THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 35, pp. 29837–29850, August 24, 2012

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*

Received for publication, April 18, 2012, and in revised form, June 29, 2012 Published, JBC Papers in Press, July 9, 2012, DOI 10.1074/jbc.M112.373654

Sung Ho Moon[‡], Christopher M. Jenkins[‡], Michael A. Kiebish[‡], Harold F. Sims[‡], David J. Mancuso[‡], and Richard W. Gross[‡§¶1]

From the Departments of [‡]Medicine and [§]Developmental Biology, Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110 and the [¶]Department of Chemistry, Washington University, St. Louis, Missouri 63130

Background: The composition and regulation of the mitochondrial permeability transition pore (mPTP) are incompletely understood.
Results: Calcium-induced mPTP opening was markedly inhibited in iPLA$_2\gamma^{-/-}$ mice but was robustly activated by acyl-CoA in wild-type mice.
Conclusion: iPLA$_2\gamma$ is a critical mechanistic participant in mPTP opening in a process that is modulated by cellular lipids.
Significance: iPLA$_2\gamma$ in conjunction with acyl-CoA integrates mPTP opening with cellular bioenergetics.

Herein, we demonstrate that calcium-independent phospholipase $A_2\gamma$ (iPLA$_2\gamma$) is a critical mechanistic participant in the calcium-induced opening of the mitochondrial permeability transition pore (mPTP). Liver mitochondria from iPLA$_2\gamma^{-/-}$ mice were markedly resistant to calcium-induced swelling in the presence or absence of phosphate in comparison with wild-type littermates. Furthermore, the iPLA$_2\gamma$ enantioselective inhibitor (*R*)-(*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one ((*R*)-BEL) was markedly more potent than (*S*)-BEL in inhibiting mPTP opening in mitochondria from wild-type liver in comparison with hepatic mitochondria from iPLA$_2\gamma^{-/-}$ mice. Intriguingly, low micromolar concentrations of long chain fatty acyl-CoAs and the non-hydrolyzable thioether analog of palmitoyl-CoA markedly accelerated Ca^{2+}-induced mPTP opening in liver mitochondria from wild-type mice. The addition of L-carnitine enabled the metabolic channeling of acyl-CoA through carnitine palmitoyltransferases (CPT-1/2) and attenuated the palmitoyl-CoA-mediated amplification of calcium-induced mPTP opening. In contrast, mitochondria from iPLA$_2\gamma^{-/-}$ mice were insensitive to fatty acyl-CoA-mediated augmentation of calcium-induced mPTP opening. Moreover, mitochondria from iPLA$_2\gamma^{-/-}$ mouse liver were resistant to Ca^{2+}/*t*-butyl hydroperoxide-induced mPTP opening in comparison with wild-type littermates. In support of these findings, cytochrome *c* release from iPLA$_2\gamma^{-/-}$ mitochondria was dramatically decreased in response to calcium in the presence or absence of either *t*-butyl hydroperoxide or phenylarsine oxide in comparison with wild-type littermates. Collectively, these results identify iPLA$_2\gamma$ 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 mitochondria-mediated necrotic cell death is the opening of the mitochondrial permeability transition pore (mPTP)[2] (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 the phosphate ion (P_i). However, the mechanism by which

* This work was supported, in whole or in part, by National Institutes of Health Grants 5PO1HL57278 and RO1HL41250. R. W. G. has financial relationships with LipoSpectrum and Platomics.
[1] To whom correspondence should be addressed: Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8020, St. Louis, MO 63110. Tel.: 314-362-2690; Fax: 314-362-1402; E-mail: rgross@wustl.edu.

[2] The abbreviations used are: mPTP, mitochondrial permeability transition pore; ANT, adenine nucleotide translocase; CPT, carnitine palmitoyltransferase; CyPD, cyclophilin D; CsA, cyclosporine A; iPLA$_2\gamma$, calcium-independent phospholipase A$_2\gamma$; PAO, phenylarsine oxide; PiC, phosphate carrier; Pyr, pyrrolidine; ROS, reactive oxygen species; VDAC, voltage-activated anion channel; TBH, *t*-butyl hydroperoxide; BEL, (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one.

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 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 cardiolipin (15, 16). Cytochrome *c* is known to bind cardiolipin through both charge-pairing and hydrophobic interactions (17, 18), and calcium displaces cytochrome *c* from cardiolipin, 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 localized to the mitochondrial matrix, which functions as an essential component of the mitochondrial permeability transition pore (2, 8). Cyclosporine A (CsA), an immunosuppressant that inhibits the protein phosphatase calcineurin, tightly binds CyPD in the mitochondrial matrix, resulting in potent desensitization of the mPTP to Ca^{2+}, P_i, 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 peptidyl prolyl *cis-trans* isomerase activity and/or its interaction with the 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 Ca^{2+}- and 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 their mechanisms of action are unknown (35–37). Notably, L-carnitine has been proposed to protect against fatty acyl-CoA augmentation of mPTP opening by facilitating removal of fatty acyl-CoA by carnitine palmitoyltransferase-1 (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 (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL) blocked the Ca^{2+}-induced increase in swelling of rat liver mitochondria (41). Supporting these results, Schnellmann and co-workers (42, 43) identified a racemic BEL-sensitive iPLA$_2$ in rabbit kidney cortex mitochondria responsible for Ca^{2+}-induced mitochondrial swelling. These sets of data both indicated that a member of the calcium-independent phospholipase A$_2$ family was probably 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 because all members of the iPLA$_2$ 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 iPLA$_2\gamma$. Through the use of a genetic iPLA$_2\gamma$ loss of function model in conjunction with enantioselective pharmacologic inhibition of iPLA$_2\gamma$, we now identify the fundamental role of iPLA$_2\gamma$ in calcium-induced mPTP opening and its modulation by fatty acid and fatty acyl-CoA. Collectively, the present study integrates alterations in mitochondrial bioenergetic function with mPTP opening in which iPLA$_2\gamma$ plays a central mechanistic role.

EXPERIMENTAL PROCEDURES

Reagents—1-Palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (55 mCi/mmol) was purchased from PerkinElmer Life Sciences. Racemic BEL was obtained from Cayman Chemical Co. (Ann Arbor, MI). The (*R*)- and (*S*)-enantiomers of BEL were prepared as described previously (48). *N*-[(2*S*,4*R*)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl]-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl] acrylamide (pyrrolidine; 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*-butyl hydroperoxide (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, and *S*-hexadecyl-CoA was prepared in our laboratory as described previously (49). All other reagents were purchased from either Sigma-Aldrich or Fisher.

Generation and Affinity Purification of a Rabbit Polyclonal Antibody against Human iPLA$_2\gamma$—All procedures for generation of iPLA$_2\gamma$ antibody were performed by Open Biosystems of Thermo Fisher Scientific. Briefly, male white New Zealand rabbits were initially immunized with 500 μg of the 20-mer peptide (CKINDWIKLKSDMYEGLPFF conjugated to keyhole limpet hemocyanin). After three booster immunizations at 2-week intervals, serum was collected, and iPLA$_2\gamma$ antibody was puri-

fied by affinity chromatography using the peptide covalently bound to resin.

Animal Studies and Generation of iPLA$_2\gamma^{-/-}$ 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 iPLA$_2\gamma$ 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-month-old male wild-type littermates with male iPLA$_2\gamma$ knock-out mice.

Isolation of Hepatic Mitochondria—Wild-type and iPLA$_2\gamma^{-/-}$ 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 (0.21 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 rpm. Next, the homogenate was centrifuged for 5 min at 850 × *g*, and the supernatant was collected and centrifuged for 12,000 × *g* for 10 min. The pellet was resuspended in mitochondrial isolation buffer without BSA and centrifuged at 7,200 × *g* for 10 min, and the pellet was resuspended in mitochondrial isolation buffer 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 mPTP opening, isolated mitochondria from wild-type and iPLA$_2\gamma^{-/-}$ 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 presence of 1 mM KH_2PO_4 (for experiments with inorganic phosphate). Intact mitochondria were equilibrated with swelling buffer at 23 °C for 10 min. For experiments examining the effect of PLA$_2$ inhibitors, mitochondria were preincubated with either 5 μM (*R*)-BEL, 5 μM (*S*)-BEL, 1 μM Pyr, or DMSO vehicle alone (1%, v/v). Mitochondrial swelling was initiated by the addition of 70 μM $CaCl_2$ (final) with comparisons with the 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 the initiation of swelling by Ca^{2+}. Decreases in the absorbance (540 nm) of the mitochondria indicative of swelling were measured every 15 s using a SpectraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA).

Phospholipase A$_2$ Activity Assay—The mitochondrial pellet isolated from WT and iPLA$_2\gamma^{-/-}$ mouse liver was resuspended in ice-cold HEPES buffer (10 mM HEPES (pH 7.4), 1 mM DTT, 10% glycerol) and sonicated (10 1-s pulses) at 30% power. For the measurement of mitochondrial phospholipase activity with radiolabeled substrate, mitochondrial homogenates (0.1 mg of protein) in 300 μl of HEPES buffer were incubated with radiolabeled phosphatidylcholine substrates (~1 × 10^5 dpm of 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine) in the presence of 4 mM EGTA or 1 mM Ca^{2+} for 10, 20, and 30 min. Phospholipase reactions were terminated by the addition of 2 ml of chloroform/methanol/acetic acid (50:48:2, v/v/v) followed by the addition of 700 μl of water. Lipids were extracted into the chloroform and loaded onto channeled Partisil LK6D silica gel 60 Å 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 described previously (51).

