamate/malate. Mitochondria from CMiPLA2 γKO 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 CMiPLA2 γKO mice (Fig. 2). These results demonstrate the ability of mitochondria from the CMiPLA2 γKO to respire normally and efficiently synthesize ATP.
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**TABLE 1**

| Type  | Body wt | HR | LV PWd | IVSD | LVIDd | LV PW | IVS | LVID | LVM | LVMI | RWT | FS  |
|-------|---------|----|--------|------|-------|-------|-----|------|-----|------|-----|-----|
| WT    | 30.5±1.7 | 638.7±1.8 | 0.93±0.06 | 0.99±0.04 | 3.59±0.25 | 1.56±0.20 | 1.67±0.14 | 1.60±0.20 | 124.7±9.4 | 41.2±0.28 | 0.54±0.05 | 55.3±5.0 |
| KO    | 31.2±2.9 | 651.0±11.8 | 0.95±0.06 | 0.96±0.03 | 3.72±0.25 | 1.63±0.14 | 1.68±0.12 | 1.59±0.24 | 131.1±7.6 | 42.2±0.34 | 0.51±0.06 | 57.2±4.9 |

**FIGURE 2.** High resolution respirometry of mitochondria from wild-type and cardiac myocyte-specific iPLA$_2$γ knock-out (KO) mice. Heart mitochondria isolated from wild-type Cre$^+$ (WT) and cardiac myocyte-specific iPLA$_2$γ 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 of protein in the presence of: A, pyruvate and malate (Pyr M); B, palmitoyl carnitine and malate (Pc M); C, pyruvate, glutamate, and malate (Pyr G M). ADP (1.25 mM), succinate (5 mM), rotenone (Rot, 0.5 μM), and oligomycin (O, 2.5 μM) were sequentially added. D, ATP to oxygen (P/O) ratios for WT and CMiPLA$_2$γKO (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$_2$γKO mouse myocardium as determined by Student’s test.

**Lipidomic Analyses of Myocardium from WT and CMiPLA$_2$γKO Mice**—To determine alterations in the myocardial lipidome 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, 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 CMiPLA₂γKO myocardium, were nearly identical. Modest decreases in the levels of 18:2–18:2–18:2–22:6 CL and 18:2–18:2–18:2–22:4 CL were observed in CMiPLA₂γKO myocardium. CMiPLA₂γKO mouse myocardium 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 7S,8Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid (RVD-1), 19,20-di-hydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid, and 7-hydroxy-4Z,8E,10Z,13Z,16Z-docosahexaenoic acid (7-HDoHE) (Fig. 5). These results identify iPLA₂γ as a prominent enzymic mediator for the generation of signaling oxidized fatty acids in myocardium.

**Decreased Susceptibility of mPTP Opening in Myocardium from CMiPLA₂γKO Mice in Comparison with Wild-type Mice**—Recent work in our laboratory led to the identification of iPLA₂γ as an important modulator of the Ca²⁺-induced opening of the mPTP in mitochondria isolated from liver (9). To determine the contribution of iPLA₂γ to the opening of the cardiac myocyte mPTP, we compared Ca²⁺-induced mitochondrial swelling in WT versus CMiPLA₂γKO 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 CMiPLA₂γKO mice (Fig. 6). Ca²⁺-induced swelling of mitochondria from both WT and CMiPLA₂γKO 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 iPLA2 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 iPLA2 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+1/2 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 CMiPLA2 KO (KO) mouse myocardium (n = 6). **, p < 0.01.

**FIGURE 5.** Cardiac oxidized fatty acids in wild-type and cardiac myocyte-specific iPLA2 KO myocardial tissue. Myocardial tissue was isolated from wild-type (WT) and CMiPLA2 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; oxoODE, o xo-octadecadienoic acid; EpOME, epoxyoctadecenoic acid; LTb, leukotriene B; TXb, thromboxane B; PG, prostaglandin; RVD, resolvin; DihDoHE, dihydroxydocosahexaenoic acid; DihDPa, dihydroxydocosapentaenoic acid; and HDoHE, hydroxydocosa hexaenoic acid.
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 tetrathenylphosphonium (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-EE, PGE$_2$, 9-oxoODE, or 8-HDoHE, all of which were dramatically increased during I/R (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-EE, 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 $\mu$M calcium ion by dissipating the electric potential across the membrane resulting in no further depolarization upon addition of an uncoupling agent, trifluoromethoxy carbonyl cyanide 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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**FIGURE 7.** Oxidized metabolites of arachidonic acid, linoleic acid, and docosahexaenoic acid in non-ischemic and ischemic border zones of myocardium from wild-type and cardiac myocyte-specific iPLA$_2^\gamma$ knock-out mice following ischemia/reperfusion. Oxidized fatty acid metabolites from non-ischemic and ischemic myocardial zones of wild-type (WT) and CMiPLA$_2^\gamma$KO (KO) mice were extracted, isolated by solid phase extraction, derivatized with AMPP, and quantitated 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 C18 column. Significant decreases in the production of multiple identified oxidized metabolites of arachidonic acid (A and B), linoleic acid (C), and docosahexaenoic acid (D) in ischemic border zones are present as a result of cardiac myocyte-specific ablation of iPLA$_2^\gamma$. Significant increases in the production of multiple identified oxidized metabolites in ischemic border zones compared with non-ischemic zones were also demonstrated. Values presented are the means ± S.E. Comparisons were made using Student’s t test (n = 6). *, p < 0.05 versus WT non-ischemic. §, p < 0.05 when compared with WT, ischemic. Refer to Fig. 5 legend for oxylipin abbreviations.

