# Accumulation of Long-Chain Acylcarnitine and 3-Hydroxy Acylcarnitine Molecular Species in Diabetic Myocardium: Identification of Alterations in Mitochondrial Fatty Acid Processing in Diabetic Myocardium by Shotgun Lipidomics†

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ABSTRACT: Diabetic cardiomyopathy is the result of maladaptive changes in energy homeostasis. However, the biochemical mechanisms underlying dysfunctional lipid metabolism in diabetic myocardium are incompletely understood. Herein, we exploit shotgun lipidomics to demonstrate a 4-fold increase in acylcarnitines in diabetic myocardium, which was reversible upon insulin treatment. Analysis of acylcarnitine molecular species in myocardium unexpectedly identified acylcarnitine molecular species containing a mass shift of 16 amu in comparison to the anticipated molecular species. Synthesis of 3-hydroxy acylcarnitine identified the natural products as the 3-hydroxylated acylcarnitines through comparisons of diagnostic fragmentation patterns of synthetic and naturally occurring constituents using tandem mass spectrometry. Diabetes induced an increase of both calcium-independent phospholipase $A_2$ ($iPLA_2$) mRNA and $iPLA_2$ activity in rat myocardium. Cardiac ischemia in myocardium genetically engineered to overexpress $iPLA_2$ dramatically increased the amount of acylcarnitine present in myocardium. Moreover, mechanism-based inactivation of $iPLA_2$ in either wild-type or transgenic myocardium ablated a substantial portion of the acylcarnitine increase. Collectively, these results identify discrete insulin remediable abnormalities in mitochondrial fatty acid processing in diabetic myocardium and identify $iPLA_2$ as an important enzymatic contributor to the pool of fatty acids that can be used for acylcarnitine synthesis and energy production in myocardium.

Fatty acids provide the majority of energy for myocardial hemodynamic function through the precisely regulated extraction of fatty acids from serum that is tightly coordinated to meet metabolic demands (*1*−*3*). This process is accomplished by the lipoprotein-lipase-mediated hydrolysis of serum triacylglycerols (TAGs)[1] and phospholipids followed by protein-facilitated sarcolemmal fatty acid transport and the subsequent intracellular trapping of the carboxylic acid through formation of the thioesters catalyzed by endofacial sarcolemmal acyl CoA synthases (*4*−*8*). Subsequently, aliphatic chains extracted into cardiac myocytes are channeled into either anabolic (e.g., lipid storage) or catabolic fates (e.g., oxidation) depending upon the metabolic history and thermodynamic energy requirements of the organism as a whole. The large majority of fatty acids that enter a myocyte are channeled into mitochondrial oxidative pathways employing a multistep process designed to transport fatty acyl CoA through the mitochondrial double-membrane bilayer (outer and inner mitochondrial membranes) by the sequential actions of carnitine palmitoyl transferase I (CPT-I), carnitine:acylcarnitine translocase (CACT), and the regeneration of acyl CoA in the mitochondrial matrix catalyzed by CPT-II (*9*, *10*). The fatty acyl CoA in the mitochondrial matrix is either utilized for fatty acid $\beta$ oxidation or is hydrolyzed by mitochondrial thioesterases to modulate uncoupling protein (UCP) function (*11*−*15*). In normal myocardium, the steady-state concentration of acylcarnitine is low because the transformation of cytosolic acyl CoA into acylcarnitine is tightly coupled to myocardial energy demand through cytosolic malonyl CoA-mediated inhibition of CPT-I (*16*). During physiologic conditions, the synthesis of acylcarnitine is the first committed and rate-determining step in the oxidation of fatty acids (*17*).

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[1] Abbreviations: AC*m*:*n*, acylcarnitine containing *m* carbons and *n* double bonds in acyl chain; BEL, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; CACT, carnitine:acylcarnitine translocase; CPT, carnitine palmitoyltransferase; ESI/MS, electrospray ionization mass spectrometry; ESI/MS/MS, electrospray ionization tandem mass spectrometry; FAO, fatty acid oxidation; FFA, free fatty acid; $iPLA_2$, calcium-independent phospholipase $A_2$; LCAC, long-chain acylcarnitine; OHAC*m*:*n*, 3-hydroxy acylcarnitine containing *m* carbons and *n* double bonds in acyl chain; $PLA_2$, phospholipase $A_2$; PPAR$\alpha$, peroxisome proliferator activated receptor $\alpha$; TAG, triacylglycerol; UCP, uncoupling protein.

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However, during myocardial ischemia, acylcarnitines increase dramatically because of the block in fatty acid oxidation caused by the absence of $O_2$ and the accumulation of reducing equivalents in the mitochondrial matrix compartment of ischemic myocardium (*18*, *19*).

In ischemic myocardium, both oxygen and fatty acid substrates do not reach afflicted myocytes and their combined absence results in varying amounts of cellular apoptosis, necrosis, and functional damage depending upon the severity and duration of the ischemic insult (*20*, *21*). The resultant accumulation of acylcarnitines and other amphiphilic metabolites leads to alterations in membrane molecular dynamics and alters the kinetics of ion channels that adversely influence the electrophysiologic properties of the ischemic heart predisposing to lethal ventricular tachyarrhythmias and sudden death (*22*−*24*). In glucose-perfused Langendorf hearts (perfused in the absence of fatty acid or TAG substrates), the induction of myocardial ischemia also results in acylcarnitine accumulation (*18*), demonstrating that at least a portion of the fatty acyl moieties in the accumulating acylcarnitine pool must be derived from endogenous lipid stores. However, the biochemical mechanisms providing the fatty acid substrate for acyl CoA production used in the synthesis of acylcarnitines in ischemic myocardium are not known with certainty. Previously, it had been thought that TAG hydrolysis in myocardium was largely responsible (*25*, *26*), but recent electrospray ionization mass spectrometry (ESI/MS) evidence demonstrated that extremely low levels of endogenous TAGs are present in normal myocardium (*27*) that cannot quantitatively account for the concentrations of acylcarnitines found during the ischemic insult at least in glucose-perfused Langendorf hearts.

Calcium-independent phospholipases $A_2$ (iPLA$_2$) are an important group of intracellular phospholipases $A_2$ (PLA$_2$) that catalyze the release of free fatty acids (FFAs) from membrane phospholipids in response to agonist stimulation, changes in intracellular calcium ion homeostasis, and alterations in cellular energy requirements (*28*, *29*). Acute myocardial ischemia results in the activation of PLA$_2$ activity. Characterization of intracellular myocardial PLA$_2$ activities demonstrated that myocardial phospholipases were predominantly calcium-independent PLA$_2$s (*30*−*32*). Recently, we have constructed a transgenic mouse overexpressing the major phospholipase $A_2$ activity in myocardium (as ascertained by both mass and measurable phospholipase $A_2$ activity), termed iPLA$_2\beta$, and demonstrated that myocardial ischemia results in the dramatic activation of iPLA$_2\beta$ leading to robust increases in fatty acid release and lysolipid production (*32*). Accordingly, these results suggested that a substantial portion of the acylcarnitine mass present in ischemic myocardium could be due to the ischemia-induced activation of iPLA$_2$ through the provision of a fatty acid substrate, at least a portion of which was destined for acylcarnitine generation.

Recent advances in lipidomics make it feasible to analyze the molecular species of acylcarnitines in biological samples by direct infusion of organic extracts utilizing ESI/MS (*18*, *33*, *34*). Herein, we report the accumulation of long-chain acylcarnitines (LCACs) and the unanticipated accumulation of 3-hydroxy acylcarnitines in rat myocardium rendered diabetic for 6 weeks by streptozotocin treatment (a model of type-I diabetes), which are both remediable by insulin treatment. We also identify iPLA$_2$ as an important contributor to the generation of these acylcarnitine molecular species by providing a substantial portion of fatty acid substrates used for acylcarnitine synthesis. Thus, through the combined power of chemistry, molecular biology, and physiology, we now identify acylcarnitines as biomarkers for dysfunctional lipid and mitochondrial fatty acid metabolism in diabetic myocardium to identify a novel strategy potentially suitable for pharmaceutical modification of diabetes-induced cardiomyopathy through alteration of myocardial iPLA$_2$ activity.