Immunoblot Analyses—Mitochondrial proteins were separated by SDS-PAGE (10–15% polyacrylamide gels), transferred to polyvinylidine fluoride membranes by electroelution, and blocked with 5% nonfat 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% nonfat 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 respirometer (Innsbruck, Austria) with substrate and inhibitor additions as described previously (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 KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 1 g/liter 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 of O_2/ml at 105 kilopascals 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) plus glutamate (10 mM) and malate (5 mM) (state 2), followed by ADP (1.25 mM) (state 3), succinate (5 mM) (state 3 max), rotenone (0.5 μM), oligomycin (1 μM) (state 4), antimycin A (1 μM), and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (5 μM) with ascorbate (0.5 mM) (to measure cytochrome *c* oxidase activity).

Statistical Analyses—Values are expressed as mean ± S.E. The significance of experimental observations was determined by Student's *t* test, and results were considered significant at $p < 0.05$.

RESULTS

Genetic Ablation of iPLA$_2\gamma$ Results in Decreased Ca^{2+}-dependent PLA$_2$ Activity in Mouse Liver Mitochondria—To examine whether iPLA$_2\gamma$ was present in isolated murine liver mitochondria, we performed Western analyses of wild-type and iPLA$_2\gamma^{-/-}$ liver mitochondrial proteins using a custom affinity-purified antibody directed against the C terminus of iPLA$_2\gamma$ (CKINDWIKLKSDMYEGLPFF). Genetic ablation of iPLA$_2\gamma$ (50) resulted in complete elimination of immunoreactive bands ranging from 63 to 87 kDa, a predominant band at 52 kDa, and a single band at 45 kDa (Fig. 1*A*) using this highly sensitive antibody possessing the greatest specificity for iPLA$_2\gamma$ identified to date to the best of our knowledge. Only the protein

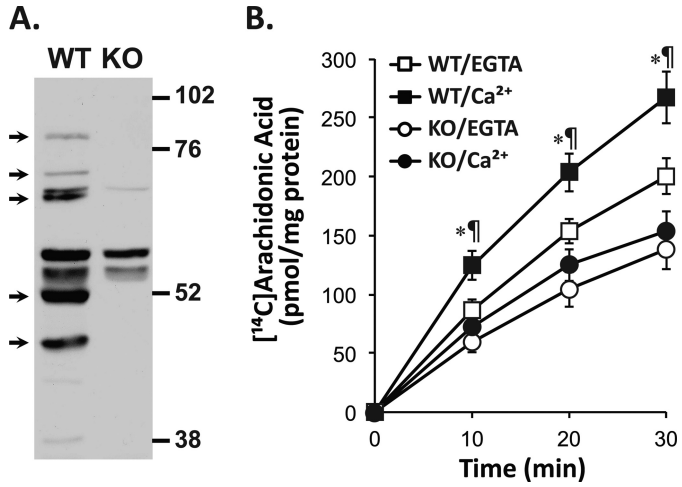

FIGURE 1. **Genetic ablation of murine PNPLA8 results in elimination of multiple isoforms of iPLA$_2\gamma$ in hepatic mitochondria and decreased Ca^{2+}-stimulated PLA$_2$ activity.** Hepatic mitochondria from WT and iPLA$_2\gamma^{-/-}$ (KO) mice were isolated by differential centrifugation and briefly sonicated as described under "Experimental Procedures." *A*, mitochondrial proteins (50 μg protein/lane) from WT and KO mice were resolved by SDS-PAGE (10% gel), transferred to PVDF membranes by electroelution, and probed with a rabbit polyclonal antibody directed against the C terminus of iPLA$_2\gamma$ for Western blot analysis utilizing an anti-rabbit IgG HRP conjugate and ECL reagents to visualize iPLA$_2\gamma$ protein. *B*, calcium-dependent and calcium-independent PLA$_2$ activities in WT and KO liver mitochondria. Exogenous 1-palmitoyl-2-[^{14}C]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 the 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 under "Experimental Procedures." Values are the average of four independent preparations ± S.E. (*error bars*). *, $p < 0.05$ when comparing EGTA *versus* Ca^{2+} treatment. ¶, $p < 0.005$ when comparing WT *versus* KO.

band at 58 kDa and a faint doublet at ~55 kDa are nonspecific cross-reacting proteins that are observable. All of the bands observed by Western blotting utilizing our antibody directed against the C terminus of iPLA$_2\gamma$ are typically detected in liver tissue homogenates and are completely blocked by the cognate peptide. The observed iPLA$_2\gamma$ polypeptides are most consistent with the use of alternate in-frame ATG start codons to generate the 87.4- and 73.6-kDa isoforms. Although there are ATG codons in the mRNA that could be predicted to produce the 62.4- 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 intramitochondrial proteolytic processing or mitochondria-associated membrane proteins (*e.g.* peroxisomal proteins). Additionally, because there is neither an alternatively spliced transcript nor an alternate translational ATG start site that could encode the observed 52- and 45-kDa isoforms, presumably they are generated through proteolytic processing. It is interesting to note that all of the identified mitochondrial iPLA$_2\gamma$ polypeptides are of sufficient length to possess both the C-terminal KINDWIKLKSDMY-EGLPFF sequence recognized by the antibody and the GVSTG active site. Thus, these results specifically demonstrate the loss of multiple isoforms of iPLA$_2\gamma$ in liver mitochondria in the iPLA$_2\gamma^{-/-}$ mouse.

Next, we measured both calcium-dependent and calcium-independent PLA$_2$ activities in WT and iPLA$_2\gamma^{-/-}$ liver mitochondria. Although iPLA$_2\gamma$ does not require calcium ion for membrane association or catalysis, we have recently demonstrated that iPLA$_2\gamma$ 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-[^{14}C]arachidonoyl-*sn*-glycerophosphorylcholine resulted in a time-dependent increase in the release of [^{14}C]arachidonic acid, which was enhanced by the presence of calcium ion (Fig. 1*B*). In contrast, iPLA$_2\gamma^{-/-}$ liver mitochondrial sonicates exhibited ~30–40% lower PLA$_2$ activity in the presence of EGTA and ~50% lower PLA$_2$ activity in incubations containing Ca^{2+} in comparison with wild-type controls (Fig. 1*B*). More importantly, calcium-facilitated PLA$_2$ activity was virtually absent in iPLA$_2\gamma^{-/-}$ liver mitochondria. These results demonstrate a significant decrease in PLA$_2$ activity in iPLA$_2\gamma^{-/-}$ liver mitochondria, indicating a loss of iPLA$_2\gamma$ function that cannot be compensated by increased expression of other intracellular phospholipases A$_2$.

The (R)-BEL-inhibitable Ca^{2+}/Phosphate-induced Swelling Present in Mitochondria from Wild-type Mouse Liver Is Dramatically Attenuated in Mitochondria Prepared from iPLA$_2\gamma^{-/-}$ Mice—Previous work by Pfeiffer and colleagues (41) indicated that a calcium-independent phospholipase A$_2$ was probably responsible for the Ca^{2+}-mediated swelling of rat liver mitochondria because pretreatment with racemic BEL blocked mitochondrial swelling with a concomitant ablation of free fatty acid release. To further investigate the role(s) of iPLA$_2$(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 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 CsA (Fig. 2*A*). 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. 2, *A* and *C*). In contrast, the cPLA$_2\alpha$-selective inhibitor Pyr did not affect calcium-induced mitochondrial swelling (Fig. 2, *A* and *C*).

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 in the presence of phosphate. The rate of calcium-induced mitochondrial swelling at early time points (2 and 5 min) was markedly attenuated relative to wild-type mitochondria (Fig. 2, *B* and *C*). As was the case in wild-type mitochondria, mPTP opening was cyclophilin D-dependent because 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