**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$^{2+}$-induced opening of the mPTP that is facilitated by Ca$^{2+}$ activation of iPLA$_2^\gamma$ causes irreversible dissipation of the mitochondrial 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 iPLA$_2^\gamma$ 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 iPLA$_2^\gamma$ 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$_2^\gamma$ as an important regulator of the calcium-induced opening of the mPTP in liver mitochondria (9), myocardial mitochondria from the CMiPLA$_2^\gamma$KO mouse demonstrate the regulatory role of cardiac iPLA$_2^\gamma$ 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$_2^\gamma$. This is particularly relevant because iPLA$_2^\gamma$ 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-lysophosphatidylethanolamines, 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 Ca$^{2+}$ depletion and reactive oxygen species generation (47, 48) and by acting as an endogenous ionophore (49). Supporting these mechanisms, deletion of mitochondrial membrane-associated iPLA$_2^\gamma$ led to the remarkable and robust salvage of damaged regions of myocardium after I/R, which emphasizes a prominent role of car-
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
successful exclusion of false-positive identification through high mass accuracy analysis of informative product ions (36). Although the myocardial lipidome of CMiPLA₂γKO mice is relatively unaltered in comparison with WT, the decrease in numerous low abundance oxidized free fatty acids was evident in CMiPLA₂γKO mouse myocardium under basal conditions. The presence of large amounts of oxlams in WT murine myocardium was unanticipated and suggests their previously unknown roles in myocardial signaling. The observation that oxlams were so prominent suggests that their oxidation occurred predominantly in the mitochondrial compartment that is rich in 18:2 fatty acids esterified to cardiolipin. Moreover, the finding of dramatic increases in multiple oxidized lipid second messengers present in the infarct border zone after I/R, which were substantially reduced in the CMiPLA₂γKO mouse, identifies iPLA₂γ as the rate-determining step for the pathological production of these oxylipins during I/R injury.

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 (ΔΨₘ) in the presence of multiple oxidized lipid metabolites to determine their effects on Ca²⁺-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 Ca²⁺. In contrast, hydroxylated polyunsaturated fatty acids (e.g. 12-HETE, 20-HETE, and 8-HDöHE), 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 Ca²⁺ overload resulting in abnormal oxidative stress and mitochondrial dysfunction (44, 49). Therefore, the results of this study suggest that iPLA₂γ 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 iPLA₂γ 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 iPLA₂γ in the Ca²⁺-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 iPLA₂γ 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 CMiPLA₂γKO 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). Oxylipins, 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 iPLA₂γ was obtained from Sigma. Anti-iPLA₂γ 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 insertedloxP-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'-TATAGAGATGCCAACACCGATGGAAGCGGC-3' and 5'-AGTTGCTAGTGTATGACTGACT-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 Echocardiographic analyses were performed under light anesthesia as described previously (57, 58). Following euthanasia by cervical dislocation, and their hearts were removed and placed in ice-cold mitochondria isolation buffer (MIB: 0.23M mannitol, 0.07M sucrose, 3 mM HEPES, 0.1 mM 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® 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), 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 ℃ 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 (ΔΨ_m) measurement was performed using OROBOROS® Oxygraph 2K equipped with a TPP⁺ ion-selective electrode. Mitochondria isolated from C57BL/6 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 ℃. 0.1 mM KH₂PO₄ and oxidized fatty acids (1 μM 12-HETE, 20-HETE,
iPLA$_2^\gamma$ Knock-out Decreases Eicosanoids during I/R

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 CmipiLA$_2^\gamma$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 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 of the reperfusion time. After 24 h, the mice were given heparin (100 units, i.p.) and re-anesthetized with ketamine/xylazine, of the 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 CmipiLA$_2^\gamma$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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