GENETIC ABLATION OF CALCIUM-INDEPENDENT PHOSPHOLIPASE A₂γ (iPLA₂γ) ATTENUATES CALCIUM-INDUCED OPENING OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE AND RESULTANT CYTOCHROME C RELEASE

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Running Title: iPLA₂γ-mediated mPTP Opening

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Background: The composition and regulation of the mitochondrial permeability transition pore (mPTP) are incompletely understood.

Results: Calcium-induced mPTP opening was markedly inhibited in iPLA₂γ⁻/⁻ mice but was robustly activated by acyl-CoA in wild-type mice.

Conclusion: iPLA₂γ is a critical mechanistic participant in mPTP opening in a process that is modulated by cellular lipids.

Significance: iPLA₂γ in conjunction with acyl-CoA integrates mPTP opening with cellular bioenergetics.

SUMMARY

Herein, we demonstrate that calcium-independent phospholipase A₂γ (iPLA₂γ) is a critical mechanistic participant in the calcium-induced opening of the mitochondrial permeability transition pore (mPTP). Liver mitochondria from iPLA₂γ⁻/⁻ mice were markedly resistant to calcium-induced swelling in the presence or absence of phosphate in comparisons to wild-type littermates. Furthermore, the iPLA₂γ enantioselective inhibitor (R)-BEL was markedly more potent than (S)-BEL in inhibiting mPTP opening in mitochondria from wild-type liver in comparison to hepatic mitochondria from iPLA₂γ⁻/⁻ mice. Intriguingly, low micromolar concentrations of long chain fatty acyl-CoAs and the non-hydrolyzable thioether analog of palmitoyl-CoA markedly accelerated Ca²⁺-induced mPTP opening in liver mitochondria from wild-type mice. Addition of L-carnitine enabled the metabolic channeling of acyl-CoA through carnitine palmitoyl transferases (CPT I/II) and attenuated the palmitoyl-CoA-mediated amplification of calcium-induced mPTP opening. In contrast, mitochondria from iPLA₂γ⁻/⁻ mice were insensitive to fatty acyl-CoA mediated augmentation of calcium-induced mPTP opening. Moreover, mitochondria from iPLA₂γ⁻/⁻ mouse liver were resistant to Ca²⁺/β-butyldihydroperoxide (TBH) induced mPTP opening in comparison to wild-type littermates. In support of these findings, cytochrome c release from iPLA₂γ⁻/⁻ mitochondria was dramatically decreased in response to calcium in the presence or absence of either TBH or phenylarsine oxide (PAO) in comparisons to wild-type littermates. Collectively, these results identify iPLA₂γ as an important mechanistic component of the mPTP, define its downstream products as potent regulators of mPTP opening and demonstrate the integrated roles of mitochondrial bioenergetics and lipidomic flux in modulating mPTP opening promoting the activation of necrotic and necroapoptotic pathways of cell death.
The first committed step leading to mitochondrial-mediated necrotic cell death is the opening of the mitochondrial permeability transition pore (mPTP) (1-4). Opening of the mPTP results in mitochondrial depolarization, swelling and the release of cytochrome c, which collectively precipitate cell death through necrosis and necroapoptosis leading to cell dropout that ultimately compromises organ function (5-7). Although some of the components mediating mPTP opening and their mechanisms of regulation have been identified (2,8), the ensemble of molecular constituents that comprise the mPTP and the processes that regulate its opening are largely unknown.

Mitochondria play critical roles in cellular bioenergetics and signaling functions in which calcium is an important regulator. Mitochondrial matrix calcium is required for the opening of the mPTP, functioning as a permissive factor for all pore inducers including oxidative stress, acyl-CoA, phosphate, and adenine nucleotide depletion (9). One of the critical factors in the calcium-induced modulation of mPTP opening is the presence of phosphate ion (P). However, the mechanism by which phosphate ions regulate the opening of the mPTP is complex. Notably, the phosphate carrier (PiC) has been demonstrated to be a likely component of the mPTP, facilitating pore opening (10), but its role in pore formation and its functional interaction with other mPTP constituents remains at an elementary level of understanding.

Deleterious consequences of the opening of the mPTP include the release of membrane impermeable reactive oxygen species (ROS), matrix antioxidants (e.g. glutathione) and pro-apoptotic factors (e.g. cytochrome c, apoptosis inducing factor, endonuclease G, etc.) into the cytosol that precipitate the activation of multiple proteolytic cascades that lead to necrosis and/or necroapoptosis (2,11). Excessive generation of ROS is known to have multiple deleterious effects on mitochondrial function including peroxidation of highly unsaturated cardiolipin (CL) in the inner membrane (12,13). Numerous downstream apoptotic events have been demonstrated to be initiated by the release of cytochrome c from mitochondria such as caspase activation, the interaction with cytosolic apoptosis protease activating factor-1 (APAF-1) inducing the formation of the apoptosome, and further propagation of mitochondrial damage with the localized release of calcium ion, free radicals and other toxic moieties (11,14). It has been proposed that the release of cytochrome c is dependent upon peroxidation and/or hydrolysis of CL (15,16). Cytochrome c is known to bind CL through both charge-pairing and hydrophobic interactions (17,18) and calcium displaces cytochrome c from CL resulting in compromise of electron transport chain function and cellular bioenergetic efficiency (19-26).

Mitochondrial cyclophilin D (CyPD) is a peptidyl prolyl cis-trans isomerase F (PPIase F) localized to the mitochondrial matrix which functions as an essential component of the mitochondrial permeability transition pore (2,8). Cyclosporine A (CsA), an immunosuppressant which inhibits the protein phosphatase calcineurin, tightly binds CyPD in the mitochondrial matrix resulting in potent desensitization of the mPTP to Ca²⁺, inorganic phosphate (P) and oxidative stress (27-31). Studies with non-immunosuppressive cyclosporine analogs have suggested that CsA protects mitochondria from the formation of the mPTP by inhibition of its PPIase activity and/or its interaction with pore complex, but not by inhibition of CyPD immunosuppressive activity (30,32). Approaches utilizing genetic ablation of CyPD in mice have established that CyPD is required for mediating Ca²⁺ and reactive oxygen species (ROS)-induced cell death, but is dispensable in the Bcl-2 family member mediated cell death pathway (33,34).

Long chain fatty acyl-CoA as well as fatty acid has previously been demonstrated to be a potent modulator facilitating the opening of the mitochondrial permeability transition pore but its mechanism of action is unknown (35-37). Similarly, L-carnitine has been proposed to protect against fatty acyl-CoA augmentation of mPTP opening by facilitating removal of fatty acyl-CoA by CPT-1 mediated transport of fatty acids across the mitochondrial outer membrane for subsequent matrix β-oxidation (36,37). Calcium stimulates the synthesis and flux of acyl-CoA from fatty acids into β-oxidation pathways to meet the energetic demands of the cell (38-40).

Previously, Pfeiffer et al. demonstrated that racemic BEL blocked the Ca²⁺-induced increase in swelling of rat liver mitochondria (41).
Supporting these results, Schnellmann et al. identified a racemic BEL-sensitive iPLA2 in rabbit kidney cortex mitochondria responsible for Ca\(^{2+}\)-induced mitochondrial swelling (42,43). These data both indicated that a member of the calcium-independent phospholipase A\(_2\) family was likely responsible for the calcium-mediated mitochondrial swelling. However, the molecular identity of the BEL-sensitive enzyme participating in mPTP opening cannot be determined from those studies alone since all members of the iPLA2 family are inhibited by BEL as are other serine hydrolases and thiol esterases (e.g. aldehyde dehydrogenase) (44-47).