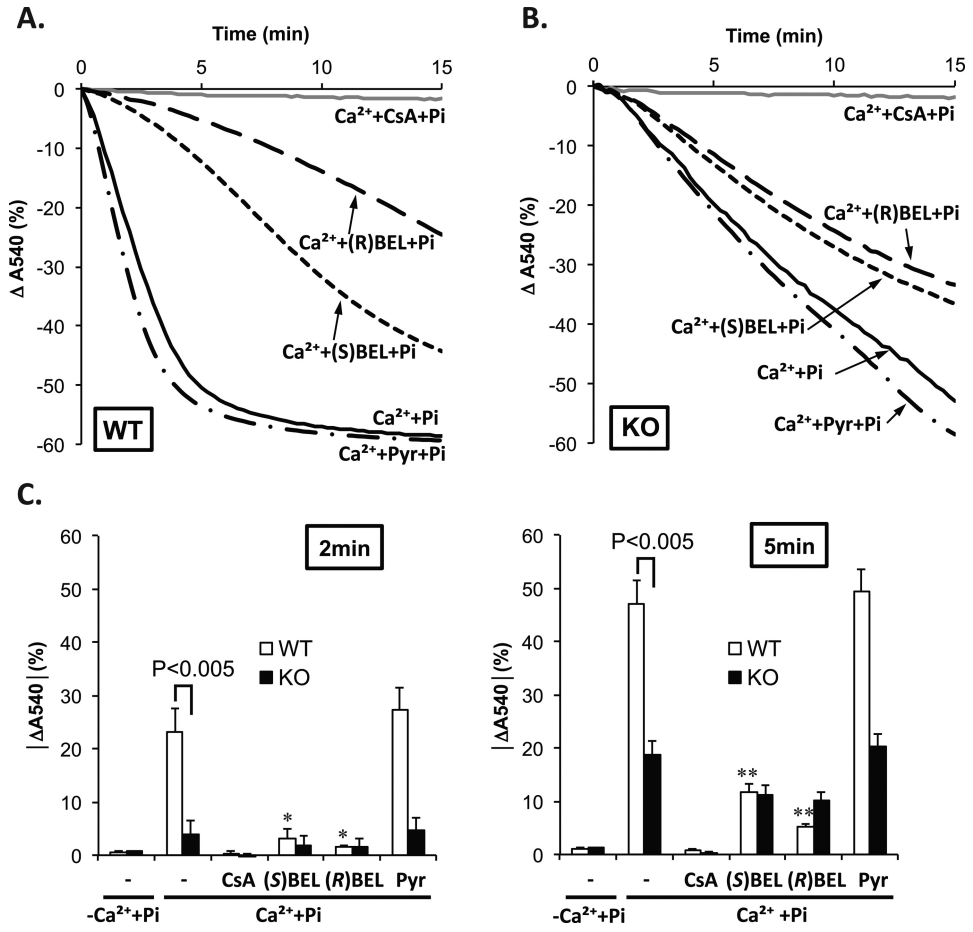

FIGURE 2. **Alterations in the kinetics of the calcium-induced swelling of liver mitochondria from wild-type and iPLA$_2\gamma^{-/-}$ mice in the presence of phosphate by pharmacologic inhibition of different phospholipases A$_2$.** Hepatic mitochondria were isolated by differential centrifugation from WT and iPLA$_2\gamma^{-/-}$ (KO) mice and resuspended in swelling buffer containing 0.23 M mannitol, 70 mM sucrose, 1 mM KH_2PO_4, 5 mM succinate, and 2.5 μM rotenone. Following preincubation with either 5 μM (*S*)-BEL, 5 μM (*R*)-BEL, 1 μM Pyr, 1 μM cyclosporine A (CsA), or DMSO vehicle alone (1%, v/v) for 10 min at 23 °C prior to exposure to either 70 μM Ca^{2+} or 10 μM 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* (*, $p < 0.005$; **, $p < 0.0005$ when compared with Ca^{2+} + P_i treatment without inhibitors). Values are the average of four independent preparations ± S.E. (*error bars*).

and (*S*)-BEL were equipotent in inhibiting the calcium-mediated swelling of iPLA$_2\gamma^{-/-}$ liver mitochondria (Fig. 2*B*), indicating the loss of an (*R*)-BEL-inhibitable component (iPLA$_2\gamma$) and the likely involvement of one or more components that are 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 comparison with incubations containing exogenous phosphate ion (Fig. 3*A* compared with Fig. 2*A*). In contrast to the virtually complete inhibition of Ca^{2+}-induced mitochondrial swelling by CsA in the presence of phosphate ion (Fig. 2*A*), CsA partially abolished swelling at later time points when P_i was omitted (Fig. 3*A*). In the absence of calcium ion, liver mitochondria from iPLA$_2\gamma^{-/-}$ mice displayed similar amounts of spontaneous swelling as their wild-type littermates (Fig. 3, compare *A* and *B*). 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. 3*B*). 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. Although no significant differences in VDAC and ANT protein levels were manifest in iPLA$_2\gamma^{-/-}$ liver mitochondria relative to wild-type control,

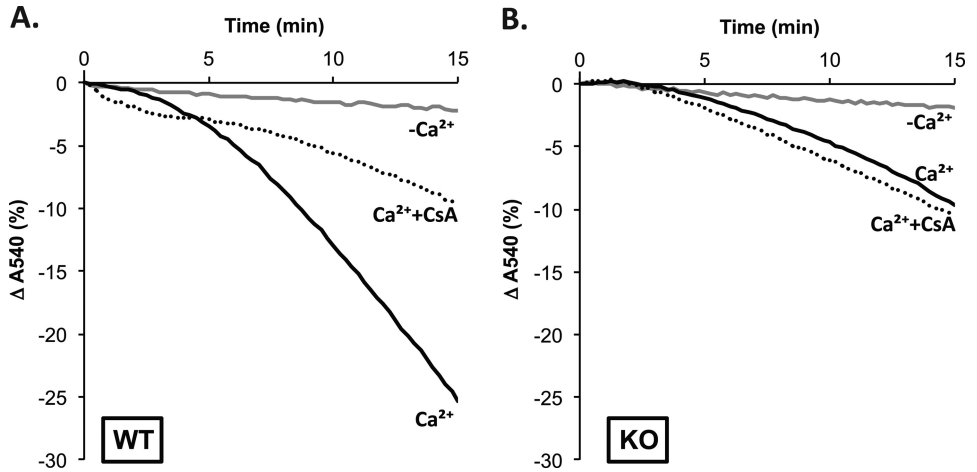

FIGURE 3. **Resistance of iPLA$_2\gamma^{-/-}$ hepatic mitochondria to Ca^{2+}-induced swelling in the absence of phosphate.** Liver mitochondria were isolated by differential centrifugation from WT and iPLA$_2\gamma^{-/-}$ (KO) mice and resuspended in swelling buffer containing 0.23 M mannitol, 70 mM sucrose, 5 mM succinate, and 2.5 μM rotenone without P_i as described under "Experimental Procedures." Intact mitochondria were then preincubated with either 1 μM CsA or DMSO vehicle alone (1%, v/v) for 10 min 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 light scattering at 540 nm at 15-s intervals at 23 °C. Representative traces from four independent preparations are shown.

CyPD content was modestly increased (~1.8-fold higher in iPLA$_2\gamma^{-/-}$ liver mitochondria than in their wild-type counterparts) (Fig. 4). This moderately increased CyPD protein level mediated by up-regulated expression and/or decreased degradation in iPLA$_2\gamma^{-/-}$ 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$_2\gamma^{-/-}$ mitochondria to Ca^{2+}/P_i-induced swelling, we measured iPLA$_2\gamma^{-/-}$ liver mitochondrial swelling in the presence of 5 or 10 μM palmitic acid. 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. 5*A*). To determine if the observed palmitic acid-rescued swelling of iPLA$_2\gamma^{-/-}$ mitochondria was mediated by CsA-sensitive mPTP opening, we preincubated hepatic mitochondria from iPLA$_2\gamma^{-/-}$ mice with 1 μM CsA. The Ca^{2+}/P_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. 5*B*).

Fatty Acyl-CoA and Its Non-hydrolyzable Thioether Analog Activate Ca^{2+}-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). The addition of a submicellar concentration of palmitoyl-CoA (5 μM) to wild-type liver mitochondria in the absence of P_i facilitated calcium-induced mitochondrial swelling (Fig. 6). Both oleoyl-CoA and

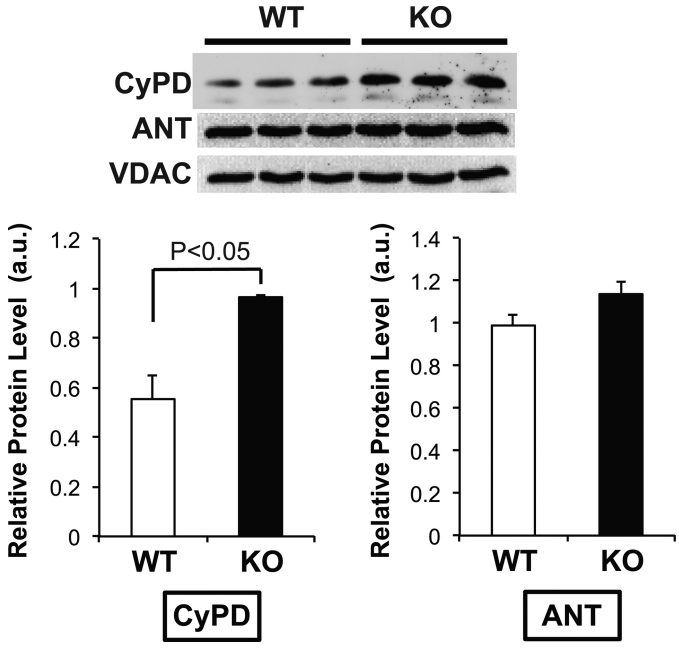

FIGURE 4. **Increased expression of cyclophilin D, but not the ANT and VDAC, in iPLA$_2\gamma^{-/-}$ liver mitochondria.** Liver mitochondria isolated from WT and iPLA$_2\gamma^{-/-}$ (KO) mice by differential centrifugation were sonicated, and mitochondrial proteins were resolved by SDS-PAGE prior to Western blot analysis as described under "Experimental Procedures." Immunoblots of CyPD, ANT, and VDAC are shown in the *top panel* (three separate preparations each of WT and KO mice). Relative protein expression levels of CyPD and ANT were determined by densitometry and normalized against VDAC in the *bottom panel*. Values are the means of normalized blot intensities in arbitrary units (*a.u.*) with S.E. (*error bars*).

arachidonoyl-CoA induced similar increases in Ca^{2+}-mediated mPTP opening (Fig. 6, *right*). In contrast, 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, 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 is required to effect fatty acyl-CoA-mediated modulation of mPTP opening.