## EXPERIMENTAL PROCEDURES

*Materials.* Dodecanoylcarnitine (AC12:0, used as an internal standard) was purchased from Sigma Chemical Co. (St. Louis, MO). [1-$^{14}$C]-Palmitoyl-L-carnitine hydrochloride was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All solvents were at least HPLC-grade and were obtained from Fisher Scientific (Pittsburgh, PA). Streptozotocin, insulin, and most other reagents were of analytical grade and were bought from Sigma (St. Louis, MO).

*Treatment and Maintenance of Control and Diabetic Rats.* Male Sprague Dawley rats (350−450 g of body weight) were purchased from Charles River Labs (Wilmington, MA). Rats were housed separately, kept on a 12-h light−dark cycle, and given free access to food (Pico-Vac standard lab rodent diet containing 20% protein and 5% fat) and water. Diabetes was induced by a single intravenous injection (tail vein) of streptozotocin (35 mg/kg body weight in 0.2 mL of 0.1 M citrate buffer at pH 4.5) as described previously (*27*). All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996) and were approved by the Animal Study Committee at Washington University.

*Preparation of Lipid Extracts from Rat Myocardium.* Rats were sacrificed by asphyxiation by carbon dioxide. The hearts were excised quickly and immersed in ice-cold buffer (250 mM sucrose and 25 mM imidazole at pH 8.0 and 4 °C). After extraneous tissue and epicardial fat were removed, each heart was quickly dried and immediately freeze-clamped at the temperature of liquid nitrogen. Myocardial wafers were pulverized into a fine powder with a stainless steel mortar and pestle. A tissue sample (≈50 mg) was weighed from each rat heart. Lipids of each individual myocardial sample were extracted by a modified Bligh and Dyer technique (*35*) utilizing 5% (by vol) acetic acid in the aqueous layer in the presence of AC12:0 (1700 pmol/mg of protein, used as an internal standard for the quantification of acylcarnitine and its analogue). This molecular species of acylcarnitine represents <1% of the total endogenous acylcarnitine mass. The lipid extracts were dried under a nitrogen stream, dissolved in chloroform, filtered with 0.2 $\mu$m Gelman acrodisc CR PTFE syringe filters (Gelman Science, Ann Arbor, MI), re-extracted, and then dried under a nitrogen stream. The final lipid residue was resuspended in 0.2 mL of 1:1 chloroform/methanol for ESI/MS analyses of acylcarnitines and their hydroxy analogues.

*ESI/MS of Acylcarnitines.* ESI-MS analyses were performed utilizing a Finnigan TSQ-7000 Spectrometer (Finnigan MAT, San Jose, CA) equipped with an ESI source and controlled by Finnigan ICIS software operated on a DEC α

workstation as described previously (*27*, *33*). Typically, a 5-min period of signal averaging in the profile mode was employed for each spectrum of lipid extract from rat heart. All samples of heart-tissue lipid extracts were appropriately diluted in 1:2 chloroform/methanol prior to direct infusion into the ESI source using a Harvard syringe pump at a flow rate of 1 $\mu$L/min. Acylcarnitines and their hydroxy analogues in the diluted chloroform lipid extracts of rat hearts were analyzed by ESI/MS in the positive-ion mode and quantitated by comparison of the individual ion peak intensity with that of the internal standard (i.e., AC12:0) after correction for $^{13}$C isotope content (*18*, *27*). Identification of ion peaks was achieved utilizing tandem mass spectrometry in both precursor ion scanning and product-ion scanning mode. The linear relationships between different acylcarnitine molecular species mass and ion current have been previously demonstrated (*18*, *36*). Experiments examining the response factors of nonhydroxy and 3-hydroxy acylcarnitines by electrospray ionization demonstrated that both were equal within experimental error (±10%) (*36*). The product-ion tandem mass spectrometry was similarly conducted as described previously (*37*). The tandem mass spectrometry in precursor-ion scanning mode was performed through scanning and passage of the molecular ion(s) of acylcarnitines from the first quadrupole into the second quadrupole, where dissociation was induced through collisional activation with argon gas. Specific product ions of *m*/*z* 85 and *m*/*z* 145 were monitored after passage of all resultant product ions into the third quadrupole. A mass window of 0.7 amu was selected. The degree of collisional activation was adjusted through variation of the DC offset voltage and collision gas pressure. During this study, a collision energy of 25 eV and a collision gas pressure of 2.5 mTorr were used.

*Phospholipase Assay with a Fluorogenic Probe.* The fluorogenic probe, arachidonoyl-3-cyano-7-hydroxycoumarin (ACHC), was synthesized as described (*38*) using DCC as the coupling reagent. Hydrolysis of ACHC was monitored on a SLM 4800C spectrofluorometer (SLM Instrument, Urbana, IL) and followed by the increase in fluorescence intensity at 450 nm (excitation of 351 nm at pH 7.3) because of the production of the highly fluorescent 3-CHC. ACHC was disolved in ethanol to give a 2 mM stock solution. To 3 mL of pre-warmed assay buffer (100 mM Tris-HCl at pH 7.3 and 5 mM EGTA) was added ACHC solution (7.5 $\mu$L) to give a final concentration of 5 $\mu$M. The solvolysis was monitored for 100 s before the initiation of hydrolysis with cytosolic or microsomal proteins.

*Reverse Transcription and Real-Time Quantitative Polymerase Chain Reaction (PCR).* Total RNA was purified from mouse hearts utilizing a RNeasy Mini Kit from Qiagen (Valencia, CA) according to the instructions of the manufacturer. For cDNA preparation, 250 pmol of random hexamers were hybridized by incubation for 10 min at 25 °C and extended by incubation for 30 min at 48 °C in the presence of 125 units of reverse transcriptase in 100 $\mu$L of PCR buffer (5.5 mM $MgCl_2$, 0.5 mM of each dNTP, and 40 units of RNase inhibitor). Reverse transcriptase was inactivated by incubation at 95 °C for 5 min. Amplification of each target cDNA was performed with TaqMan PCR reagent kits and quantified by the ABI PRISM 7700 detection system according to the protocol provided by the manufacturer (Applied Biosystems, Foster City, CA). GAPDH cDNA was measured and used as an internal standard. Oligonucleotide primer pairs and probes specific for iPLA$_2\beta$ (5′-CCTTCCATTACGCTGTGCAA/ 5′-GCGTCAGCCCTTGGTTGTT/ 5′-CCAGGTGCTACAGCTCCTAGGAAAGAATGC) and iPLA$_2\gamma$ (5′-GAGGAGAAAAAGCGTGTGTTACTTC/ 5′-GGTTGTTCTTCTTAAGGCCTGAA/5′-TCTGTTATCAATACTCACTCTTGCAATA) were employed.