Accordingly, in the present work we used combined genetic and pharmacologic approaches to definitively assign the observed functional alterations in mPTP opening to iPLA2\(_\gamma\). Through the use of a genetic iPLA2\(_\gamma\) loss of function model in conjunction with enantioselective pharmacologic inhibition of iPLA2\(_\gamma\), we now identify the fundamental role of iPLA2\(_\gamma\) in calcium-induced mPTP opening and its modulation by fatty acyl-CoA. Collectively, the present study integrates alterations in mitochondrial bioenergetic function with mPTP opening in which iPLA2\(_\gamma\) plays a central mechanistic role.

**EXPERIMENTAL PROCEDURES**

**Reagents** – 1-Palmitoyl-2-[1-\(^{14}\)C]arachidonoyl-sn-glycero-3-phosphocholine (55 mCi/mmols) was purchased from PerkinElmer Life Sciences. Racemic (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL) was obtained from Cayman Chemical Co. (Ann Arbor, MI). The (R)- and (S)-enantiomers of BEL were prepared as previously described (48). N-[2S,4R]-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl]-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl] acrylamide (Pyr) was purchased from EMD Biosciences (Billerica, MA). Anti ANT, anti cytochrome c, anti VDAC, anti cyclophilin D (PPIF), anti- mouse HRP -IgG and anti-rabbit HRP-IgG antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Acyl-CoAs (Acetyl-, 16:0-, 18:1-, and 20:4-CoA), palmitic acid, free CoASH, L-carnitine, t-butylhydroperoxide (TBH), phenylarsine oxide (PAO), mitochondria respiration substrates (pyruvate, glutamate, palmitoyl-L-carnitine, succinate, cytochrome c, and mitochondria complex inhibitors (antimycin A, oligomycin, and rotenone) were purchased from Sigma-Aldrich Co. (St. Louis, MO) and S-hexadecyl-CoA (S-HD-CoA) was prepared in our laboratory as described previously (49). All other reagents were purchased from either Sigma Aldrich or Fisher Scientific.

**Generation and Affinity Purification of a Rabbit Polyclonal Antibody Against Human iPLA2\(_\gamma\)** - All procedures for generation of iPLA2\(_\gamma\) antibody were performed by Open Biosystems of Thermo Fisher Scientific. Briefly, male white New Zealand rabbits were initially immunized with 500\(\mu\)g of the 20-mer peptide (CKINDWIKLKSMDYELPFF conjugated to KLH). After 3 booster immunizations at 2 week intervals, serum was collected and iPLA2\(_\gamma\) antibody was purified by affinity chromatography using the covalently bound peptide sequence.

**Animal Studies and Generation of iPLA2\(_\gamma^{-/-}\) Mice** – All procedures were conducted in accordance with the National Institutes of Health guidelines for humane treatment of animals and were reviewed and approved by the Animal Studies Committee of Washington University. Mice null for iPLA2\(_\gamma\) were generated in our laboratory as described previously (50). Heterozygous offspring were interbred to generate homozygous knockouts and wild-type littermates. All experiments in this study were performed by comparisons of 10-15 week old male wild type littermates to male iPLA2\(_\gamma\) knockout mice.

**Isolation of Hepatic Mitochondria** –Wild-type and iPLA2\(_\gamma^{-/-}\) mice were euthanized by cervical dislocation after which liver tissue was immediately excised and washed extensively in ice cold isolation buffer. After dissection and mincing with a razor blade on ice (4°C ambient temperature) in mitochondrial isolation buffer (MIB; 0.2 M mannitol, 70 mM sucrose, 0.1 mM potassium-EDTA, 1 mM EGTA, 10 mM Tris-HCl, 0.5% BSA, pH 7.4) the liver tissue was homogenized using 12-15 passes with a Teflon
homogenizer using a rotation speed of 120 revolutions per minute. Next, the homogenate was centrifuged for 5 min at 850 x g, and the supernatant was collected and centrifuged for 12,000 x g for 10 min. The pellet was resuspended in MIB without BSA, centrifuged for 7,200 x g for 10 min. and the pellet was resuspended in MIB without BSA. Mitochondrial protein content was determined using a BCA protein assay (Thermo Fisher Scientific, San Jose, CA).

**Determination of Mitochondrial Swelling** — For determination of mitochondrial permeability transition pore (mPTP) opening, isolated mitochondria from wild-type and iPLA2γ−/− mouse livers were placed in mitochondrial swelling buffer (0.23 M mannitol, 70 mM sucrose, 5 mM succinate, 2.5 μM rotenone in the absence or the presence of 1 mM KH2PO4 (for experiments with inorganic phosphate). Intact mitochondria were equilibrated with swelling buffer at 23°C for 10 min. For experiments examining the effect of PLA2 inhibitors, mitochondria were preincubated with either 5 μM (R)-BEL, 5 μM (S)-BEL, 1 μM pyrrolidine (Pyr) or DMSO vehicle alone (1 % v/v). Mitochondrial swelling was initiated by addition of 70 μM CaCl2 (final) with comparisons to addition of 10 μM EGTA as control. In experiments examining the effects of free fatty acid and fatty acyl-CoA, palmitic acid or acyl-CoAs were added prior to initiation of swelling by Ca2+. Decreases in the absorbance of the mitochondria indicative of swelling at 540nm were measured every 15 s using a SpetraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA).

**Phospholipase A2 Activity Assay** — The mitochondrial pellet isolated from WT and iPLA2γ−/− mouse liver was resuspended in ice cold HEPES buffer (10 mM HEPES (pH 7.4), 1 mM DTT, 10 % glycerol) and sonicated (10 × 1 sec pulses) at 30 % power. For the measurement of mitochondrial phospholipase activity with radiolabeled substrate, mitochondrial homogenates (0.1 mg protein) in 300 μL of HEPES buffer were incubated with radiolabeled phosphatidylcholine substrates (~1×10⁵ dpm of 1-palmitoyl-2-[1-14C]arachidonoyl-sn-glycero-3-phosphocholine) in the presence of 4 mM EGTA or 1 mM Ca2⁺ for 10, 20, and 30 min. Phospholipase reactions were terminated by addition of 2 mL of chloroform/methanol/acetic acid (50:48:2, v/v/v) followed by addition of 700 μL of water. Lipids were extracted into the chloroform and loaded onto channeled Partisil LK6 silica gel 60Å plates and resolved using petroleum ether/ethyl ether/glacial acetic acid (70:30:1, v/v/v) with oleic acid as a fatty acid standard as previously described (51).

**Immunoblot Analyses** — Mitochondrial proteins were separated by SDS-PAGE (10-15% polyacrylamide gels), transferred to polyvinylidene fluoride membranes by electroelution, and blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Membranes were probed using the indicated primary antibodies diluted in TBS-T containing 1% bovine serum albumin, washed with TBS-T, and then incubated with the appropriate secondary HRP conjugate (diluted 1:2000 in TBS-T containing 5% non-fat dry milk or 1% BSA).