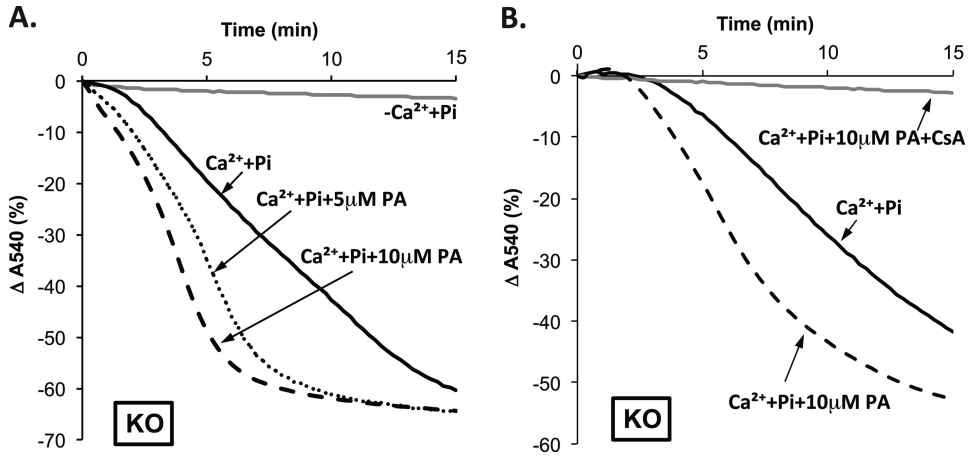

FIGURE 5. **Reconstitution of Ca^{2+}/phosphate-induced swelling of iPLA$_2\gamma^{-/-}$ mitochondria by palmitic acid.** Intact mitochondria isolated from iPLA$_2\gamma^{-/-}$ mouse liver were placed in swelling buffer with 1 mM P_i and equilibrated for 10 min at 23 °C. The indicated concentrations of palmitic acid or ethanol vehicle alone (0.5% final, v/v) were added prior to measuring mitochondrial swelling (ΔA_{540}) initiated by the addition of 70 μM Ca^{2+} or 10 μM EGTA ($-Ca^{2+}$) (*A*). Ca^{2+}/P_i-induced iPLA$_2\gamma^{-/-}$ hepatic mitochondrial swelling was monitored in the presence of 10 μM palmitic acid after pretreatment with 1 μM CsA or DMSO vehicle alone (1%, v/v) (*B*).

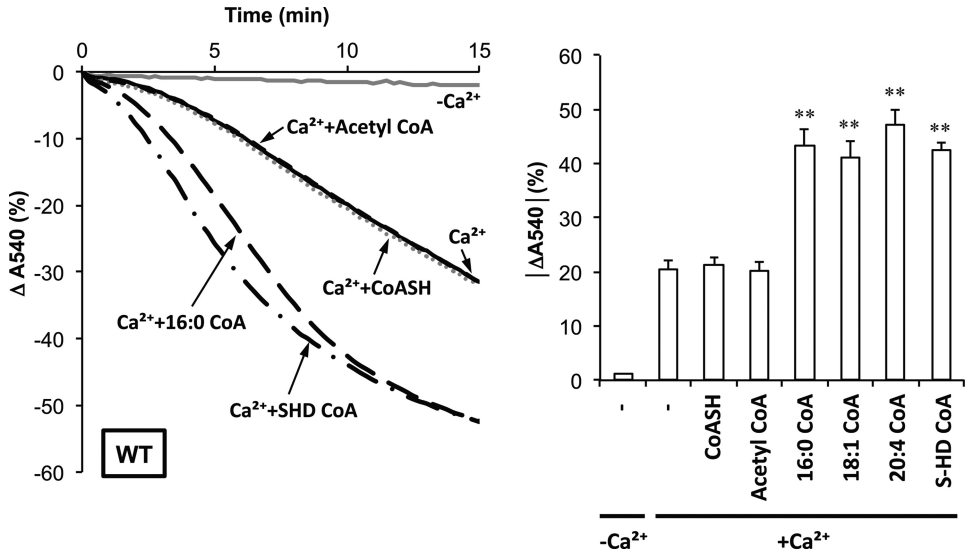

FIGURE 6. **Augmentation of Ca^{2+}-mediated mitochondrial swelling by fatty acyl-CoA molecular species but not short-chain acyl-CoA or CoASH.** Intact mitochondria from WT mouse liver were placed in swelling buffer without phosphate and equilibrated for 10 min at 23 °C. The impact of the addition of either acetyl-, palmitoyl (16:0)-, oleoyl (18:1)-, arachidonoyl (20:4)-, *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*). Net changes in absorbance at 540 nm for 10 min are shown on the *right*. **, $p < 0.00005$ when compared with Ca^{2+} treatment without acyl CoA or free CoASH. Values are the average of 5–6 preparations ± S.E. (*error bars*).

Sensitivity of Ca^{2+}-induced Fatty Acyl-CoA-augmented Mitochondrial Swelling to CsA Inhibition—Next, we sought to determine whether the fatty acyl-CoA enhancement of Ca^{2+}-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, preincubation of wild-type liver mitochondria with CsA resulted in nearly complete inhibition of mPTP opening mediated by Ca^{2+} alone (Fig. 7*A*). In addition, CsA potently inhibited the accelerated swelling of wild-type mitochondria enhanced by palmitoyl-CoA in the presence of calcium ion (Fig. 7*A*). Dramatic differences in the rates of mitochondrial swelling are apparent in iPLA$_2\gamma^{-/-}$ mitochondria exposed to Ca^{2+} in the presence or absence of palmitoyl-CoA in comparison with wild-type mitochondria (Fig. 7*B*), 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$_2\gamma^{-/-}$ mitochondria at early time points (Fig. 7*B*), 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$_2\gamma^{-/-}$ in calcium-induced mPTP opening and its modulation by palmitoyl-CoA.

Effects of L-Carnitine through CPT-1 on Ca^{2+}-induced Mitochondrial Swelling—Utilization of fatty acyl-CoA substrates for mitochondrial β-oxidation requires L-carnitine for conjugation of the fatty acyl thioester to carnitine catalyzed by CPT-1 to

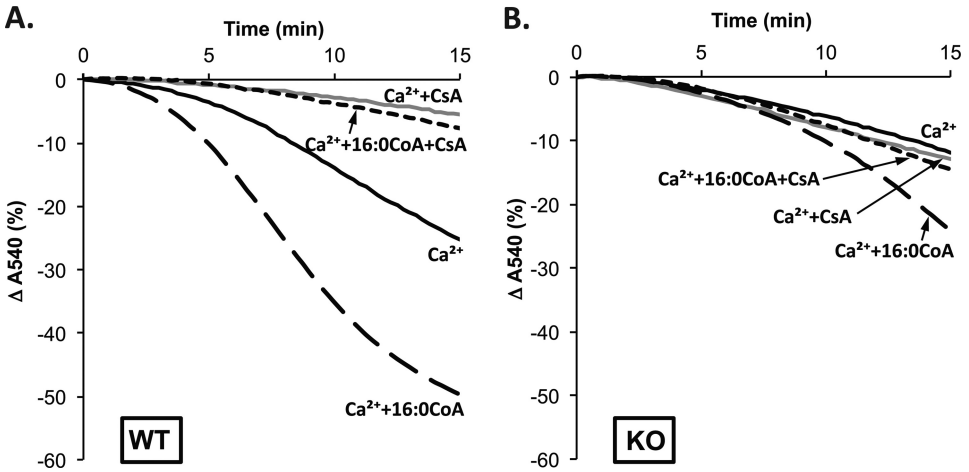

FIGURE 7. **Resistance of iPLA$_2\gamma^{-/-}$ hepatic mitochondria to fatty acyl-CoA-enhanced swelling in the presence of Ca^{2+}.** Intact mitochondria from WT and iPLA$_2\gamma^{-/-}$ (KO) mice were placed in swelling buffer without P_i and preincubated with 1 μM CsA or DMSO vehicle alone (1% final, 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 traces from four independent preparations are shown.

form acylcarnitine. To assess whether increased flux of fatty acyl-CoAs into mitochondria influenced the impact of fatty acyl-CoA-mediated augmentation of mitochondrial swelling, we examined the effects of supplementation with L-carnitine on mPTP opening in wild-type mitochondria. In control experiments, L-carnitine alone or palmitoyl-L-carnitine had no effect on the Ca^{2+}-mediated swelling of wild-type liver mitochondria (Fig. 8). However, interestingly, the addition of L-carnitine largely reversed the palmitoyl-CoA-enhanced swelling after calcium challenge. In marked contrast, L-carnitine was unable to prevent the mitochondrial swelling induced by the non-hydrolyzable thioether analog of palmitoyl-CoA, *S*-hexadecyl-CoA (Fig. 8), indicating that cleavage and conjugation of the acyl moiety to L-carnitine was necessary for the observed protection. These results suggest that metabolic channeling of fatty acyl-CoA into acyl-carnitine for β-oxidative pathways by L-carnitine and CPT-1 attenuates the fatty acyl-CoA augmentation of mPTP opening. Collectively, these results demonstrate the integrated roles of mitochondrial metabolic flux with mPTP opening.

Genetic Ablation of iPLA$_2\gamma$ Confers Resistance to Tertiary Butyl Hydroperoxide- 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 iPLA$_2\gamma^{-/-}$ 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 iPLA$_2\gamma^{-/-}$ liver mitochondria. In control experiments with liver mitochondria isolated from WT mice, calcium-induced mitochondrial swelling was significantly augmented by 1 mM TBH, especially in the later phase (Fig. 9*A*). In contrast, mPTP opening of liver mitochondria from iPLA$_2\gamma^{-/-}$ mice was insensitive to TBH (Fig. 9*B*). Next, we determined whether PAO, a potent inducer of mPTP opening through oxidation of components of the mPTP, was able to augment mPTP opening in mitochondria from iPLA$_2\gamma^{-/-}$ 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 iPLA$_2\gamma^{-/-}$ mitochondria (Fig. 9). These results demonstrate that PAO-mediated mitochondrial swelling is not affected by the absence of iPLA$_2\gamma$ and demonstrate that the mPTP machinery is functional in hepatic mitochondria prepared from the iPLA$_2\gamma^{-/-}$ mouse.