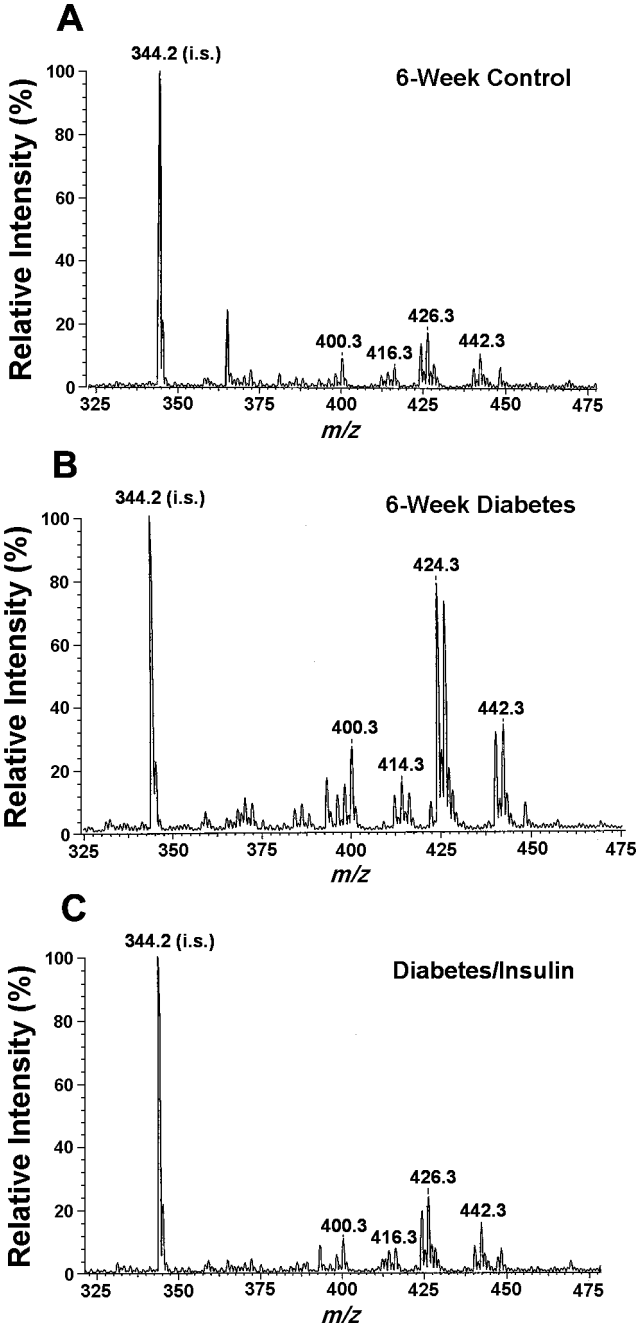


FIGURE 1: Identification and quantitation of acylcarnitine molecular species in rat myocardium utilizing ESI/MS. Lipids of rat hearts from 6-week control (A), 6-week diabetic (B), and 6 weeks of diabetes followed by 6 weeks of insulin treatment (C) were extracted from freeze-clamped myocardium by a modified Bligh and Dyer technique utilizing 5% acetic acid in the aqueous phase as described in the Experimental Procedures. Acylcarnitine molecular species were analyzed and subsequently quantitated by ESI/MS in the positive ion mode as their protonated molecular ions. Mass spectra were normalized to the most intense peak corresponding to internal standard (i.s., AC12:0) in each individual spectrum.

Table 1: Quantitation of Individual Acylcarnitine Molecular Species in Rat Hearts[a]

| *m*/*z* | molecular species | control ($n = 6$) | 6-week diabetes ($n = 5$) | 12-week diabetes ($n = 4$) | diabetes/insulin ($n = 4$) | control/insulin ($n = 5$) |
|---|---|---|---|---|---|---|
| 368 | AC14:2 | 53 ± 8 | 125 ± 16 | 88 ± 11 | 66 ± 9 | 41 ± 8 |
| 370 | AC14:1 | 67 ± 8 | 161 ± 29 | 105 ± 25 | 97 ± 27 | 45 ± 11 |
| 372 | AC14:0 | 99 ± 11 | 171 ± 33 | 125 ± 26 | 126 ± 38 | 94 ± 25 |
| 384 | OHAC14:2 | 31 ± 4 | 128 ± 28 | 57 ± 8 | 44 ± 10 | 22 ± 7 |
| 386 | OHAC14:1 | 56 ± 11 | 145 ± 28 | 78 ± 12 | 58 ± 13 | 36 ± 10 |
| 388 | OHAC14:0 | 51 ± 11 | 100 ± 15 | 64 ± 10 | 60 ± 15 | 28 ± 9 |
| 396 | AC16:2 | 49 ± 6 | 187 ± 42 | 121 ± 20 | 68 ± 11 | 35 ± 7 |
| 398 | AC16:1 | 75 ± 10 | 235 ± 51 | 173 ± 34 | 138 ± 34 | 64 ± 14 |
| 400 | AC16:0 | 164 ± 23 | 521 ± 139 | 350 ± 90 | 277 ± 81 | 185 ± 48 |
| 412 | OHAC16:2 | 58 ± 11 | 225 ± 41 | 114 ± 12 | 69 ± 6 | 32 ± 9 |
| 414 | OHAC16:1 | 74 ± 16 | 255 ± 45 | 142 ± 20 | 93 ± 22 | 52 ± 15 |
| 416 | OHAC16:0 | 90 ± 16 | 240 ± 44 | 161 ± 27 | 132 ± 40 | 56 ± 19 |
| 422 | AC18:3 | 26 ± 4 | 151 ± 28 | 111 ± 15 | 65 ± 7 | 26 ± 7 |
| 424 | AC18:2 | 206 ± 35 | 1506 ± 364 | 758 ± 163 | 177 ± 51 | 216 ± 69 |
| 426 | AC18:1 | 262 ± 52 | 1293 ± 286 | 759 ± 217 | 405 ± 119 | 268 ± 77 |
| 428 | AC18:0 | 131 ± 18 | 256 ± 56 | 245 ± 52 | 222 ± 53 | 145 ± 29 |
| 440 | OHAC18:2 | 85 ± 18 | 589 ± 121 | 319 ± 42 | 108 ± 25 | 57 ± 17 |
| 442 | OHAC18:1 | 129 ± 37 | 585 ± 100 | 354 ± 43 | 196 ± 56 | 68 ± 22 |
| 448 | AC20:4 | 133 ± 17 | 201 ± 32 | 95 ± 15 | 61 ± 9 | 109 ± 24 |
| | total | 1838 ± 295 | 7073 ± 1371 | 4218 ± 720 | 2462 ± 417 | 1578 ± 300 |
| | OHAC | 573 ± 115 | 2267 ± 390 | 1288 ± 161 | 759 ± 164 | 351 ± 108 |
| | non-OHAC | 1265 ± 177 | 4806 ± 976 | 2930 ± 601 | 1703 ± 373 | 1227 ± 264 |
| | saturated AC | 535 ± 72 | 1288 ± 267 | 945 ± 191 | 817 ± 202 | 508 ± 121 |
| | unsaturated AC | 1304 ± 221 | 5786 ± 1108 | 3274 ± 561 | 1645 ± 319 | 1069 ± 255 |

[a] Individual molecular species of acylcarnitine were identified and quantified from rat hearts as described in the Experimental Procedures. Each value represents the mean ± SEM. The values are in pmol/mg protein.

*Generation of Transgenic Mice Overexpressing iPLA$_2\beta$ in a Cardiac Myocyte-Specific Manner.* Cardiac myocyte-specific expression of iPLA$_2\beta$ in transgenic mice was accomplished by cloning the full-length 2.4 kb coding region of the wild-type Chinese hamster iPLA$_2\beta$ into the *Sal*I site of the α myosin heavy chain (αMHC) vector downstream from the αMHC promoter as previously described (*32*). Transgenic founders, generated by microinjection of a *Not*I-linearized fragment containing the αMHC promoter−iPLA$_2\beta$ DNA sequence directly into the pronucleic of mouse zygotes, were mated with C57B1/J6 mice to establish transgenic lines. Second and third generation heterozygous mice, typically 3−4 month of age, were used for all studies.

*Induction of Mouse Myocardial Ischemia.* Mouse hearts were perfused retrograde via the aorta (Langendorf perfused) with modified Kreb−Henseleit buffer consisting of 137 mM NaCl, 4.7 mM KCl, 3 mL $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 0.5 mM NaEDTA, 15 mM $NaHCO_3$, and 11 mM of glucose equilibrated with $O_2/CO_2$ (95:5). Langendorf-perfused hearts were either control-perfused at 60 mmHg or rendered ischemic for selected intervals. To determine the effects of BEL on acylcarnitine accumulation, hearts were perfused with buffer containing 10 $\mu$M BEL beginning 5 min before coronary artery ligation and during the ischemic interval. At the end of each perfusion interval, hearts were rapidly freeze-clamped and myocardial tissue was pulverized to a fine power at the temperature of liquid nitrogen. Acylcarnitines were extracted by a modified Bligh and Dyer technique utilizing 5% acetic acid in the aqueous phase as described above.