**Mitochondrial High Resolution Respirometry** — High resolution respirometry was performed using 100 μg of liver mitochondrial protein per 2 mL chamber of an OROBOROS® Oxygraph 2K (Innsbruck, Austria) with substrate and inhibitor additions as previously described (52,53). Briefly, liver mitochondria were incubated in 2 mL of respiration buffer (MiRO5; 110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 20 mM HEPES, 10 mM KH2PO4, 3 mM MgCl2, 0.5 mM EGTA, 1 g/L BSA (Fraction V), pH 7.1) for high resolution respirometry. Respiratory measurements were performed at 30°C in MiRO5 buffer with 500 rpm stir bar rotation. The oxygen concentration at air saturation was determined to be 230 nmol O2 per ml at 105 kPa barometric pressure. Oxygen calibration and slope calculations were performed routinely to ensure accuracy of the flux measurements. Oxygen flux was calculated as a time derivative of oxygen concentration using the DatLab4.3 Analysis software (OROBOROS®, Innsbruck, Austria). Respiration was started by the addition of glutamate (10 mM) or palmitoyl-L-carnitine (20 μM) or pyruvate (5 mM) or pyruvate (5 mM)/glutamate (10 mM), and malate (5 mM) [State 2] followed by ADP (1.25 mM) [State 3],
iPLA2 performed Western analyses of wild-type and iPLA2 and a single band at 45 kDa (Fig. 1A) using this from 63-87 kDa, a predominant band at 52 kDa, elimination of immunoreactive bands ranging specificity for iPLA2 highly sensitive antibody possessing the greatest cross-reacting proteins that are observable. All the antibody directed against the C-terminus of bands observed by Western blotting utilizing our iPLA2 and a faint doublet at ~55 kDa are non-specific of our knowledge. Only the protein band at 58 kDa

Statistical Analyses – Values are expressed as mean ± SEM. The significance of experimental observations were determined by a Student’s t-test and results were considered significant at P < 0.05.

RESULTS

Genetic ablation of iPLAγ results in decreased Ca2+-dependent PLA2 activity in mouse liver mitochondria – To examine whether iPLAγ was present in isolated murine liver mitochondria, we performed Western analyses of wild-type and iPLAγ liver mitochondrial proteins using a custom affinity purified antibody directed against the C-terminus of iPLAγ (CKINDWIKLKSMDMYELPFF). Genetic ablation of iPLAγ (50) resulted in complete elimination of immunoreactive bands ranging from 63-87 kDa, a predominant band at 52 kDa, and a single band at 45 kDa (Fig. 1A) using this highly sensitive antibody possessing the greatest specificity for iPLAγ identified to date to the best of our knowledge. Only the protein band at 58 kDa and a faint doublet at ~55 kDa are non-specific cross-reacting proteins that are observable. All the bands observed by Western blotting utilizing our antibody directed against the C-terminus of iPLAγ are typically detected in liver tissue homogenates and are completely blocked by the cognate peptide. The observed iPLAγ polypeptides are most consistent with the use of alternate in-frame ATG start codons to generate the 87.4 kDa and 73.6 kDa isoforms. Although there are ATG codons in the mRNA which could be predicted to produce the 62.4 kDa and 56.8 kDa isoforms, they would not possess the mitochondrial import signal present in the larger isoforms and therefore most likely arise from either intra-mitochondrial proteolytic processing or mitochondrial-associated membrane proteins (e.g. peroxisomes). Additionally, since there is neither an alternatively spliced transcript nor an alternate translational ATG start site which could encode the observed 52 kDa and 45 kDa isoforms, presumably they are generated through proteolytic processing. It is interesting to note that all of the identified mitochondrial iPLAγ polypeptides are of sufficient length to possess both the C-terminal KINDWIKLKSMDMYELPFF sequence recognized by the antibody and the GVSTG active site. Thus, these results specifically demonstrate the loss of multiple isoforms of iPLAγ in liver mitochondria in the iPLAγ−/− mouse.

Next, we measured both calcium-dependent and calcium-independent PLA2 activities in WT and iPLAγ−/− liver mitochondria. Although iPLAγ does not require calcium ion for membrane association or catalysis, we have recently demonstrated that iPLAγ present in myocardial mitochondria can be activated in the presence of low micromolar concentrations of free calcium ions (54). Incubation of wild-type liver mitochondrial sonicates with 1-palmitoyl-2-[14C]arachidonoyl-sn-glycerophosphorylcholine ([14C]PAPC) resulted in a time-dependent increase in the release of [14C]arachidonic acid which was enhanced by the presence of calcium ion (Fig. 1B). In contrast, iPLAγ−/− liver mitochondrial sonicates exhibited approximately 30-40% lower PLA2 activity in the presence of EGTA and approximately 50% lower PLA2 activity in incubations containing Ca2+ in comparison to wild-type controls (Fig. 1B). More importantly, calcium-facilitated PLA2 activity was virtually absent in iPLAγ−/− liver mitochondria. These results demonstrate a significant decrease in PLA2 activity in iPLAγ−/− liver mitochondria indicating loss of iPLAγ function which cannot be compensated by increased expression of other intracellular phospholipases A2.

The (R)-BEL inhibitable Ca2+/phosphate-induced swelling present in mitochondria from wild-type mouse liver is dramatically attenuated in mitochondria prepared from iPLAγ−/− mice. Previous work by Pfeiffer and colleagues indicated that a calcium-independent phospholipase A2 was likely responsible for the Ca2+-mediated swelling of rat liver mitochondria since pretreatment with racemic BEL blocked mitochondrial swelling with a concomitant ablation of free fatty acid (FFA) release (41). To further investigate the role(s) of iPLAγ(s) in facilitating the opening of the mPTP, we utilized both genetic and enantioselective
pharmacologic approaches to determine whether iPLA$_2^\gamma$ was the enzyme mediating the Ca$^{2+}$-induced swelling of liver mitochondria. As anticipated, Ca$^{2+}$ challenge of wild-type mitochondria in the presence of phosphate ion (P$_i$) induced a rapid and dramatic swelling demonstrated by a rapid decrease in the absorbance at 540 nm which was completely blocked by inclusion of cyclosporine A (CsA) (Fig. 2A). Pretreatment with the iPLA$_2^\gamma$-selective inhibitor (R)-BEL markedly attenuated the initial rapid phase of mitochondrial swelling at early time points (2 and 5 min) and was more effective than the iPLA$_2^\beta$ selective inhibitor (S)-BEL in inhibiting this process (Fig. 2A and 2C). In contrast, the cPLA$_2^\alpha$ selective inhibitor pyrrolidine (Pyr) did not affect calcium-induced mitochondrial swelling (Fig. 2A and 2C).

Although experiments with pharmacologic inhibitors can provide important insight into the chemical mechanisms mediating a biologic process, off target effects of pharmacologic agents can be misleading. Moreover, BEL inhibits all known members of the iPLA family and identification of the enzyme responsible for the observed effects cannot be made through pharmacologic approaches alone. Accordingly, we used a genetic iPLA$_2^\gamma$ loss of function model to unambiguously identify the role of this enzyme in calcium-induced mPTP opening. Liver mitochondria were isolated from iPLA$_2^\gamma^{-/-}$ mice and challenged with calcium ion. The rate of calcium-induced mitochondrial swelling at early time points (2 and 5 min) was markedly attenuated relative to wild-type mitochondria (Fig. 2B and 2C). As was the case in wild-type mitochondria, mPTP opening was cyclophilin D-dependent since complete ablation of mitochondrial swelling in iPLA$_2^\gamma^{-/-}$ mitochondria was accomplished with CsA. In contrast to the experiments with wild-type mitochondria, (R)-BEL and (S)-BEL were equipotent in inhibiting the calcium-mediated swelling of iPLA$_2^\gamma^{-/-}$ liver mitochondria (Fig. 2B) indicating the loss of an (R)-BEL inhibitable component (iPLA$_2^\gamma$) and the likely involvement of one or more components which is equally sensitive to both (R)-BEL and (S)-BEL. Collectively, these results identify an important role for iPLA$_2^\gamma$ in mediating calcium-induced mPTP opening in liver mitochondria using synergistic genetic and pharmacologic approaches.