Previously, it has been demonstrated that the opening of mPTP facilitates release of cytochrome *c* from the inner mitochondria membrane, triggering the execution of the intrinsic pathway of apoptosis. Next, we examined if the observed alterations in the swelling of iPLA$_2\gamma^{-/-}$ liver mitochondria were accompanied by cytochrome *c* release. Expression levels of cytochrome *c* protein were not significantly different in wild-type *versus* iPLA$_2\gamma^{-/-}$ liver mitochondria (Fig. 10*A*). However, in wild-type controls, marked release of cytochrome *c* was observed after calcium ion challenge. The addition of TBH or PAO further increased calcium-induced cytochrome *c* release (Fig. 10*B*). In sharp contrast, virtually no release of cytochrome *c* was observed in iPLA$_2\gamma^{-/-}$ liver mitochondria in response to calcium or TBH, whereas modest cytochrome *c* release was elicited by PAO in the presence of phosphate ion (Fig. 10*B*). Collectively, these results demonstrate the important roles of mitochondrial iPLA$_2\gamma$ 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 iPLA$_2\gamma^{-/-}$ Hepatic Mitochondria—Functional analysis of oxygen consumption in hepatic mitochondria revealed a consistent deficiency in complex I-mediated substrate utilization in liver mitochondria from iPLA$_2\gamma^{-/-}$ mice compared with wild-type littermates. The data demonstrated a 50% decrease in state 3 respiration stimulated by pyruvate, palmitoyl-L-carnitine, glutamate, or pyruvate/glutamate (Fig. 11, *A–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. 11*C*). This result is most likely due to the greater deficiency in glutamate-stimulated respiration found in the iPLA$_2\gamma^{-/-}$ mice. Analysis of complex IV activity by tetramethyl-*p*-phenylenediamine and ascorbate treatment revealed

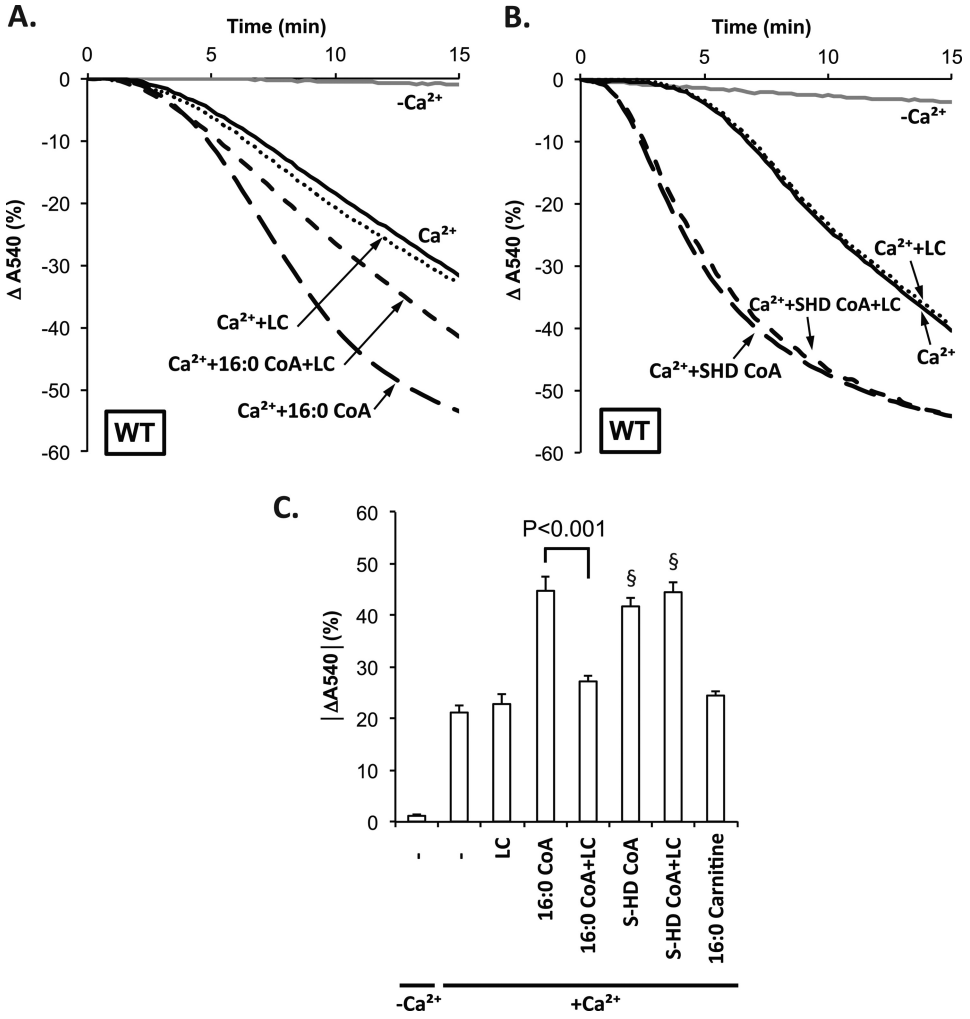

FIGURE 8. **Impact of L-carnitine on fatty acyl-CoA and fatty acyl thioether CoA enhanced swelling of wild-type liver mitochondria.** Intact mitochondria from WT mice were placed in swelling buffer without phosphate and preincubated with 1 mM L-carnitine (*LC*) or buffer alone for 10 min at 23 °C. Palmitoyl (16:0)-CoA, non-hydrolyzable acyl thioether CoA (*SHD CoA*), or 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 (*A* and *B*). Net changes in A_{540} for 10 min are shown in *C* (§, $p < 10^{-5}$ when compared with Ca^{2+} treatment). Values are the average of 4–6 separate preparations ± S.E. (*error bars*).

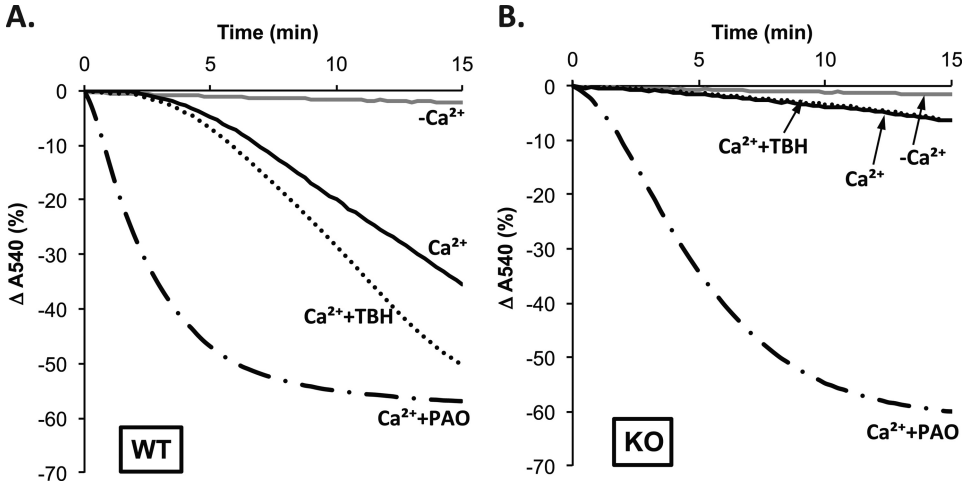

FIGURE 9. **Sensitivity of iPLA$_2\gamma^{-/-}$ hepatic mitochondria to phenylarsine oxide- but not *t*-butyl hydroperoxide-mediated swelling in the presence of calcium ion.** Intact mitochondria isolated from WT and iPLA$_2\gamma^{-/-}$ (KO) mouse liver were placed in swelling buffer without phosphate and preincubated with either 1 mM TBH or buffer alone for 10 min at 23 °C. For experiments with 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.

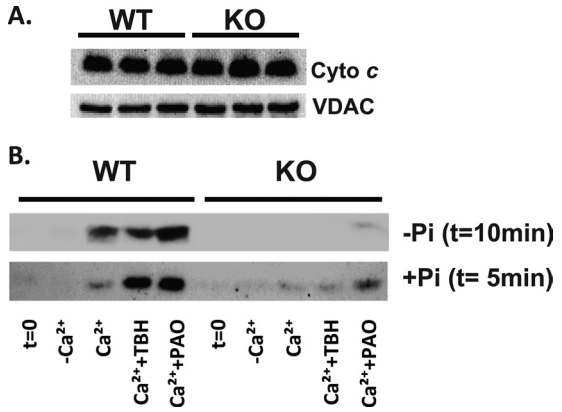

FIGURE 10. **Attenuated release of cytochrome *c* from iPLA$_2\gamma^{-/-}$ liver mitochondria in response to *t*-butyl hydroperoxide and phenylarsine oxide in the presence of calcium ion.** *A*, hepatic mitochondria from WT and iPLA$_2\gamma^{-/-}$ (KO) mice ($n = 3$ each) were isolated by differential 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 WT and iPLA$_2\gamma^{-/-}$ (KO) mouse livers were placed in swelling buffer with/without P_i and preincubated with 1 mM TBH or buffer alone for 10 min at 23 °C. For the experiments with 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 the addition of either 70 μM Ca^{2+} or 10 μM 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.