*Miscellaneous.* Protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard. All data were normalized to the protein content and are presented as the mean ± standard error of the mean (SEM). Statistically significant differences between mean values were determined by unpaired Student's *t* tests.

## RESULTS

*Determination of the Accumulation of Acylcarnitine and 3-Hydroxy Acylcarnitine Molecular Species in Streptozotocin-Treated Rat Myocardium by Shotgun Lipidomics.* Direct infusion of chloroform extracts of rat myocardium into the ion source of a triple quadrupole mass spectrometer operating in the positive-ion mode demonstrated multiple peaks corresponding to acylcarnitine molecular species, which were quantified by comparisons to the internal standard (*m*/*z* 344, AC12:0). Major molecular species of acylcarnitines were present at *m*/*z* 400 (AC16:0), *m*/*z* 424 (AC18:2), *m*/*z* 426 (AC18:1), and *m*/*z* 448 (AC20:4) (Figure 1). Comparisons of ESI mass spectra of control and rat myocardium rendered diabetic for 6 weeks by streptozotocin treatment (a traditional model of type-I diabetes) demonstrated a dramatic increase in acylcarnitine molecular species (parts A and B of Figure 1 and Table 1). An increase in the mass of acylcarnitines from 1838 to 7073 pmol/mg protein was present. It should be noted that acylcarnitines at these elevated concentrations induce eletrophysiologic dysfunction and diabetic patients suffer an increased risk of sudden death (*39*).

Tandem mass spectrometry confirmed the identities of each of the acylcarnitine molecular species (Figure 2). For instance, the ESI tandem mass spectra of synthetic palmitoyl-L-carnitine (*m*/*z* 400) displayed a prominent product ion at *m*/*z* 85, corresponding to protonated γ-crotonolactone resulting from the loss of an acyl group and trimethylamine from acylcarnitine, identifying the presence of a carnitine moiety present in the selected molecular ion. There were also several minor product ions at *m*/*z* 341, 257, 239, and 144 present in the spectrum corresponding to $[M\text{-}NMe_3]^+$, protonated fatty acid, $RC{\equiv}O^+$, and the loss of the fatty acid from the

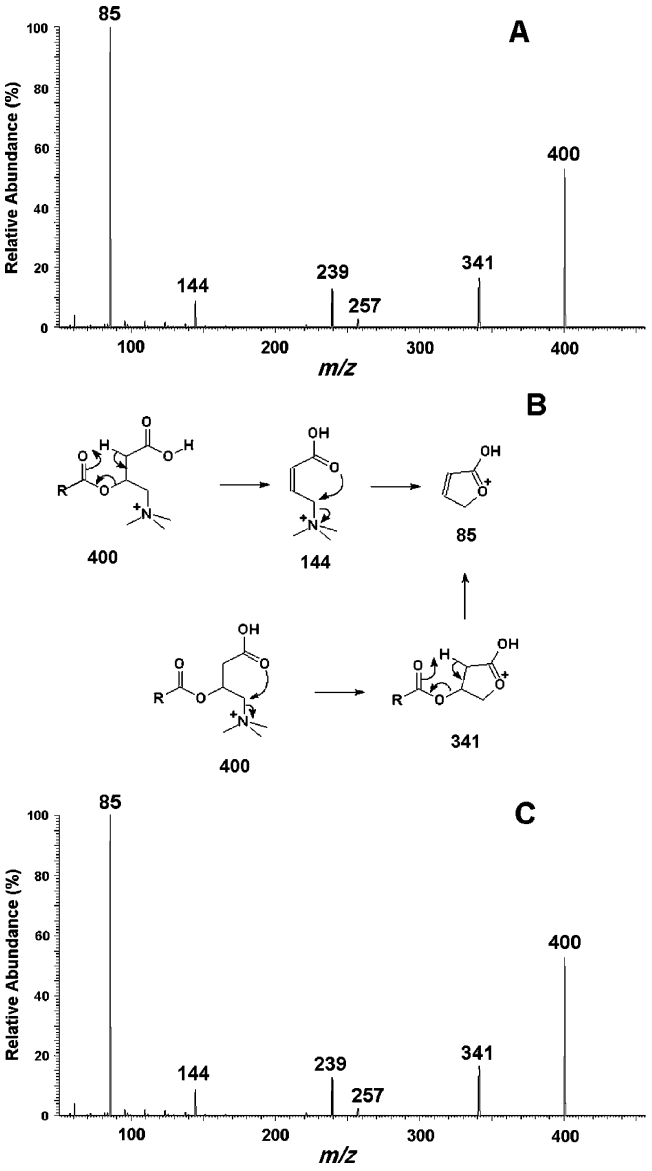


FIGURE 2: Positive-ion electrospray ionization tandem mass spectra of protonated acylcarnitines. Lipids were extracted from freeze-clamped rat myocardium using a modified Bligh and Dyer technique with 5% acetic acid in the aqueous phase as described in the Experimental Procedures. Tandem mass spectrometry of protonated 3-hydroxy acylcarnitines were performed utilizing collision energy of 25 eV and a collision gas pressure of 2.5 mTorr as described in the Experimental Procedures. (A) Tandem mass spectrometry of protonated palmitoyl-L-carnitine (*m*/*z* 400) standard. (B) Fragmentation pathway of protonated palmitoyl-L-carnitine (*m*/*z* 400). (C) Tandem mass spectrometry of protonated palmitoyl-L-carnitine (*m*/*z* 400) in chloroform extract from rat myocardium.

pseudomolecular ion, respectively (parts A and B of Figure 2). The ESI tandem mass spectrum of *m*/*z* 400 in the rat myocardial chloroform extract demonstrated a predominant product ion at *m*/*z* 85 and the presence of ions at *m*/*z* 341, 257, 239, and 144 indicating that the acyl moiety in the acylcarnitine ion at *m*/*z* 400 was palmitoyl (16:0) (Figure 2C).

Surprisingly, ESI/MS analyses of chloroform extracts of rat myocardium in the positive-ion mode also displayed the presence of multiple ion peaks at *m*/*z* 414, 440, and 442 corresponding to 16 amu mass shifts from the anticipated acylcarnitines previously demonstrated in myocardium (Figure 1). These results suggest either odd chain-length acyl moieties with different unsaturation or the presence of an O atom in the acyl moieties. The recent demonstration (*40*) of the presence of substantial amounts of fatty acid α oxidation and odd chain-length fatty acids in a mammalian cell line required determination of the detailed chemical structures of the unanticipated acylcarnitine molecular species. Because mitochondrial fatty acid *β* oxidation represents the major fatty acid catabolic pathway in myocardium and CPT II may reversibly catalyze the generation of acylcarnitines from acylCoA *β*-oxidation intermediates (*41*), we hypothesized that the observed unanticipated acylcarnitine molecular species could be 3-hydroxy acylcarnitines. Accordingly, we synthesized 3-hydroxypalmitoyl carnitine utilizing a novel and broadly applicable synthetic strategy for generation of individual molecular species of acylcarnitine with labile aliphatic chains (Figure 3 and also see the Supporting Information).