Ca$^{2+}$-induced mitochondrial swelling in the absence of phosphate ion – Previously, phosphate ion has been shown to be a prominent factor in facilitating the opening of the CsA-sensitive component of the mPTP (55). As anticipated, calcium-challenge of mitochondria from WT livers in the absence of phosphate resulted in a lag phase of 2-3 min during which time no swelling was manifest followed by a slower rate of opening in comparisons to incubations containing exogenous phosphate ion (Fig. 3A compared to Fig. 2A). In contrast to the virtually complete inhibition of Ca$^{2+}$-induced mitochondrial swelling by CsA in the presence of phosphate ion (Fig. 2A), only modest decreases in swelling were manifest at later time points when P$_i$ was omitted (Fig. 3A). In the absence of calcium ion, liver mitochondria from iPLA$_2^\gamma^{-/-}$ mice displayed similar amounts of spontaneous swelling as their wild-type littermates (compare Figs. 3A and 3B). However, in marked contrast to wild-type mitochondria, hepatic mitochondria prepared from iPLA$_2^\gamma^{-/-}$ mice were remarkably resistant to Ca$^{2+}$-mediated swelling (Fig. 3B). Furthermore, inclusion of CsA failed to prevent the residual calcium-dependent swelling at later time points, indicating that Ca$^{2+}$-mediated CsA-sensitive (through CyPD) mitochondrial swelling is dependent on the presence of iPLA$_2^\gamma$ in the absence of phosphate ion.

Expression of VDAC, ANT, and CyPD in iPLA$_2^\gamma^{-/-}$ mitochondria – Although the precise molecular composition of the mPTP complex is not known with certainty, multiple mitochondrial proteins have been identified as potential components of the pore itself and/or as associated regulatory factors (2,8). We examined whether iPLA$_2^\gamma$ loss of function impacted the expression levels of three extensively studied mitochondrial proteins implicated in the mitochondrial permeability transition: VDAC, ANT, and cyclophilin D. While no significant differences in VDAC and ANT protein levels were manifest in iPLA$_2^\gamma^{-/-}$ liver mitochondria relative to wild-type control, CyPD content was modestly increased (~1.8-fold higher in iPLA$_2^\gamma^{-/-}$ liver mitochondria than in their wild-type counterparts) (Fig. 4).
moderately increased CyPD protein level mediated by either upregulated expression and/or decreased degradation in iPLA$_{2\gamma}^\gamma$-liver mitochondria did not increase the susceptibility of the mPTP to calcium-mediated opening in mitochondria.

Identification of palmitic acid as a modulator of iPLA$_{2\gamma}$-dependent opening of the mPTP – Previously, we have demonstrated that iPLA$_{2\gamma}$ is activated by calcium ion and exhibits robust PLA$_1$ activity utilizing phospholipid substrates containing polyunsaturated fatty acids esterified to the sn-2 position resulting in the production of both saturated fatty acids (from the sn-1 position) and 2-polyunsaturated lysolipid molecular species (54). To address whether saturated fatty acids could abrogate the resistance of iPLA$_{2\gamma}^\gamma$ mitochondria to Ca$_2^+$/Pi$_i$-induced swelling in the presence of phosphate, we measured iPLA$_{2\gamma}^\gamma$-liver mitochondrial swelling in the presence of 5 or 10 µM palmitic acid (PA). Inclusion of low micromolar concentrations of palmitic acid substantially increased the initial (0-5 min) and intermediate (5-8 min) rates of mitochondrial swelling induced by exogenous phosphate and calcium ions (Fig. 5A). To determine if the observed palmitic acid-rescued swelling of iPLA$_{2\gamma}^\gamma$-mitochondria was mediated by the classic CsA-sensitive mPTP opening, we preincubated hepatic mitochondria from iPLA$_{2\gamma}^\gamma$-mice with 1 µM CsA. The Ca$_2^+$/Pi$_i$-induced palmitate-facilitated mitochondrial swelling was nearly completely inhibited by CsA demonstrating that calcium-induced palmitate-modulated swelling was mediated by cyclophilin D-dependent mPTP opening (Fig. 5B).

Fatty acyl-CoA and its non-hydrolyzable thioether analog activate Ca$_2^+^\gamma$-induced mitochondrial swelling – To further define the roles of long chain fatty acyl-CoAs as modulators of mPTP opening, we examined the effects of palmitoyl-CoA, oleoyl-CoA, and arachidonoyl-CoA on the calcium-induced swelling of wild-type liver mitochondria (Fig. 6). Addition of a submicellar concentration of palmitoyl-CoA (5 µM) to wild-type liver mitochondria in the absence of Pi$_i$ facilitated calcium-induced mitochondrial swelling (Fig. 6). Both oleoyl-CoA and arachidonoyl-CoA induced similar increases in Ca$_2^+^\gamma$-mediated mPTP opening (Fig. 6, right panel). In contrast, non-acylated free coenzyme A (CoASH) or acetyl-CoA did not alter the kinetics of swelling manifest in wild-type mitochondria in the presence of calcium ion (Fig. 6). Importantly, the non-hydrolyzable thioether analog of palmitoyl-CoA, S-hexadecyl-CoA (SHD-CoA), was equipotent in facilitating the calcium-induced swelling of wild-type mitochondria (Fig. 6) indicating that neither hydrolysis of long chain fatty acyl-CoA nor protein acylation are required to effect fatty acyl-CoA mediated modulation of mPTP opening.

Sensitivity of Ca$_2^+^\gamma$-induced fatty acyl-CoA augmented mitochondrial swelling to CsA inhibition – Next, we sought to determine whether the fatty acyl-CoA enhancement of Ca$_2^+^\gamma$-induced mitochondrial swelling was sensitive to the CyPD antagonist, CsA. It should be noted that the following experiments were performed in the absence of phosphate ion (as in Fig. 3) resulting in an attenuated rate of mitochondrial swelling in order to more accurately examine the mechanism of fatty acyl-CoA facilitated mPTP opening. As anticipated, pre-incubation of wild-type liver mitochondria with CsA resulted in near complete inhibition of mPTP opening mediated by Ca$_2^+^\gamma$ alone (Fig. 7A). In addition, CsA potentely inhibited the accelerated swelling of wild-type mitochondria enhanced by palmitoyl-CoA in the presence of calcium ion (Fig. 7A). Dramatic differences in the rates of mitochondrial swelling are apparent in iPLA$_{2\gamma}^\gamma$-mitochondria exposed to Ca$_2^+^\gamma$ in the presence or absence of palmitoyl-CoA in comparison to wild-type mitochondria (Fig. 7B) indicating an obligatory role for iPLA$_{2\gamma}$ in these processes. In contrast to wild-type mitochondria, palmitoyl-CoA and CsA had little or no effect on the calcium-induced swelling observed in iPLA$_{2\gamma}^\gamma$-mitochondria at early time point (Fig. 7B) indicating the importance of iPLA$_{2\gamma}$ in the initiation and propagation of the early stages of mPTP pore opening. Collectively, these results demonstrate the fundamental role of iPLA$_{2\gamma}^\gamma$ in calcium-induced mPTP opening and its modulation by palmitoyl-CoA.