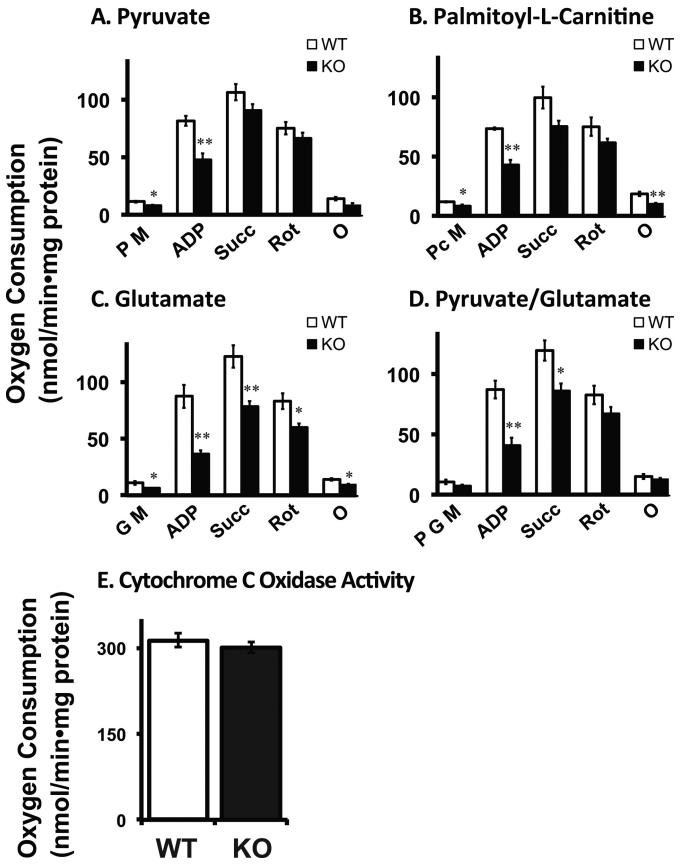

FIGURE 11. **Decreased respiratory capacity of iPLA$_2\gamma^{-/-}$ hepatic mitochondria utilizing multiple substrates.** Respiration of mitochondria isolated from WT (*open bars*) and iPLA$_2\gamma^{-/-}$ (KO) (*solid bars*) mouse livers was determined by oxygen consumption in the presence of the indicated mitochondrial substrates and inhibitors utilizing an OROBOROS apparatus as described under "Experimental Procedures." Oxygen consumption was monitored in the presence of pyruvate/malate (*P M*) (*A*), palmitoyl-L-carnitine/malate (*Pc M*) (*B*), glutamate/malate (*G M*) (*C*), or pyruvate/glutamate/malate (*P G 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 five separate preparations are presented as means ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$ when compared with WT under the same conditions. *E*, cytochrome *c* oxidase activity in WT and KO mitochondria was determined by measuring oxygen consumption following the addition of ascorbate and tetramethyl-*p*-phenylenediamine in the presence of the indicated substrates, ADP, and inhibitors, including rotenone, oligomycin, and antimycin A.

no change in cytochrome *c* oxidase activity in isolated hepatic mitochondria in iPLA$_2\gamma^{-/-}$ compared with wild-type mice (Fig. 11*E*). 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 iPLA$_2\gamma^{-/-}$ mice demonstrate a compromised capacity to efficiently utilize the primary tricarboxylic acid cycle anapleurotic 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 the mitochondrial permeability transition pore (mPTP), has been implicated in multiple mechanisms of mitochondrial dysfunction, including alterations of mitochondria-driven energy metabolism and ion homeostasis (56), production of reactive oxygen species (2, 11, 57), and the release of proapoptotic factors (2, 11). Although previous studies using chemical inhibitors have suggested mitochondrial phospholipase A_2 activity to be involved in the induction of mPTP opening (36, 41, 42), the identity of the PLA_2(s) responsible for mitochondrial permeability transition and its mechanism of activation is largely unknown. In the current study, we have identified iPLA$_2\gamma$ as a critical component of the mPTP by genetic ablation and confirmed its importance through enantioselective mechanism-based inhibition. Furthermore, we have demonstrated that iPLA$_2\gamma$-mediated mPTP 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 about 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*-butyl hydroperoxide, was capable of inducing mPTP opening utilizing hepatic mitochondria from iPLA$_2\gamma^{-/-}$ mice. These results identify the substantial protection rendered by iPLA$_2\gamma$ loss of function and suggest that the selective inhibition of iPLA$_2\gamma$ could have salutary effects on certain pathophysiologic conditions resulting from ROS-medi-

ated damage. Collectively, the results of this study unambiguously demonstrate that iPLA$_2\gamma$ is a critical mechanistic component in the mPTP opening.

The mPTP is a Ca^{2+}-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 that is inhibitable by BEL could modulate Ca^{2+}-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 iPLA$_2\gamma$ function resulted in the remarkable resistance of liver mitochondria to Ca^{2+}-activated swelling in the presence or absence of phosphate ion. Furthermore, exogenous palmitate, which is a major fatty acid produced by the PLA$_1$ activity of iPLA$_2\gamma$, restored iPLA$_2\gamma^{-/-}$ 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 iPLA$_2\gamma^{-/-}$ liver mitochondria is largely reversible by CsA.

Recently, we have demonstrated the divalent cation-dependent activation of iPLA$_2\gamma$ 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 second 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 that 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 β-oxidation, then this response provides a physiologic adaptation 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 necro-apoptotic 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 β-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-enhanced but not *S*-hexadecyl-CoA-enhanced mitochondrial swelling selectively without affecting that 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 (65) has demonstrated that CPT-1 interacts with acyl-CoA synthetase and VDAC, forming hetero-oligomeric complexes in the mitochondrial outer membrane. Although knock-out 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).

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 because 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 pathophysiologic 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.* see Ref. 73). Notably, iPLA$_2\gamma^{-/-}$ mitochondria are markedly more resistant to Ca^{2+}-mediated swelling than their wild-type counterparts. Thus, the up-regulation 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 tricarboxylic acid 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 ROS by accelerating uncoupling and the loss of antioxidants, such as reduced glutathione, after mPTP opening (2, 11). Previously, 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 iPLA$_2\gamma^{-/-}$ liver mitochondria are markedly resistant to TBH-induced cytochrome *c* release in the presence of Ca^{2+}, which is consistent with the observed resistance to mPTP opening in mitochondria from iPLA$_2\gamma^{-/-}$ mice.

In contrast, PAO is a well established inhibitor of phosphotyrosine phosphatases. PAO has been shown to cause a rapid

and irreversible decrease in the mitochondrial free Ca^{2+} concentration (82). In addition, PAO has been shown to inhibit the mitochondrial ANT through cross-linking of vicinal cysteine residues in the ANT (positions 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 and co-workers (10) has provided evidence that the mitochondrial PiC may play a key role in mPTP formation and regulation. In this study, CyPD was demonstrated to bind to PiC in a CsA-dependent manner, and cross-linking of cysteine residues on PiC by PAO correlated with mPTP opening (10). Potent sensitization of liver mitochondrial swelling to Ca^{2+} by PAO in our study was observed in both WT and iPLA$_2\gamma^{-/-}$ 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, probably is not affected by ablation of iPLA$_2\gamma$ activity. However, interestingly, iPLA$_2\gamma$ is probably a participant in PAO-accelerated cytochrome *c* release because PAO facilitation of Ca^{2+}-induced cytochrome *c* release was significantly reduced in mitochondria from iPLA$_2\gamma^{-/-}$ livers in comparison with WT littermates.

Collectively, the results of the present study demonstrate the prominent roles of iPLA$_2\gamma$ 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 conspires to initiate the execution of cell death pathways after pathologic alterations in calcium, ROS, and/or toxic lipid metabolites.