To unambiguously identify the specific fragmentation of 3-hydroxy acylcarnitine molecular species and assign the chemical structures of acylcarnitines in rat myocardium, ESI tandem mass spectra of synthetic 3-hydroxypalmitoyl-L-carnitine were obtained. Similar to palmitoyl-L-carnitine, 3-hydroxypalmitoyl-L-carnitine displayed a predominant product ion at *m*/*z* 85, which corresponds to protonated *γ*-crotonolactone (parts A and B of Figure 4). The product ions at *m*/*z* 357 and 144, also present in the spectrum, corresponded to $[M\text{-}NMe_3]^+$ and the loss of a fatty acid from the molecular ion, respectively (parts A and B of Figure 4). The loss of $H_2O$ from *m*/*z* 357 gives *m*/*z* 339 and indicates the presence of hydroxyl group in the molecule. The presence of the hydroxyl group in the aliphatic chain was substantiated by the identification of the product ions at *m*/*z* 237 and 255, which correspond to $RCH{=}CHC{\equiv}O^+$ and protonated unsaturated fatty acid, respectively. Pleasingly, *γ*-hydrogen rearrangement of *m*/*z* 357 gives a specific product ion at *m*/*z* 145, which can be exploited in the tandem mass spectrum of 3-hydroxy acylcarnitines (parts A and B of Figure 4) and is absent in spetra of other acylcarnitines (parts A and B of Figure 2). The predominant product ion at *m*/*z* 85 in the tandem mass spectra of *m*/*z* 416 and 440 in rat myocardial chloroform extracts demonstrated their identities as acylcarnitines (parts C and D of Figure 4). Moreover, the presence of *m*/*z* 145 in their tandem mass spectra demonstrated the presence of 3-hydroxyl group in their acyl chains (parts C and D of Figure 4). Collectively, these results demonstrated the identities of *m*/*z* 416 and 440 as 3-hydroxypalmitoyl-L-carnitine (OHAC16:0) and 3-hydroxylinoleoyl-L-carnitine (OHAC18:2), respectively.

Tandem mass spectrometry in the precursor-ion scanning mode for *m*/*z* 85 profiles the molecular species of all acylcarnitines (e.g., hydroxy or non-hydroxy) directly from chloroform extracts (Figure 5). Because only 3-hydroxy acylcarnitines gives rise to an ion at *m*/*z* 145, profiles for the molecular species of 3-hydroxy acylcarnitines present (*m*/*z* 416, 440, 442, and 444) can be easily obtained utilizing tandem mass spectrometry in the precursor-ion scanning mode for *m*/*z* 145 (Figure 5). We cannot exclude the possibility that other unknown compounds in the extraction mixture could also give a fragment at *m*/*z* 145. However, the likelihood that an unknown compound of even modest abundance will give nearly the exact same molecular weight and exact mass of two specific fragments (*m*/*z* 85 and 145)

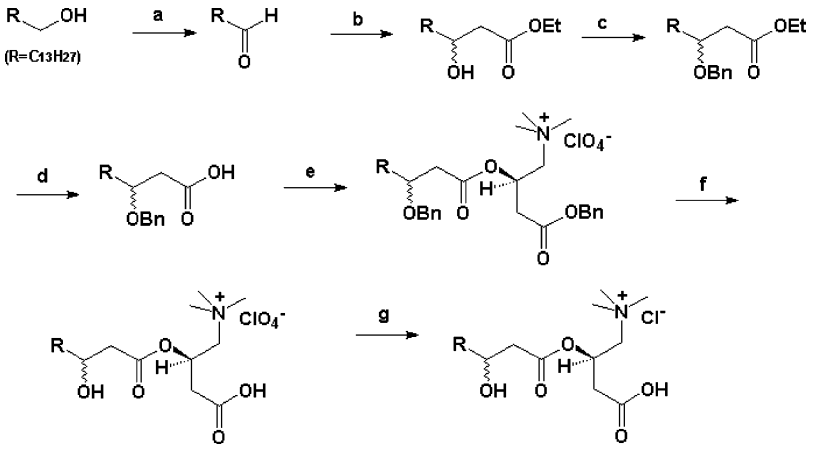

FIGURE 3: Synthesis of 3-hydroxypalmitoyl-L-carnitine. (a) Oxalyl chloride, DMSO. (b) Ethyl acetate, lithium bis(trimethylsilyl)amide/THF. (c) Benzyl 2,2,2-trichloroacetimidate, trifluoromethanesulfonic acid. (d) 4N LiOH/methanol/THF, 1:2:3. (e) DCC/THF. (f) $H_2$, 10% Pd/C; (g) anion exchange.

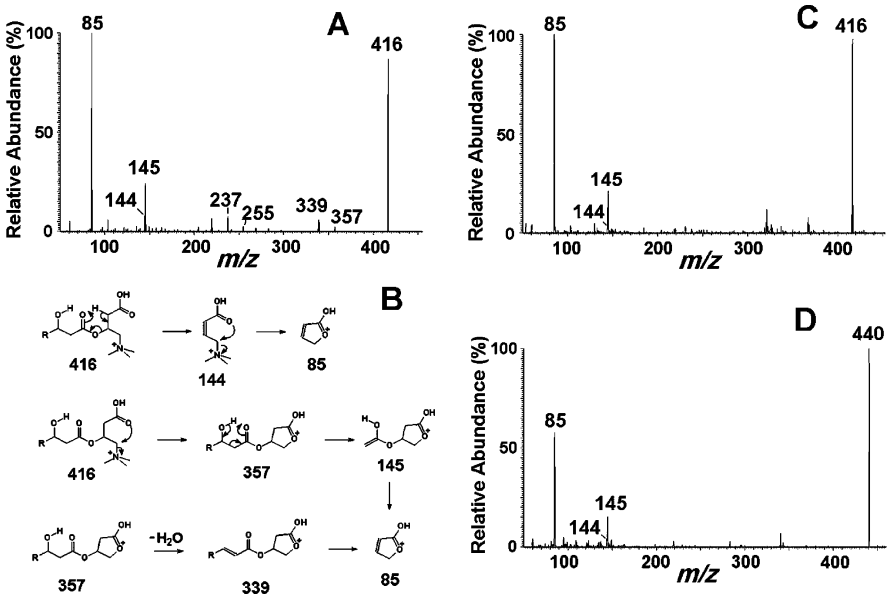


FIGURE 4: Positive-ion electrospray ionization tandem mass spectra of protonated 3-hydroxy acylcarnitines. Lipids were extracted from freeze-clamped rat myocardium by a modified Bligh and Dyer technique utilizing 5% acetic acid in the aqueous phase as described in the Experimental Procedures. Tandem mass spectrometry of protonated 3-hydroxy acylcarnitines were performed utilizing collision energy of 25 eV and a collision gas pressure of 2.5 mTorr as described in the Experimental Procedures. (A) Tandem mass spectrometry of protonated 3-hydroxypalmitoyl-L-carnitine (*m*/*z* 416) standard. (B) Fragmentation pattern of protonated 3-hydroxypalmitoyl-L-carnitine. (C) Tandem mass spectrometry of protonated 3-hydroxypalmitoyl-L-carnitine (*m*/*z* 416) in chloroform extract from rat myocardium. (D) Tandem mass spectrometry of protonated 3-hydroxylinoleoyl-L-carnitine (*m*/*z* 440) in chloroform extract from rat myocardium.

in the given ratios for 3-hydroxy acylcarnitine is very low. Collectively, the results identify a specific method for profiling 3-hydroxy acylcarnitines in complex biological mixtures directly from their organic extracts.

The predominant molecular species in the acylcarnitine pool in diabetic myocardium were molecular species with 18:1 and 18:2 aliphatic chains, which increased 6- and 7-fold, respectively (Table 1). The total 3-hydroxy acylcarnitine mass present in myocardium of rats rendered diabetic increased 4-fold from 573 to 2267 pmol/mg protein (Table 1). This mass increase was mainly due to the 5- and 7-fold increases in OHAC18:1 and OHAC18:2 molecular species mass, respectively. The total 3-hydroxy acylcarnitine mass represented 32% of total acylcarnitine mass present in diabetic rat myocardium. Strikingly, unsaturated acylcarnitines increased 4.4-fold in the myocardium of rats rendered diabetic, while saturated acylcarnitines only increased 2.4-fold (Table 1). This result suggests that pathways reflecting preferential release of unsaturated fatty acids were selectively activated in diabetic myocardium.