Effects of L-carnitine through CPT-1 on Ca$_2^+^\gamma$-induced mitochondrial swelling - Utilization of fatty acyl-CoA substrates for mitochondrial β-
Genetic ablation of iPLA2γ confers resistance to tertiary butylhydroperoxide but not phenylarsine oxide facilitated swelling of liver mitochondria—Reactive oxygen species have been previously demonstrated to be important mediators promoting mPTP opening. Considering the resistance of iPLA2γ−/− hepatic mitochondria to various inducers/promoters of mitochondrial swelling, we sought to determine if reactive oxygen donors could promote opening of the mPTP in WT or iPLA2γ−/− liver mitochondria. In control experiments with liver mitochondria isolated from WT mice, calcium-induced mitochondrial swelling was significantly augmented by 1 mM tert-butylhydroperoxide (TBH) especially in the later phase (Fig. 9A). In contrast, mPTP opening of liver mitochondria from iPLA2γ−/− mice was insensitive to TBH (Fig. 9B). Next, we determined whether phenylarsine oxide (PAO), a potent inducer of mPTP opening through oxidation of components of the mPTP, was able to augment mPTP opening in mitochondria from iPLA2γ−/− mice. Although significant differences in the induction profiles of swelling at early-phase time points were present, PAO was found to dramatically enhance opening of the mPTP in both WT and iPLA2γ−/− mitochondria (Fig. 9). These results demonstrate that PAO-mediated mitochondrial swelling is not affected by the absence of iPLA2γ and demonstrate that the mPTP machinery is functional in hepatic mitochondria prepared from the iPLA2γ−/− mouse.

Previously, it has been demonstrated that the opening of mPTP facilitates release of cytochrome c from inner mitochondria membrane triggering the execution of the intrinsic pathway of apoptosis. Next, we examined if the observed alterations in the swelling of iPLA2γ−/− liver mitochondria were accompanied by cytochrome c release. Expression levels of cytochrome c protein were not significantly different in wild-type versus iPLA2γ−/− liver mitochondria (Fig. 10A). However, in wild-type controls, marked release of cytochrome c was observed after calcium ion challenge. Addition of TBH or PAO further increased calcium-induced cytochrome c release (Fig. 10B). In sharp contrast, virtually no release of cytochrome c was observed in iPLA2γ−/− liver mitochondria in responses to calcium or TBH while modest cytochrome c release was elicited by PAO in the presence of phosphate ion (Fig. 10B). Collectively, these results demonstrate the important roles of mitochondrial iPLA2γ in facilitating the calcium-mediated release of cytochrome c augmented by ROS and phosphate that is coupled to the bioenergetic status of the mitochondrion.

High resolution respirometry of wild-type and iPLA2γ−/− hepatic mitochondria—Functional analysis of oxygen consumption in hepatic mitochondria revealed a consistent deficiency in complex I mediated substrate utilization in liver mitochondria from iPLA2γ−/− mice compared to wild-type littermates. The data demonstrated a 50% decrease in state 3 respiration stimulated by pyruvate, palmitoyl-L-carnitine, glutamate, or pyruvate/glutamate (Fig. 11A-D). However, utilization of succinate (complex II substrate) did not reveal a consistent deficiency in respiration in rotenone treated samples, except in isolated
respiring mitochondria initially incubated with glutamate (Fig. 11C). This result is most likely due to the greater deficiency in glutamate-stimulated respiration found in the iPLA2γ−/− mice. Analysis of complex IV activity by TMPD and ascorbate, treatment revealed no change in cytochrome c oxidase activity in isolated hepatic mitochondria in iPLA2γ−/− compared to wild-type mice (Fig. 11E). Thus, the decrease in complex I mediated substrate utilization was not the result of lower oxygen consumption regulated by cytochrome c oxidase. Therefore, liver mitochondria isolated from iPLA2γ−/− mice demonstrate a compromised capacity to efficiently utilize the primary TCA cycle anaplerotic substrates coupled to Complex I mediated proton generation for electron transport chain function.

DISCUSSION

Calcium-induced opening of the mitochondrial high-conductance non-selective anion channel, known as mitochondrial permeability transition pore (mPTP), has been implicated in multiple mechanisms of mitochondrial dysfunction including alterations of mitochondrial-driven energy metabolism and ion homeostasis (56), production of reactive oxygen species (2,11,57), and the release of pro-apoptotic factors (2,11). Although previous studies using chemical inhibitors have suggested mitochondrial phospholipase A2 activity to be involved in the induction of mPTP opening (36,41,42), the identity of the PLA2(s) responsible for mitochondrial permeability transition and its mechanism of activation are largely unknown. In the current study, we have identified iPLA2γ as a critical component of the mPTP by genetic ablation and confirmed its importance through enantioselective mechanism-based inhibition. Furthermore, we have demonstrated that mPTP pore opening is accompanied by the loss of cytochrome c from intact mitochondria.

In addition, the results of the current study underscore the importance of fatty acyl-CoA in modulating calcium-induced mPTP opening and provide important evidence on the mechanisms that mediate acyl-CoA augmentation of this process. Through the use of the non-hydrolyzable thioether analog, S-hexadecyl-CoA, we demonstrate that acyl-CoA modulation of the mPTP is not due to palmitoylation of mPTP channel constituents, alterations in mitochondrial bioenergetics through fatty acid β-oxidation or the use of free fatty acids for membrane synthesis, remodeling or other anabolic processes.

Furthermore, in the present study, we demonstrate that the highly thiol-selective reagent phenylarsine oxide, but not the potent general oxidant t-butylhydroperoxide, was capable of inducing mPTP opening utilizing hepatic mitochondria from iPLA2γ−/− mice. These results identify the substantial protection rendered by iPLA2γ loss of function and suggest that the selective inhibition of iPLA2γ could have salutary effects on certain pathophysiologic conditions resulting from ROS-mediated damage. Collectively, the results of this study unambiguously demonstrate that iPLA2γ is a critical mechanistic component in the mPTP opening.

The mPTP is a Ca2+-dependent channel in the mitochondrial inner membrane whose prolonged opening initiates cell death programs (1-4). Previous reports by others have demonstrated that mitochondrial phospholipase activity which is inhibitable by BEL could modulate Ca2+-induced opening of mPTP (41,43). However, the synergistic use of genetic ablation and pharmacologic inhibition are necessary to avoid confounding conclusions that result from compensatory alterations in genetically engineered loss of function models or from off-target effects of pharmacologic agents. Thus, the multiple approaches utilized in this study have demonstrated that loss of iPLA2γ function resulted in the remarkable resistance of liver mitochondria to Ca2+-activated swelling in the presence or absence of phosphate ion. Furthermore, exogenous palmitate, which is a major fatty acid produced by the PLA1 present in iPLA2γ, restored iPLA2γ−/− hepatic mitochondrial swelling mimicking that observed in WT control mitochondria. Palmitate has been previously proposed to activate both CsA-sensitive and CsA-insensitive pores in rat liver mitochondria (58,59). In this study, we found that palmitate-induced swelling in iPLA2γ−/− liver mitochondria is largely reversible by CsA.

Recently, we have demonstrated the divalent-cation dependent activation of iPLA2γ...
phospholipase activity in myocardial mitochondria (54). Consistent with our previous report, we also demonstrate the Ca\(^{2+}\)-dependent activation of iPLA\(_2\gamma\) in WT hepatic mitochondria as evidenced by the loss of calcium-activated phospholipase activity in iPLA\(_2\gamma^{-/-}\) mitochondria. These results provide strong evidence that iPLA\(_2\gamma\) is a major phospholipase activity in both hepatic and myocardial mitochondria. Thus, the data in the present study identify an integrated mechanism through which calcium can activate the production of multiple lipid 2\(^{nd}\) messengers (i.e. lysolipids, arachidonic acid, and downstream eicosanoid metabolites) some of which have previously been implicated in modulating the opening of the mPTP (58,60).