REFERENCES

1. Halestrap, A. P., Kerr, P. M., Javadov, S., and Woodfield, K. Y. (1998) Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochim. Biophys. Acta* **1366,** 79–94
2. Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* **341**, 233–249
3. Halestrap, A. P., McStay, G. P., and Clarke, S. J. (2002) The permeability transition pore complex. Another view. *Biochimie* **84,** 153–166
4. Bernardi, P., Krauskopf, A., Basso, E., Petronilli, V., Blalchy-Dyson, E., Di Lisa, F., and Forte, M. A. (2006) The mitochondrial permeability transition from *in vitro* artifact to disease target. *FEBS J.* **273,** 2077–2099
5. Qian, T., Herman, B., and Lemasters, J. J. (1999) The mitochondrial permeability transition mediates both necrotic and apoptotic death of hepatocytes exposed to Br-A23187. *Toxicol Appl. Pharmacol.* **154,** 117–125
6. De Giorgi, F., Lartigue, L., Bauer, M. K., Schubert, A., Grimm, S., Hanson, G. T., Remington, S. J., Youle, R. J., and Ichas, F. (2002) The permeability transition pore signals apoptosis by directing Bax translocation and multimerization. *FASEB J.* **16,** 607–609
7. Nakayama, H., Chen, X., Baines, C. P., Klevitsky, R., Zhang, X., Zhang, H., Jaleel, N., Chua, B. H., Hewett, T. E., Robbins, J., Houser, S. R., and Molkentin, J. D. (2007) Ca^{2+}- and mitochondria-dependent cardiomyocyte necrosis as a primary mediator of heart failure. *J. Clin. Invest.* **117,** 2431–2444
8. Javadov, S., Karmazyn, M., and Escobales, N. (2009) Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases. *J. Pharmacol. Exp. Ther.* **330,** 670–678
9. Halestrap, A. P., and Pasdois, P. (2009) The role of the mitochondrial permeability transition pore in heart disease. *Biochim. Biophys. Acta* **1787,** 1402–1415
10. Leung, A. W., Varanyuwatana, P., and Halestrap, A. P. (2008) The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition. *J. Biol. Chem.* **283,** 26312–26323
11. Kroemer, G., and Reed, J. C. (2000) Mitochondrial control of cell death. *Nat. Med.* **6,** 513–519
12. Choi, S. Y., Gonzalvez, F., Jenkins, G. M., Slomianny, C., Chretien, D., Arnoult, D., Petit, P. X., and Frohman, M. A. (2007) Cardiolipin deficiency releases cytochrome c from the inner mitochondrial membrane and accelerates stimuli-elicited apoptosis. *Cell Death Differ.* **14,** 597–606
13. Bradley, J. M., Silkstone, G., Wilson, M. T., Cheesman, M. R., and Butt, J. N. (2011) Probing a complex of cytochrome *c* and cardiolipin by magnetic circular dichroism spectroscopy. Implications for the initial events in apoptosis. *J. Am. Chem. Soc.* **133,** 19676–19679
14. Tait, S. W., and Green, D. R. (2010) Mitochondria and cell death. Outer membrane permeabilization and beyond. *Nat. Rev. Mol. Cell Biol.* **11,** 621–632
15. Shidoji, Y., Hayashi, K., Komura, S., Ohishi, N., and Yagi, K. (1999) Loss of molecular interaction between cytochrome *c* and cardiolipin due to lipid peroxidation. *Biochem. Biophys. Res. Commun.* **264,** 343–347
16. Schug, Z. T., and Gottlieb, E. (2009) Cardiolipin acts as a mitochondrial signalling platform to launch apoptosis. *Biochim. Biophys. Acta* **1788,** 2022–2031
17. Hanske, J., Toffey, J. R., Morenz, A. M., Bonilla, A. J., Schiavoni, K. H., and Pletneva, E. V. (2012) Conformational properties of cardiolipin-bound cytochrome *c*. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 125–130
18. Sinibaldi, F., Howes, B. D., Piro, M. C., Polticelli, F., Bombelli, C., Ferri, T., Coletta, M., Smulevich, G., and Santucci, R. (2010) Extended cardiolipin anchorage to cytochrome *c*. A model for protein-mitochondrial membrane binding. *J. Biol. Inorg. Chem.* **15,** 689–700
19. Rytömaa, M., and Kinnunen, P. K. (1994) Evidence for two distinct acidic phospholipid-binding sites in cytochrome *c*. *J. Biol. Chem.* **269,** 1770–1774
20. Rytömaa, M., and Kinnunen, P. K. (1995) Reversibility of the binding of cytochrome *c* to liposomes. Implications for lipid-protein interactions. *J. Biol. Chem.* **270,** 3197–3202
21. Sinibaldi, F., Fiorucci, L., Patriarca, A., Lauceri, R., Ferri, T., Coletta, M., and Santucci, R. (2008) Insights into cytochrome *c*-cardiolipin interaction. Role played by ionic strength. *Biochemistry* **47,** 6928–6935
22. Kalanxhi, E., and Wallace, C. J. (2007) Cytochrome *c* impaled. Investigation of the extended lipid anchorage of a soluble protein to mitochondrial membrane models. *Biochem. J.* **407,** 179–187
23. Huang, Y., Liu, L., Shi, C., Huang, J., and Li, G. (2006) Electrochemical analysis of the effect of Ca^{2+} on cardiolipin-cytochrome *c* interaction. *Biochim. Biophys. Acta* **1760,** 1827–1830
24. Duan, Y., Gross, R. A., and Sheu, S. S. (2007) Ca^{2+}-dependent generation of mitochondrial reactive oxygen species serves as a signal for poly(ADP-ribose) polymerase-1 activation during glutamate excitotoxicity. *J. Physiol.* **585,** 741–758
25. Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., and Sheu, S. S. (2004) Calcium, ATP, and ROS. A mitochondrial love-hate triangle. *Am. J. Physiol. Cell Physiol.* **287,** C817–C833
26. Peng, T. I., and Jou, M. J. (2010) Oxidative stress caused by mitochondrial calcium overload. *Ann. N.Y. Acad. Sci.* **1201,** 183–188
27. Crompton, M., Ellinger, H., and Costi, A. (1988) Inhibition by cyclosporin A of a Ca^{2+}-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. *Biochem. J.* **255,** 357–360
28. Halestrap, A. P., and Davidson, A. M. (1990) Inhibition of Ca^{2+}-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial matrix peptidyl-prolyl *cis-trans* isomerase and preventing it interacting with the adenine nucleotide translocase. *Biochem. J.* **268,** 153–160
29. Tanveer, A., Virji, S., Andreeva, L., Totty, N. F., Hsuan, J. J., Ward, J. M., and Crompton, M. (1996) Involvement of cyclophilin D in the activation of a mitochondrial pore by Ca^{2+} and oxidant stress. *Eur. J. Biochem.* **238,** 166–172
30. Griffiths, E. J., and Halestrap, A. P. (1991) Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl *cis-trans* isomerase. Implications for the immuno-

suppressive and toxic effects of cyclosporin. *Biochem. J.* **274**, 611–614
31. Clarke, S. J., McStay, G. P., and Halestrap, A. P. (2002) Sanglifehrin A acts as a potent inhibitor of the mitochondrial permeability transition and reperfusion injury of the heart by binding to cyclophilin-D at a different site from cyclosporin A. *J. Biol. Chem.* **277,** 34793–34799
32. Waldmeier, P. C., Feldtrauer, J. J., Qian, T., and Lemasters, J. J. (2002) Inhibition of the mitochondrial permeability transition by the nonimmunosuppressive cyclosporin derivative NIM811. *Mol. Pharmacol.* **62,** 22–29
33. Baines, C. P., Kaiser, R. A., Purcell, N. H., Blair, N. S., Osinska, H., Hambleton, M. A., Brunskill, E. W., Sayen, M. R., Gottlieb, R. A., Dorn, G. W., Robbins, J., and Molkentin, J. D. (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* **434,** 658–662
34. Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., Inohara, H., Kubo, T., and Tsujimoto, Y. (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* **434,** 652–658
35. Mittnacht, S., Jr., and Farber, J. L. (1981) Reversal of ischemic mitochondrial dysfunction. *J. Biol. Chem.* **256,** 3199–3206
36. Beatrice, M. C., Palmer, J. W., and Pfeiffer, D. R. (1980) The relationship between mitochondrial membrane permeability, membrane potential, and the retention of Ca^{2+} by mitochondria. *J. Biol. Chem.* **255,** 8663–8671
37. Oyanagi, E., Yano, H., Uchida, M., Utsumi, K., and Sasaki, J. (2011) Protective action of L-carnitine on cardiac mitochondrial function and structure against fatty acid stress. *Biochem. Biophys. Res. Commun.* **412,** 61–67
38. Kim, D. K., Heineman, F. W., and Balaban, R. S. (1991) Effects of β-hydroxybutyrate on oxidative metabolism and phosphorylation potential in canine heart *in vivo*. *Am. J. Physiol.* **260,** H1767–H1773
39. Otto, D. A., and Ontko, J. A. (1978) Activation of mitochondrial fatty acid oxidation by calcium. Conversion to the energized state. *J. Biol. Chem.* **253,** 789–799
40. Ontko, J. A., and Westbrook, D. J. (1983) Dual effects of calcium on the oxidation of fatty acids to ketone bodies in liver mitochondria. *Biochem. Biophys. Res. Commun.* **116,** 173–179
41. Gadd, M. E., Broekemeier, K. M., Crouser, E. D., Kumar, J., Graff, G., and Pfeiffer, D. R. (2006) Mitochondrial iPLA2 activity modulates the release of cytochrome *c* from mitochondria and influences the permeability transition. *J. Biol. Chem.* **281,** 6931–6939
42. Kinsey, G. R., McHowat, J., Patrick, K. S., and Schnellmann, R. G. (2007) Role of Ca^{2+}-independent phospholipase A2γ in Ca^{2+}-induced mitochondrial permeability transition. *J. Pharmacol. Exp. Ther.* **321,** 707–715
43. Blum, J. L., Kinsey, G. R., Monian, P., Sun, B., Cummings, B. S., McHowat, J., and Schnellmann, R. G. (2011) Profiling of fatty acids released during calcium-induced mitochondrial permeability transition in isolated rabbit kidney cortex mitochondria. *Toxicol. In Vitro* **25,** 1001–1006
44. Jenkins, C. M., Mancuso, D. J., Yan, W., Sims, H. F., Gibson, B., and Gross, R. W. (2004) Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J. Biol. Chem.* **279,** 48968–48975
45. Daniels, S. B., Cooney, E., Sofia, M. J., Chakravarty, P. K., and Katzenellenbogen, J. A. (1983) Haloenol lactones. Potent enzyme-activated irreversible inhibitors for α-chymotrypsin. *J. Biol. Chem.* **258,** 15046–15053
46. Fuentes, L., Pérez, R., Nieto, M. L., Balsinde, J., and Balboa, M. A. (2003) Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase A2. *J. Biol. Chem.* **278,** 44683–44690
47. van Tienhoven, M., Atkins, J., Li, Y., and Glynn, P. (2002) Human neuropathy target esterase catalyzes hydrolysis of membrane lipids. *J. Biol. Chem.* **277,** 20942–20948
48. Jenkins, C. M., Han, X., Mancuso, D. J., and Gross, R. W. (2002) Identification of calcium-independent phospholipase A2 (iPLA2) β, and not iPLA2γ, as the mediator of arginine vasopressin-induced arachidonic acid release in A-10 smooth muscle cells. Enantioselective mechanism-based discrimination of mammalian iPLA2s. *J. Biol. Chem.* **277,** 32807–32814
49. Jenkins, C. M., Yang, J., Sims, H. F., and Gross, R. W. (2011) Reversible high affinity inhibition of phosphofructokinase-1 by acyl-CoA. A mechanism integrating glycolytic flux with lipid metabolism. *J. Biol. Chem.* **286,** 11937–11950
50. Mancuso, D. J., Sims, H. F., Han, X., Jenkins, C. M., Guan, S. P., Yang, K., Moon, S. H., Pietka, T., Abumrad, N. A., Schlesinger, P. H., and Gross, R. W. (2007) Genetic ablation of calcium-independent phospholipase A2γ leads to alterations in mitochondrial lipid metabolism and function, resulting in a deficient mitochondrial bioenergetic phenotype. *J. Biol. Chem.* **282,** 34611–34622
51. Moon, S. H., Jenkins, C. M., Mancuso, D. J., Turk, J., and Gross, R. W. (2008) Smooth muscle cell arachidonic acid release, migration, and proliferation are markedly attenuated in mice null for calcium-independent phospholipase A2β. *J. Biol. Chem.* **283,** 33975–33987
52. Jüllig, M., Hickey, A. J., Chai, C. C., Skea, G. L., Middleditch, M. J., Costa, S., Choong, S. Y., Philips, A. R., and Cooper, G. J. (2008) Is the failing heart out of fuel or a worn engine running rich? A study of mitochondria in old spontaneously hypertensive rats. *Proteomics* **8,** 2556–2572
53. Mancuso, D. J., Sims, H. F., Yang, K., Kiebish, M. A., Su, X., Jenkins, C. M., Guan, S., Moon, S. H., Pietka, T., Nassir, F., Schappe, T., Moore, K., Han, X., Abumrad, N. A., and Gross, R. W. (2010) Genetic ablation of calcium-independent phospholipase A2γ prevents obesity and insulin resistance during high fat feeding by mitochondrial uncoupling and increased adipocyte fatty acid oxidation. *J. Biol. Chem.* **285,** 36495–36510
54. Moon, S. H., Jenkins, C. M., Liu, X., Guan, S., Mancuso, D. J., and Gross, R. W. (2012) Activation of mitochondrial calcium-independent phospholipase A2γ (iPLA2γ) by divalent cations mediating arachidonate release and production of downstream eicosanoids. *J. Biol. Chem.* **287,** 14880–14895
55. Varanyuwatana, P., and Halestrap, A. P. (2012) The roles of phosphate and the phosphate carrier in the mitochondrial permeability transition pore. *Mitochondrion* **12,** 120–125
56. Elrod, J. W., Wong, R., Mishra, S., Vagnozzi, R. J., Sakthievel, B., Goonasekera, S. A., Karch, J., Gabel, S., Farber, J., Force, T., Brown, J. H., Murphy, E., and Molkentin, J. D. (2010) Cyclophilin D controls mitochondrial pore-dependent Ca^{2+} exchange, metabolic flexibility, and propensity for heart failure in mice. *J. Clin. Invest.* **120,** 3680–3687
57. Castilho, R. F., Kowaltowski, A. J., Meinicke, A. R., Bechara, E. J., and Vercesi, A. E. (1995) Permeabilization of the inner mitochondrial membrane by Ca2+ ions is stimulated by *t*-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria. *Free Radic. Biol. Med.* **18,** 479–486
58. Di Paola, M., Zaccagnino, P., Oliveros-Celis, C., and Lorusso, M. (2006) Arachidonic acid induces specific membrane permeability increase in heart mitochondria. *FEBS Lett.* **580,** 775–781
59. Sultan, A., and Sokolove, P. M. (2001) Palmitic acid opens a novel cyclosporin A-insensitive pore in the inner mitochondrial membrane. *Arch. Biochem. Biophys.* **386,** 37–51
60. Penzo, D., Petronilli, V., Angelin, A., Cusan, C., Colonna, R., Scorrano, L., Pagano, F., Prato, M., Di Lisa, F., and Bernardi, P. (2004) Arachidonic acid released by phospholipase A_2 activation triggers Ca^{2+}-dependent apoptosis through the mitochondrial pathway. *J. Biol. Chem.* **279,** 25219–25225
61. Schönfeld, P., and Bohnensack, R. (1997) Fatty acid-promoted mitochondrial permeability transition by membrane depolarization and binding to the ADP/ATP carrier. *FEBS Lett.* **420,** 167–170
62. Unger, R. H. (1995) Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. *Diabetes* **44,** 863–870
63. Cooney, G. J., Thompson, A. L., Furler, S. M., Ye, J., and Kraegen, E. W. (2002) Muscle long-chain acyl CoA esters and insulin resistance. *Ann. N.Y. Acad. Sci.* **967,** 196–207
64. Abdul-Ghani, M. A., Muller, F. L., Liu, Y., Chavez, A. O., Balas, B., Zuo, P., Chang, Z., Tripathy, D., Jani, R., Molina-Carrion, M., Monroy, A., Folli, F., Van Remmen, H., and DeFronzo, R. A. (2008) Deleterious action of FA metabolites on ATP synthesis. Possible link between lipotoxicity, mitochondrial dysfunction, and insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* **295,** E678–E685
65. Lee, K., Kerner, J., and Hoppel, C. L. (2011) Mitochondrial carnitine palmitoyltransferase 1a (CPT1a) is part of an outer membrane fatty acid transfer complex. *J. Biol. Chem.* **286,** 25655–25662
66. Krauskopf, A., Eriksson, O., Craigen, W. J., Forte, M. A., and Bernardi, P.