Interestingly, acylcarnitine mass did not further increase but rather decreased during prolonged diabetes, which suggests the development of time-dependent compensatory changes (Table 1). For example, total acylcarnitine mass of 4218 pmol/mg protein was present in rat myocardium rendered diabetic for 12 weeks. This represents a 2.3-fold increase from the control but is only 60% of the acylcarnitine mass present in 6-week diabetic rat myocardium ($p < 0.01$). Similar to results found in 6-week diabetic rat myocardium, the major alterations in species mass were present in molecular species containing oleoyl and linoleoyl acyl chains with approximately 3- and 4-fold increases in both hydroxyl- and non-hydroxyl-containing molecular species (Table 1). The total 3-hydroxy acylcarnitine mass accounted for 31% of total acylcarnitine mass present in 12-week diabetic rat myocardium.

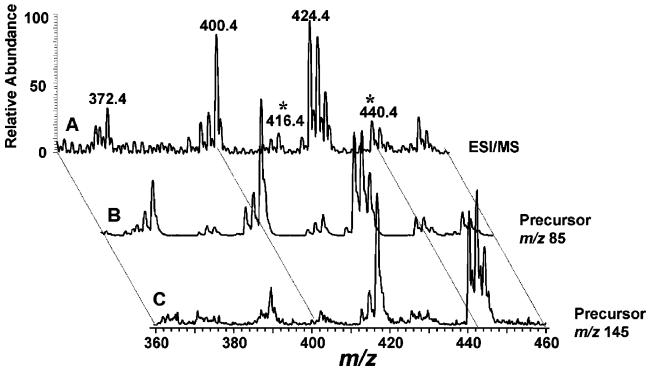


FIGURE 5: Profiling of acylcarnitine and 3-hydroxy acylcarnitines in rat myocardium. Lipids from rat hearts were extracted from freeze-clamped myocardium by a modified Bligh and Dyer technique utilizing 5% acetic acid in the aqueous phase as described in the Experimental Procedures. (A) Acylcarnitine molecular species were quantitated by ESI/MS in the positive ion mode as their protonated molecular ions. (B) Total acylcarnitines were profiled by tandem mass spectrometry in the precursor-ion scanning mode for *m*/*z* 85. (C) 3-Hydroxy acylcarnitines were profiled by tandem mass spectrometry in the precursor-ion scanning mode for *m*/*z* 145.

*The Effects of Insulin Treatment on Acylcarnitine Accumulation.* ESI/MS analyses in the positive-ion mode of chloroform extracts of insulin-treated diabetic rat myocardium demonstrated a dramatic decrease in both non-hydroxy acylcarnitines and 3-hydroxy acylcarnitines in comparison to acylcarnitine mass present in diabetic rat myocardium induced by streptozotocin treatment (Figure 1C and Table 1). The total amounts of acylcarnitines in myocardium of the control, diabetes, and diabetes/insulin were $1838 \pm 295$, $7073 \pm 1371$, and $2462 \pm 417$ pmol/mg protein, respectively. Similarly, insulin decreases the amount of 3-hydroxy acylcarnitines in diabetic myocardium from $2267 \pm 390$ pmol/mg protein to $759 \pm 164$ pmol/mg protein, which is very close to the amount of 3-hydroxy acylcarnitines in control myocardium ($573 \pm 115$ pmol/mg protein). These results demonstrated that the accumulation of acylcarnitines in diabetic myocardium was almost completely reversed by insulin treatment.

ESI/MS analyses of chloroform extracts of insulin-treated control rat myocardium in the positive-ion mode demonstrated a similar distribution of acylcarnitine molecular species in comparison to that found in untreated control rat myocardium (Table 1), although some differences of acylcarnitine mass and molecular species profiles were present. The major difference was a decrease in the total 3-hydroxy acylcarnitine mass present in insulin-treated controls compared to their untreated counterparts. Strikingly, this accounted for an approximately 40% decrease (351 versus 573 pmol/mg protein) in 3-hydroxy acylcarnitine mass, while the total mass of non-hydroxy myocardial acylcarnitine molecular species was not altered in insulin-treated nondiabetic rats (1227 versus 1265 pmol/mg protein).

*Alterations of iPLA$_2$ Activities in Diabetic Rat Myocardium.* Once acylcarnitines are synthesized, they can be hydrolyzed back to FFAs by acylcarnitine hydrolase. The measured acylcarnitine hydrolase activity does not change [assayed as FA release from palmitoyl-1-$^{14}$C−L-carnitine, $0.51 \pm 0.03$ versus $0.55 \pm 0.07$ nmol min$^{-1}$ (mg of protein)$^{-1}$] in diabetic myocardium. Thus, aberrant modulation of CPT I activity may contribute to the incremental accumulation of acylcarnitines in diabetic myocardium. Previous work demonstrated that the expression of CPT I was regulated by peroxisome proliferator activated receptor α (PPARα) and that multiple lipids are potential endogenous activators for this family of transcriptional factors (*42*−*45*). PLA$_2$ hydrolyzes phospholipids to give free fatty acids and lysophospholipids, which can be further hydrolyzed to provide additional FFA. Moreover, FFAs are proximal substrates for acylcarnitine synthesis after activation by acylCoA synthetases followed by CPT activity. Thus, PLA$_2$ may regulate acylcarnitine accumulation in diabetic myocardium by contributing to substrate availability. To determine if alterations in phospholipase activities in diabetic hearts were present, we examined enzymatic activity utilizing a fluorogenic probe, arachidonoyl-3-cyano-7-hydroxycoumarin (ACHC) that we synthesized (see the Supporting Information for synthesis). The microsomal fraction from diabetic myocardium contains twice as much ACHC esterase activity as that manifest in control hearts ($p < 0.001$) (parts A and B of Figure 6). ACHC esterase activities were readily inhibited by (*S*)-BEL (inhibitor for iPLA$_2\beta$) or (*R*)-BEL (inhibitor for iPLA$_2\gamma$) in the microsomes (Figure 6C). Thus, both major iPLA$_2$ activities are increased in diabetic myocardium. These results suggest that iPLA$_2$ activities may potentially contribute to the accumulation of the LCACs in diabetic rat myocardium. To further substantiate these alterations in measured enzymatic activity, iPLA$_2\beta$ and iPLA$_2\gamma$ mRNA levels were examined in control and 6-week diabetic mouse myocardium. Consistent with this notion, iPLA$_2\beta$ and iPLA$_2\gamma$ mRNA level increased 2.5- and 1.5-fold ($p < 0.05$), respectively, in 6-week diabetic mouse hearts (Figure 6D).

*Accumulation of Acylcarnitines in Ischemic or iPLA$_2\beta$ Overexpressing Mouse Myocardium.* To further elucidate the relationship between myocardial iPLA$_2$ activities and acylcarnitine accumulation, independent models of iPLA$_2$ induction were investigated. Previous studies demonstrated that iPLA$_2$ activities are activated in ischemic myocardium (*30*, *46*). Shotgun lipidomics demonstrated that LCACs accumulate in ischemic myocardium and that their accumulation is diminished by the mechanism-based inhibitor of iPLA$_2$, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (BEL) (Figure 7). Next, a transgenic mouse model constitutively overexpressing iPLA$_2\beta$ was examined. Direct infusion of organic extracts obtained from normally perfused myocardium of mice overexpressing iPLA$_2\beta$ in a cardiac myocyte restricted fashion again clearly demonstrated the accumulation of LCACs. The amounts of total LCACs increased 2−3-fold in mice overexpressing iPLA$_2\beta$, which were neither ischemic nor diabetic. Moreover, ischemia greatly enhances the accumulation of LCACs in iPLA$_2\beta$ overexpressing mouse myocardium, which was greatly reduced by BEL (Figure 7). Collectively, these results identify by chemical, pathophysiologic, and genetic models an important role for iPLA$_2$ in the generation of acylcarnitines in normal, ischemic, and diabetic conditions.