Previous works by others have suggested fatty acid and fatty acyl-CoA could open mPTP by mediating mitochondrial membrane depolarization (36,61). It is also well known that acyl-CoA levels are increased in several pathologic conditions such as diabetes (62-64). If mitochondria are able to effectively transduce this increased flux of acyl-CoA into energy through \(\beta\)-oxidation then this response provides a physiologic adaptive response to increased bioenergetic demands. However, if mitochondria cannot process the increased metabolic flux of acyl constituents, then acyl-CoA accumulates promoting mPTP opening leading to mitochondrial depolarization and necrotic or necroapoptotic cell death.

Elevations in acyl-CoA in diabetic tissues are largely thought to be mediated by mitochondrial dysfunction through their inability to effectively oxidize increased rates of fatty acids necessary to fuel contractile function in the absence of glucose for energy production. Alternatively, in other pathologic conditions, such as ischemia or hypoxia, acyl-CoA is increased in mitochondria due to the absence of sufficient oxygen to promote \(\beta\)-oxidation of fatty acids. In either case, regardless of the etiology underlying acyl-CoA elevations, mPTP opening is facilitated by acyl-CoA leading to increased cellular necrosis contributing to end-organ failure. Importantly, the current study revealed that L-carnitine could reverse fatty acyl-CoA, but not S-hexadecyl-CoA, enhanced mitochondrial swelling selectively without affecting swelling mediated by Ca\(^{2+}\) alone. These results suggest that CPT-1 is an important participant in mPTP opening through modulating acyl-CoA levels. Recent work utilizing multiple approaches by Hoppel and colleagues has demonstrated that CPT-1 interacts with acyl-CoA synthetase and VDAC forming hetero-oligomeric complexes in mitochondrial outer membrane (65). Although knockout studies have eliminated an essential role for both VDAC and ANT as obligatory constituents of the mPTP (66-68), a regulatory role for ANT has been confirmed (68). It is now well established that the ANT is potently inhibited by fatty acyl-CoAs and this represents a likely mechanism through which mitochondrial iPLA\(_2\gamma\) regulates mPTP opening (69,70).

Cyclophilin D (CyPD) present in the mitochondrial matrix encoded by the PPIF gene is a critical non-structural element of the mPTP complex modulating the opening probability of the channel. The interaction of cyclosporine A with CyPD has been well established to potently desensitize the mPTP in response to provocative maneuvers such as calcium challenge. However, CyPD is not obligatory for mPTP opening since CyPD\(^{-/-}\) mitochondria exhibited CsA-insensitive swelling albeit at somewhat higher concentrations of Ca\(^{2+}\) (33). Although studies with CyPD\(^{-/-}\) animals or pharmacologic inhibition have indicated a detrimental role for CyPD in pathophysologic states (e.g. ischemia/reperfusion injury) via mPTP opening (33,34,71,72), other evidence has linked CyPD overexpression with increased resistance to apoptosis through binding to Bcl-2 in an mPTP-independent pathway (e.g. (73)). Notably, iPLA\(_2\gamma^{-/-}\) mitochondria are markedly more resistant to Ca\(^{2+}\)-mediated swelling than their wild-type counterparts. Thus, the upregulation of CyPD in the iPLA\(_2\gamma^{-/-}\) mouse may be a necessary response to desensitize cells to apoptotic signals mediated by iPLA\(_2\gamma\) loss of function.

Although the beneficial effects of physiologic Ca\(^{2+}\) uptake into mitochondria (e.g. resulting in TCA cycle activation (74,75) and stimulation of oxidative phosphorylation) are well known (76,77), mitochondrial Ca\(^{2+}\) overload has been demonstrated to result in the generation of toxic reactive oxygen species (ROS) by accelerating uncoupling and the loss of antioxidants such as reduced glutathione after mPTP opening (2,11). Previously, \(\gamma\)-
butyldihydroperoxide (TBH) has been demonstrated to cause oxidative stress in cells and tissues by generating toxic free radicals leading to peroxidation of lipids or other critical oxidatively labile moieties (78,79). Consistent with this notion, it has been well established that TBH induces cell death by facilitating the opening of the mPTP and the subsequent release of cytochrome c (80,81). The current study provides evidence that iPLA2γ−/− liver mitochondria are markedly resistant to TBH-induced cytochrome c release in the presence of Ca2+ which is consistent with the observed resistance to mPTP opening in mitochondria from iPLA2γ−/− mice. In contrast, phenylarsine oxide (PAO) is a well-established inhibitor of phosphotyrosine phosphatases. PAO has been shown to cause a rapid and irreversible decrease in the mitochondrial free Ca2+ concentration (82). In addition, PAO has been shown to inhibit the mitochondrial ANT through cross-linking of vicinal cysteine residues in the ANT (160 and 257) that inhibits ADP binding and enhances CyPD binding to the “c” conformation of the ANT. Similarly, binding of acyl-CoA to the ANT induces the “c” conformation which preferentially binds CyPD (83). Recently, work by Halestrap et al. has provided evidence that the mitochondrial phosphate carrier (PiC) may play a key role in mPTP formation and regulation (10). In this study, CyPD was demonstrated to bind to PiC in a CsA-dependent manner and crosslinking of cysteine residues on PiC by PAO correlated with mPTP opening (10). Potent sensitization of liver mitochondrial swelling to Ca2+ by PAO in our study was observed in both WT and iPLA2γ−/− mice suggesting that PAO-stimulated mPTP opening by oxidation of thiol group(s) of a pore protein component or regulator such as ANT or PiC is likely not affected by ablation of iPLA2γ activity. However, interestingly, iPLA2γ is likely a participant in PAO-accelerated cytochrome c release since PAO facilitation of Ca2+-induced cytochrome c release was significantly reduced in mitochondria from iPLA2γ−/− livers in comparison to WT littermates.

Collectively, the results of the present study demonstrate the prominent roles of iPLA2γ in mediating calcium-induced mPTP opening that is modulated by oxidative stress and lipid metabolites. The results reveal the mechanistic integration of mitochondrial bioenergetics with the mPTP that regulates adaptive alterations during physiologic perturbations, but conspire to initiate the execution of cell death pathways after pathologic alterations in calcium, ROS or toxic lipid metabolites.

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**FOOTNOTES**

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**ABBREVIATIONS**

AA, arachidonic acid; Acyl-CoA, acyl-coenzyme A; ANT, adenine nucleotide translocase; CPT, carnitine palmitoyl transferase; CyPD, cyclophilin D; CsA, cyclosporine A; iPLA2γ, calcium-independent phospholipase A2γ; mPTP, mitochondrial permeability transition pore; PA, palmitic acid; PAO, phenyl arsine oxide; P, phosphate; PiC, phosphate carrier; Pyr, pyrrolidine; ROS, reactive oxygen species; S-HD-CoA, S-hexadecyl-coenzyme A; VDAC, voltage activated anion channel.

**FIGURE LEGENDS**

Figure 1. Genetic ablation of murine PNPLA8 results in elimination of multiple isoforms of iPLA2γ in hepatic mitochondria and decreased Ca2+-stimulated PLA2 activity.

Hepatic mitochondria from wild-type (WT) and iPLA2γ−/− (KO) mice were isolated by differential centrifugation and briefly sonicated as described in “Experimental Procedures”. A, Mitochondrial proteins (50 µg protein/lane) from WT and KO were resolved by SDS-PAGE (10% gel), transferred to PVDF membranes by electrolution, and probed with a rabbit polyclonal antibody directed against the C-terminus of iPLA2γ for Western blot analysis utilizing an anti-rabbit IgG HRP conjugate and ECL reagents to visualize iPLA2γ protein. B, Calcium-dependent and calcium-independent PLA2 activities in
WT and KO liver mitochondria. Exogenous 1-palmitoyl-2-[14C]arachidonoyl-sn-glycero-3-phosphocholine was added to mitochondrial sonicates in the presence of either 4 mM EGTA or 1 mM Ca\(^{2+}\) and incubated for up to 30 min at 35°C. Reactions were terminated by addition of chloroform/methanol (1:1, v/v) and radiolabeled arachidonate extracted into the chloroform layer was resolved by TLC and quantified by scintillation counting as described in “Experimental Procedures”. Values are the average of 4 independent preparations ± SEM. *, P<0.05 when comparing EGTA versus Ca\(^{2+}\) treatment. ¶, P<0.005 when comparing WT versus KO.