(2006) Properties of the permeability transition in VDAC1$^{-/-}$ mitochondria. *Biochim. Biophys. Acta* **1757,** 590–595
67. Baines, C. P., Kaiser, R. A., Sheiko, T., Craigen, W. J., and Molkentin, J. D. (2007) Voltage-dependent anion channels are dispensable for mitochondria-dependent cell death. *Nat. Cell Biol.* **9,** 550–555
68. Kokoszka, J. E., Waymire, K. G., Levy, S. E., Sligh, J. E., Cai, J., Jones, D. P., MacGregor, G. R., and Wallace, D. C. (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* **427,** 461–465
69. Lerner, E., Shug, A. L., Elson, C., and Shrago, E. (1972) Reversible inhibition of adenine nucleotide translocation by long chain fatty acyl coenzyme A esters in liver mitochondria of diabetic and hibernating animals. *J. Biol. Chem.* **247,** 1513–1519
70. Ciapaite, J., Van Eikenhorst, G., Bakker, S. J., Diamant, M., Heine, R. J., Wagner, M. J., Westerhoff, H. V., and Krab, K. (2005) Modular kinetic analysis of the adenine nucleotide translocator-mediated effects of palmitoyl-CoA on the oxidative phosphorylation in isolated rat liver mitochondria. *Diabetes* **54,** 944–951
71. Dube, H., Selwood, D., Malouitre, S., Capano, M., Simone, M. I., and Crompton, M. (2012) A mitochondria-targeted cyclosporin A with high binding affinity for cyclophilin D yields improved cytoprotection of cardiomyocytes. *Biochem. J.* **441,** 901–907
72. Schinzel, A. C., Takeuchi, O., Huang, Z., Fisher, J. K., Zhou, Z., Rubens, J., Hetz, C., Danial, N. N., Moskowitz, M. A., and Korsmeyer, S. J. (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 12005–12010
73. Eliseev, R. A., Malecki, J., Lester, T., Zhang, Y., Humphrey, J., and Gunter, T. E. (2009) Cyclophilin D interacts with Bcl2 and exerts an anti-apoptotic effect. *J. Biol. Chem.* **284,** 9692–9699
74. Hansford, R. G., and Castro, F. (1985) Role of Ca^{2+} in pyruvate dehydrogenase interconversion in brain mitochondria and synaptosomes. *Biochem. J.* **227,** 129–136
75. Denton, R. M., and McCormack, J. G. (1990) Ca^{2+} as a second messenger within mitochondria of the heart and other tissues. *Annu. Rev. Physiol.* **52,** 451–466
76. McCormack, J. G., and Denton, R. M. (1993) Mitochondrial Ca^{2+} transport and the role of intramitochondrial Ca^{2+} in the regulation of energy metabolism. *Dev. Neurosci.* **15,** 165–173
77. Territo, P. R., Mootha, V. K., French, S. A., and Balaban, R. S. (2000) Ca^{2+} activation of heart mitochondrial oxidative phosphorylation. Role of the F_0/F_1-ATPase. *Am. J. Physiol. Cell Physiol.* **278,** C423–C435
78. Sánchez, A., Alvarez, A. M., Benito, M., and Fabregat, I. (1996) Apoptosis induced by transforming growth factor-β in fetal hepatocyte primary cultures. Involvement of reactive oxygen intermediates. *J. Biol. Chem.* **271,** 7416–7422
79. Palomba, L., Sestili, P., and Cantoni, O. (2001) *tert*-Butyl hydroperoxide induces peroxynitrite-dependent mitochondrial permeability transition leading PC12 cells to necrosis. *J. Neurosci. Res.* **65,** 387–395
80. Nieminen, A. L., Byrne, A. M., Herman, B., and Lemasters, J. J. (1997) Mitochondrial permeability transition in hepatocytes induced by *t*-BuOOH. NAD(P)H and reactive oxygen species. *Am. J. Physiol.* **272,** C1286–C1294
81. Imberti, R., Nieminen, A. L., Herman, B., and Lemasters, J. J. (1993) Mitochondrial and glycolytic dysfunction in lethal injury to hepatocytes by *t*-butyl hydroperoxide. Protection by fructose, cyclosporin A, and trifluoperazine. *J. Pharmacol. Exp. Ther.* **265,** 392–400
82. Vay, L., Hernández-SanMiguel, E., Lobatón, C. D., Moreno, A., Montero, M., and Alvarez, J. (2009) Mitochondrial free [Ca^{2+}] levels and the permeability transition. *Cell Calcium* **45,** 243–250
83. McStay, G. P., Clarke, S. J., and Halestrap, A. P. (2002) Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. *Biochem. J.* **367,** 541–548