## DISCUSSION

LCACs are essential metabolic intermediates in fatty acid catabolism, which are utilized in biologic systems to transport the aliphatic moiety of membrane impermeable acyl CoA molecular species into the mitochondrial matrix for oxidation (*10*). The accumulation of acylcarnitines in muscle has

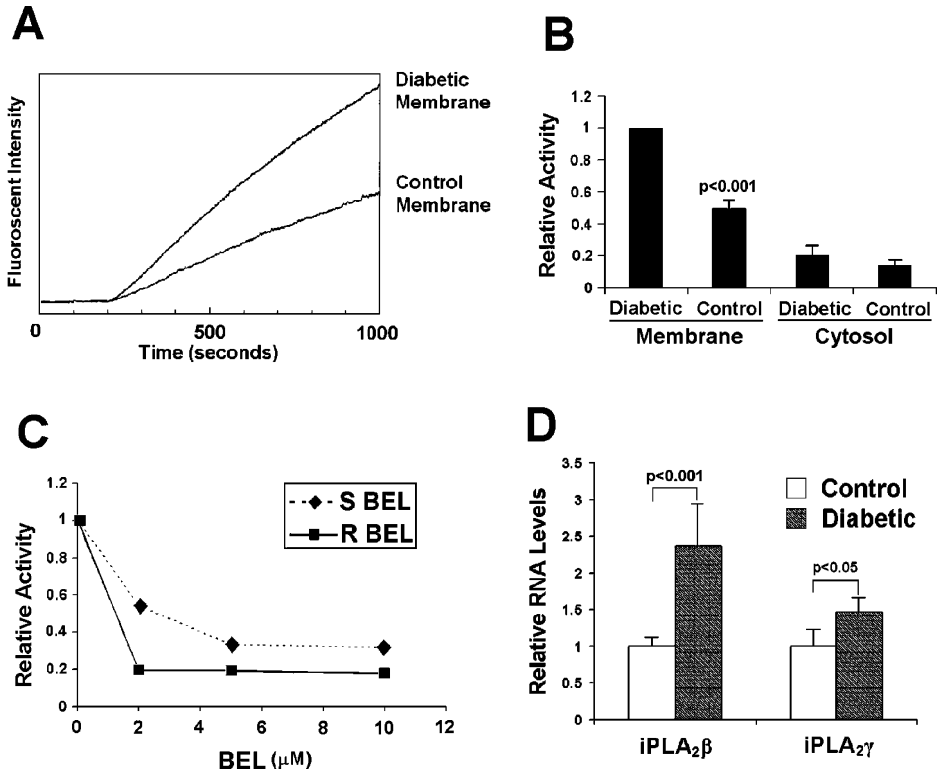


FIGURE 6: Modulation of iPLA$_2$ activities in control and diabetic myocardium. The cytosolic amd microsomal fractions of control and diabetic myocardium were prepared as described in the Experimental Procedures. Hydrolysis of ACHC was followed by the increase in fluorescent intensity at 450 nm (excitation of 351 nm at pH = 7.3) because of the production of the high fluorescent 3-cyano-7-hydroxycoumarin as described in the Experimental Procedures. Blanks were monitored for 100 s before the initiation of the hydrolysis with cytosolic or microsomal proteins. (A) Comparison of ACHC hydrolase activity in diabetic and control microsomal fractions. (B) Relative ACHC hydrolase activity in cytosolic and microsomal fractions from control and diabetic rat myocardium. (C) Effects of (*R*)-BEL and (*S*)-BEL on ACHC hydrolase activity in the microsomal fraction of diabetic rat myocardium. (D) Messenger RNA levels of iPLA$_2\beta$ and iPLA$_2\gamma$ in control and 6-week diabetic mouse myocardium.

previously been observed during dysfunctional mitochondial FAO either because of inherited enzyme defects or insufficient oxygen supply (e.g., ischemia) (*18*, *47*, *48*). In the diabetic heart, the enzymes involved in mitochondrial FAO are upregulated because the cell relies predominantly on FAO for energy production because of diminished glucose uptake and utilization (*1*, *49*, *50*). The formation of LCAC is catalyzed by carnitine palmitoyl transferase I (CPT-I), which is tightly regulated at transcriptional, translational, and post-translational levels because of its central role in modulating cellular energy production. Typically, acylcarnitines are delivered into mitochondrial matrix by carnitine-acylcarnitine translocase, where they are rethioesterified back to acyl CoA by CPT-II prior to utilization in energy-producing fatty acid $\beta$ oxidative pathways. Recent studies have also suggested involvement of acylcarnitines in the transport of acyl moieties into peroxisomes or ER (*41*, *51*−*53*), which indicate the potential complexity of roles of acylcarnitines in intracellular fatty acid trafficking.

Once acylcarnitines are synthesized, they can be hydrolyzed back to FFAs and carnitine and diffuse to different intracellular organelles or be transported by specific transporters for fatty acid oxidation or TAG synthesis (*10*, *51*). Because measured acylcarnitine hydrolase activity does not change during diabetes and consumption of acylcarnitines by FAO is increased in diabetic hearts, it seems likely that the accumulation of acylcarnitine is likely due to an increase in the acylcarnitine synthetic rates, which cannot be accommodated by accelerated flux through oxidative pathways in

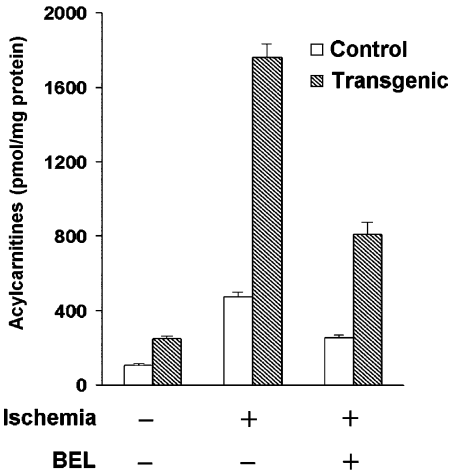


FIGURE 7: Effects of ischemia, iPLA$_2\beta$ overexpression, and BEL on the accumulation of LCACs. Langendorf-perfused hearts were either control-perfused or rendered ischemic as described in the Experimental Procedures. To determine the effects of BEL on acyl carnitine accumulation, hearts were perfused with buffer containing 10 $\mu$M BEL beginning 5 min before coronary artery ligation and during the ischemic interval. At the end of each perfusion interval, hearts were rapidly freeze-clamped and myocardial tissue was pulverized to a fine power at the temperature of liquid nitrogen. Lipids were extracted by a modified Bligh and Dyer technique utilizing 5% acetic acid in the aqueous phase. Acylcarnitines were quantified in positive ion mode utilizing ESI/MS by comparison of their protonated ion peak intensity to that of the internal standard (AC12:0). The total amount of LCAC was calculated by adding up the amounts of all individual molecular species. Each value represents mean ± SEM from at least three determinations.

diabetic myocardium. Indeed, multiple studies have demonstrated CPT mRNA and activity increase in diabetic states (*54*−*56*). It has been demonstrated that CPT-I exits as two isoforms: liver-type L-CPT-I (or CPT-I$\alpha$), which is enriched in liver, and muscle-type M-CPT-I (or CPT-I$\beta$), which is expressed abundantly in heart, skeletal muscle, and brown adipose tissue (BAT) (*57*−*59*). Rat hearts express both forms of CPT-I. Regulation of the expression of M-CPT-I is complex and as yet incompletely understood. Although FFAs have been shown to activate the transcription of M-CPT-I gene in cardiac myocytes via PPAR$\alpha$ (*43*, *60*) (increasing of M-CPT-I gene transcription and presumably CPT-I protein and activity levels), no alterations in M-CPT-I mRNA levels were observed in diabetic rat hearts (*54*). In contrast, the L-CPT-I mRNA level in rat hearts instead was shown to increase during diabetic dysfunction (*54*). Additionally, CPT-I activity is regulated by the reversible binding to malonyl CoA, and the $IC_{50}$ of M-CPT-I for malonyl CoA is 100 times lower than that of L-CPT-I (*16*, *61*). M-CPT-I activity may be altered because of malonyl CoA regulation even if transcription is unchanged in diabetic rat myocardium. This conclusion is substantiated by results identifying that the decrease of intracellular levels of malonyl CoA is associated with increased FAO in the diabetic state (*62*, *63*). Collectively, increased transcription of L-CPT-I and decreased intracellular levels of malonyl CoA in aggregate likely contribute to the upregulation of CPT-I activity in streptozotocin-treated diabetic rat hearts leading to the accumulation of acylcarnitines, which cannot be further processed by typical oxidative pathways. Moreover, the increased serum FFA delivery also provides substrates for acylcarnitine synthesis, which likely contribute to its accumulation in diabetic myocardium (*64*, *65*).