Figure 2. Alterations in the kinetics of the calcium-induced swelling of liver mitochondria from wild-type and iPLA\(_{2 \gamma}^{-/-}\) mice in the presence of phosphate by pharmacologic inhibition of different phospholipases A\(_2\).
Hepatic mitochondria were isolated by differential centrifugation from wild-type (WT) and iPLA\(_{2 \gamma}^{-/-}\) (KO) mice and resuspended in swelling buffer containing 0.23 M mannitol, 70 mM sucrose, 1 mM KH\(_2\)PO\(_4\), 5 mM succinate, and 2.5 \(\mu\)M rotenone. Following preincubation with either 5 \(\mu\)M (S)-BEL, 5 \(\mu\)M (R)-BEL, 1 \(\mu\)M pyrrolidine (Pyr), 1 \(\mu\)M cyclosporine A (CsA) or DMSO vehicle alone (1 %, v/v) for 10 min at 23°C prior to exposure to either 70 \(\mu\)M Ca\(^{2+}\) or 10 \(\mu\)M EGTA (-Ca\(^{2+}\)). WT (A) and KO (B) mitochondria were monitored for decreases in absorbance at 540 nm (indicative of mitochondrial swelling) at 15 s intervals at 23°C. Net changes in absorbance at 540 nm at 2 and 5 min in WT and KO mitochondria were calculated and compared in C where *, P<0.005 and **, P<0.0005 when compared with Ca\(^{2+}\)+Pi treatment without inhibitors. Values are the average of 4 independent preparations ± SEM.

Figure 3. Resistance of iPLA\(_{2 \gamma}^{-/-}\) hepatic mitochondria to Ca\(^{2+}\)-induced swelling in the absence of phosphate.
Liver mitochondria were isolated by differential centrifugation from wild-type (WT) and iPLA\(_{2 \gamma}^{-/-}\) (KO) mice and resuspended in swelling buffer containing 0.23 M mannitol, 70 mM sucrose, 5 mM succinate, and 2.5 \(\mu\)M rotenone without phosphate (Pi) as described in “Experimental Procedures”. Intact mitochondria were then preincubated with either 1 \(\mu\)M cyclosporine A (CsA) or DMSO vehicle alone (1 %, v/v) for 10 min prior to exposure to either 70 \(\mu\)M Ca\(^{2+}\) or 10 \(\mu\)M EGTA (-Ca\(^{2+}\)). Mitochondrial swelling of WT (A) and KO (B) was measured by monitoring light scattering at 540 nm at 15 s intervals at 23°C. Representative tracings from 4 independent preparations are shown.

Figure 4. Increased expression of cyclophilin D, but not the adenine nucleotide translocase (ANT) and the voltage-dependent anion channel (VDAC) in iPLA\(_{2 \gamma}^{-/-}\) liver mitochondria.
Liver mitochondria isolated from wild-type (WT) and iPLA\(_{2 \gamma}^{-/-}\) (KO) mice by differential centrifugation were sonicated and mitochondrial proteins were resolved by SDS-PAGE prior to Western Blot analysis as described in “Experimental Procedures”. Immunoblots of cyclophilin D (CyPD), adenine nucleotide translocase (ANT) and voltage-dependent anion channel (VDAC) are shown in the upper panel (3 separate preparations from each WT and KO). Relative protein expression levels of CyPD and ANT were determined by densitometry and normalized against VDAC in the lower panel.

Figure 5. Reconstitution of Ca\(^{2+}\)/phosphate-induced swelling of iPLA\(_{2 \gamma}^{-/-}\) mitochondria by palmitic acid.
Intact mitochondria isolated from iPLA\(_{2 \gamma}^{-/-}\) mouse liver were placed in swelling buffer with 1 mM phosphate (Pi) and equilibrated for 10 min at 23°C. The indicated concentrations of palmitic acid (PA) or ethanol vehicle alone (0.5% final, v/v) were added prior to measuring mitochondrial swelling (\(\Delta A_{540}\)) initiated by addition of 70 \(\mu\)M Ca\(^{2+}\) or 10 \(\mu\)M EGTA (-Ca\(^{2+}\)) (A). Ca\(^{2+}\)/Pi-induced iPLA\(_{2 \gamma}^{-/-}\) hepatic mitochondrial swelling was monitored in the presence of 10 \(\mu\)M palmitic acid after pretreatment with 1 \(\mu\)M CsA or DMSO vehicle alone (1%, v/v) (B).
Figure 6. Augmentation of Ca$^{2+}$-mediated mitochondrial swelling by fatty acyl-CoA molecular species, but not short-chain acyl-CoA or CoASH.
Intact mitochondria from wild-type (WT) mouse liver were placed in swelling buffer without phosphate and equilibrated for 10 min at 23°C. The impact of the addition of either acetyl-, palmitoyl (16:0)-, oleoyl (18:1)-, arachidonoyl (20:4)- or S-hexadecyl (S-HD)-CoA, or CoASH (5 µM final concentration of each) prior to exposure to either 70 µM Ca$^{2+}$ or buffer with 10 µM EGTA (-Ca$^{2+}$) on the swelling of WT mitochondria was measured by monitoring the decrease in absorbance at 540 nm at 15 s intervals at 23°C (left panel). Net changes in absorbance at 540 nm for 10 min are shown in the right panel. **, P<0.00005 when compared with Ca$^{2+}$ treatment without acyl CoA or free CoASH. Values are the average of 5-6 preparations ± SEM.

Figure 7. Resistance of iPLA2γ−/− hepatic mitochondria to fatty acyl-CoA-enhanced swelling in the presence of Ca$^{2+}$.
Intact mitochondria from wild-type (WT) and iPLA2γ−/− (KO) mice were placed in swelling buffer without phosphate (Pi) and preincubated with 1 µM cyclosporine A (CsA) or DMSO vehicle alone (1% final, v/v) for 10 min at 23°C. Palmitoyl (16:0)-CoA (5 µM final) was added prior to exposure to either 70 µM Ca$^{2+}$ or 10 µM EGTA (-Ca$^{2+}$). Swelling of WT (A) and KO (B) mitochondria was measured by monitoring the decrease in absorbance at 540 nm at 15 s intervals at 23°C. Representative tracings from 4 independent preparations are shown.

Figure 8. Impact of L-carnitine on fatty acyl-CoA and fatty acyl thioether CoA enhanced swelling of wild-type liver mitochondria.
Intact mitochondria from wild-type (WT) mice were placed in swelling buffer without phosphate and preincubated with 1 mM L-carnitine (LC) or buffer alone for 10 min at 23°C. Palmitoyl (16:0)-CoA, non-hydrolyzable acyl thioether CoA (S-HD CoA), palmitoyl (16:0)-L-carnitine (5 µM final concentration of each) was added prior to exposure to either 70 µM Ca$^{2+}$ or 10 µM EGTA (-Ca$^{2+}$). Swelling of WT mitochondria was measured by monitoring the absorbance at 540 nm at 15 s intervals at 23°C. Net changes in A$_{540}$ for 10 min are shown in C where §, P<10$^{-5}$ when compared with Ca$^{2+}$ treatment. Values are the average of 4-6 separate preparations ± SEM.

Figure 9. Sensitivity of iPLA2γ−/− hepatic mitochondria to phenylarsine oxide but not t-butylhydroperoxide mediated swelling in the presence of calcium ion.
Intact mitochondria isolated from wild-type (WT) and iPLA2γ−/− (KO) mouse liver were placed in swelling buffer without phosphate and preincubated with either 1 mM t-butylhydroperoxide (TBH) or buffer alone for 10 min at 23°C. For experiments with phenylarsine oxide (PAO), 10 µM (final) PAO or DMSO vehicle alone (1% final, v/v) was added prior to exposure to either 70 µM Ca$^{2+}$ or 10 µM EGTA (-Ca$^{2+}$). Mitochondrial swelling of WT (A) and KO (B) was measured by monitoring the change in absorbance at 540 nm at 15 s intervals at 23°C. Representative traces from 3-4 separate preparations are shown.

Figure 10. Attenuated release of cytochrome c from iPLA2γ−/− liver mitochondria in response to t-butylhydroperoxide and phenylarsine oxide in the presence of calcium ion.
A, Hepatic mitochondria from wild-type (WT) and iPLA2γ−/− (KO) mice (N=3 each) were isolated by differential centrifugation and the expression levels of total mitochondrial cytochrome c (Cyto c) were compared by Western blotting analysis with normalization against VDAC protein levels. B, Intact mitochondria isolated from wild-type (WT) and iPLA2γ−/− (KO) mouse livers were placed in swelling buffer with/without phosphate (Pi) and preincubated with 1 mM t-butylhydroperoxide (TBH) or buffer alone for 10 min at 23°C. For the experiments with phenylarsine oxide (PAO) stimulation, mitochondria were exposed to 10 µM PAO or DMSO vehicle alone (1% final, v/v) prior to initiation of swelling. Mitochondrial swelling of WT and KO was then triggered by addition of either 70 µM Ca$^{2+}$ or 10 µM EGTA.
EGTA. After 5 min (in the presence of P_i) or 10 min (in the absence of P_i) incubations, mitochondria were immediately pelleted by centrifugation at 12,000 \times g for 5 min. The resultant supernatants were collected and the amounts of cytochrome c released from the mitochondria were determined by Western blotting analysis.

**Figure 11. Decreased respiratory capacity of iPLA_2γ^−/− hepatic mitochondria utilizing multiple substrates.**

Respiration of mitochondria isolated from wild-type (WT, open bars) and iPLA_2γ^−/− (KO, solid bars) mouse livers was determined by oxygen consumption in the presence of the indicated mitochondrial substrates and inhibitors utilizing an OROBOROS apparatus as described in “Experimental Procedures.” Oxygen consumption was monitored in the presence of pyruvate (P)/malate (M) (A), palmitoyl-L-carnitine (Pc)/malate (M) (B), glutamate (G)/malate (M) (C) or pyruvate (P)/glutamate (G)/malate (M) (D). Measurement of oxygen consumption during mitochondrial respiration in the presence of the indicated substrates was performed by sequential additions of ADP (state 3), succinate (Succ, state 3 max) and the respiration inhibitors: rotenone (Rot, state 3) and oligomycin (O, state 4). Data from 5 separate preparations are presented as means ± SEM. *, P<0.05 and **, P<0.01 when compared with WT under the same condition. E, Cytochrome c oxidase activity in WT and KO mitochondria was determined by measuring oxygen consumption following addition of ascorbate and tetramethyl-p-phenylenediamine (TMPD) in the presence of the indicated substrates, ADP and inhibitors including rotenone, oligomycin, and antimycin A.
Figure 1.

A. 

B.

WT KO

CyPD

ANT

VDAC

WT KO

CyPD

ANT

VDAC

WT/EGTA

WT/Ca^{2+}

KO/EGTA

KO/Ca^{2+}

[^14]C]Arachidonic Acid (pmol/mg protein)

Time (min)

* * ¶
Figure 2.

A. Time (min)

WT

KO

B. Time (min)

WT

KO

C. WT

KO

2min

5min

P<0.005
Figure 3.

A.

B.

\[ \Delta A_{540} (\%) \]

\[ \text{Time (min)} \]

\[ \text{WT} \]

\[ \text{KO} \]

\[ \text{Ca}^{2+} \]

\[ \text{Ca}^{2+} + \text{CsA} \]

\[ -\text{Ca}^{2+} \]
Figure 4.

WT KO

CyPD

ANT

VDAC

WT/EGTA

WT/Ca²⁺

KO/EGTA

KO/Ca²⁺

Relative Protein Level (a.u.)

0 0.2 0.4 0.6 0.8 1 1.2 1.4

WT KO

CyPD

P<0.05

Relative Protein Level (a.u.)

0 0.2 0.4 0.6 0.8 1 1.2

WT KO

ANT

[¹⁴C]Arachidonic Acid (pmol/mg protein)

Time (min)

* * * * ¶ ¶ ¶ ¶
Figure 5.

A.

B.

KO

KO

- Ca²⁺ + Pi

Ca²⁺ + Pi

Ca²⁺ + Pi + 5 μM PA

Ca²⁺ + Pi + 10 μM PA

Ca²⁺ + Pi + 10 μM PA + CsA

Ca²⁺ + Pi + 10 μM PA
Figure 6.

**Diagram Description:**
- The chart illustrates the change in absorbance (ΔA540) over time (in minutes) for different conditions involving calcium ions (Ca²⁺) and various coenzyme A (CoA) derivatives.
- The x-axis represents time in minutes, ranging from 0 to 15.
- The y-axis represents ΔA540 (%), ranging from -60 to 0.
- The graph shows different lines and trends for various conditions:
  - WT (Wild Type)
  - Ca²⁺ + Acetyl CoA
  - Ca²⁺ + 16:0 CoA
  - Ca²⁺ + S-HD CoA
  - Ca²⁺ + CoASH

**Legend:**
- The y-axis also includes a legend with different symbols representing various conditions.
- The chart includes error bars to indicate the variability or uncertainty in the measured data.

**Statistical Significance:**
- The bars on the right side of the chart indicate statistical significance with ** and ** symbols, suggesting a significant difference between conditions.

**Equations/Reactions:**
- WT
- Ca²⁺ + Acetyl CoA
- Ca²⁺ + 16:0 CoA
- Ca²⁺ + S-HD CoA
- Ca²⁺ + CoASH
- Calcium concentration variation:
  - without Ca²⁺ (-Ca²⁺)
  - with Ca²⁺ (+Ca²⁺)
Figure 7.

A. 

B. 

WT

KO
Figure 8.

A. 

\[ \text{Ca}^{2+} + 16:0 \text{CoA} + \text{LC} \]

B. 

\[ \text{Ca}^{2+} + \text{SHD CoA} + \text{LC} \]

C. 

\[ \text{P}<0.001 \]
Figure 9.
Figure 10.

A.

B. -Pi (t=10min) +Pi (t=5min)
Figure 11.

A. Pyruvate

B. Palmitoyl-L-Carnitine

C. Glutamate

D. Pyruvate/Glutamate

E. Cytochrome C Oxidase Activity
Genetic ablation of calcium-independent phospholipase A$_2$$\gamma$ (iPLA$_2$$\gamma$) attenuates calcium-induced opening of the mitochondrial permeability transition pore and resultant cytochrome C release

Sung Ho Moon, Christopher M. Jenkins, Michael A. Kiebish, Harold F. Sims, David J. Mancuso and Richard W. Gross

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