The present studies demonstrate the activation of $iPLA_2$ activity in diabetic hearts and suggest that the increased $iPLA_2$ activity in diabetic myocardium contributes to the pool of endogenous fatty acids, which are utilized for the synthesis of LCACs in diabetic myocardium. This notion is supported in three independent systems where $iPLA_2$ was activated by distinct mechanisms. In ischemic hearts, acylcarnitines were dramatically increased and the increase was attenuated by pretreatment with BEL, a mechanism-based inhibitor highly selective for $iPLA_2$ versus other types of secretory and cytosolic phospholipase activities. Furthermore, acylcarnitines were also increased in myocardium by genetic engineering through cardiac myocyte restricted overexpression of $iPLA_2\beta$. Moreover, the increase in acylcarnitines was greatly enhanced by ischemia in $iPLA_2\beta$ overexpressing mice. Similarly, BEL attenuated ischemia-induced acylcarnitine accumulation in these transgenic animals. In diabetes, an endemic disease in industrialized nations, high phospholipase $A_2$ activity is present in myocardium, which is reflected by both the increased levels of mRNA encoding $iPLA_2$ and $iPLA_2$ activity with the concomitant association of increases in acylcarnitines. Finally, during cardiac ischemia, $iPLA_2$ activity and acylcarnitines increase, which are both amplified in genetically engineered mice. The biochemical mechanisms underlying $iPLA_2$-mediated activation in diabetes are still unclear but likely involve combinations of transcription and the tonic disinhibition of enzymatic activity by calmodulin and/or nucleotide-mediated alterations in $iPLA_2$ activity previously identified (*66*, *67*).

$iPLA_2$ catalyzes the hydrolysis *sn*-2 ester linkage of phospholipids to release FFAs and lysophospholipids. Lysolipids have multiple effects on biologic function including activation of ion channels, receptors, and multiple protein kinases. Previous work demonstrated that alterations in the physical properties of membranes alter the sensitivity of CPT-I to malonyl CoA inhibition (*68*). Thus, it is possible that $iPLA_2$ may alter membrane-surface properties and molecular dynamics and thus interfere with malonyl CoA-mediated inhibition of CPT-I. The most direct mechanism through which $iPLA_2$ can increase acylcarnitines is by releasing FFAs from phospholipids and providing a substrate for the CPT-I-mediated synthesis of acylcarnitines although many more complex mechanisms are also possible.

The presence of 3-hydroxy acylcarnitines in rat myocardium suggests a reversible conversion between acyl CoA and acylcarnitine pools. 3-Hydroxy acyl CoA, an intermediate of mitochondrial fatty acid $\beta$ oxidation, may directly be converted to acylcarnitine by CPT-II (*41*). Alternatively, 3-hydroxy acyl CoA can be hydrolyzed by intramitochondrial acyl CoA thioesterases. The 3-hydroxy FFA generated can diffuse out alone or may also undergo facilitated transport out of mitochondria by a pathway mediated by uncoupling protein 3 (UCP-3) (*13*, *14*). The cytosolic 3-hydroxy FFAs can thus reform thioesters and generate corresponding 3-hydroxy acylcarnitines catalyzed by CPT-I. The accumulation of 3-hydroxy acylcarnitines, instead of shorter chain-length acylcarnitine, in diabetic myocardium demonstrated that mitochondrial fatty acid $\beta$ oxidation in diabetic states may be impaired in the early cycles of degradation. The absolute mass increase of 3-hydroxy acylcarnitines is proportional to the increase of the total acylcarnitine mass in diabetic myocardium and thus demonstrates that the rate-determining step of mitochondrial fatty acid $\beta$ oxidation is downstream to the formation of 3-hydroxy acyl CoA (i.e., distal to the reaction catalyzed by long-chain 3-hydroxyacyl CoA dehydrogenase). This is the same rate-determining step in fatty acid $\beta$ oxidation as is present in control myocardium but occurs during a substantially decreased $\beta$ oxidation flux in normal compared to diabetic myocardium. This conclusion agrees with a very recent study utilizing genetically engineered long-chain acyl CoA dehydrogenase (LCAD) deficient mice (*69*). Specifically, in LCAD−/− mice, when the rate-determining step was genetically moved upstream to the formation of 3-hydroxy acyl moieties in the $\beta$-oxidation cycle, the amounts of 3-hydroxy acylcarnitine were dramatically decreased (*69*). Moreover, for patients with deficiency of long-chain 3-hydroxy acyl CoA dehydrogenase, both long-chain 3-hydroxy acylcarnitines and LCACs were elevated in plasma (*70*). Kinetic studies of the purified trifunctional $\beta$-oxidation complex from heart mitochondria also indicated that 3-hydroxy acyl CoA dehydrogenase catalyzed the slowest reaction in the sequence (*71*). One possible mechanism is that altered redox states with the consumption of $NAD^+$ without sufficient regeneration from NADH (i.e., increased negative redox state) in diabetic myocardium could compromise the completion of the fatty acid $\beta$-oxidation cycle. Another possible reason underlying the increase in 3-hydroxy acylcarnitines is to increase the reaction flux through the rate-determining step in the $\beta$ oxidation by providing more 3-hydroxy acyl CoA in critical mitochondrial compartments. This effect will be greatest if the concentration

of 3-hydroxy acyl CoAs is substantially less than the $K_m$ of this reaction in the compartment of interest. Of course, compartmentation, channeling of metabolic intermediates through the trifunctional complex, and membrane−substrate interactions are important factors in the net flux of fatty acids through $\beta$-oxidative pathways. Collectively, the increase of 3-hydroxy acylcarnitne along with non-hydroxy acylcarnitines in diabetic myocardium suggests that 3-hydroxy acyl CoA dehydrogenase catalyzes the rate-limiting step of the $\beta$-oxidation cycle in both control and diabetic myocardium, although the flux through $\beta$-oxidation pathways in diabetic myocardium is substantially larger (*50*).

The role of 3-hydroxy acylcarnitines in different physiological and pathologic conditions or as an indicator of compromised lipid metabolism in diabetic myocardium may thus represent an important biomarker of dysfunctional fatty acid metabolism. The present studies demonstrated that the molecular profiling of acylcarnitine might be a useful tool in the diagnosis of diabetes. Indeed, a recent study shows that the urinary acylcarnitine pattern can be useful in monitoring diabetes mellitus (*72*).

Collectively, the present studies demonstrated the important role of calcium-independent phospholipases $A_2$ in the accumulation of LCACs in diabetic and ischemic myocardium. Because the substantial amounts of LCACs partition into the sarcolemmal membranes, as determined by electromicroscopic autoradiography (*73*), the accumulation of LCACs in diabetic myocardium likely results in changes of membrane physical properties and electrophysiologic function potentially contributing to ischemia-induced and diabetes-induced arrhythmias. Thus, iPLA$_2$s that have pleotropic roles in myocardial energy metabolism and signaling likely contribute to the pathophysiologic sequelae of diabetes in myocardium by fostering maladaptive changes in energy homeostasis.

## SUPPORTING INFORMATION AVAILABLE

Experimental procedures for the synthesis of 3-hydroxypalmitoyl-L-carnitine chloride and arachidonoyl-3-cyano-7-hydroxycoumarin. This material is available free of charge via the Internet at http://pubs.acs.org.

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