Caloric Restriction Results in Phospholipid Depletion, Membrane Remodeling, and Triacylglycerol Accumulation in Murine Myocardium†

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ABSTRACT: Herein, we utilize the power of shotgun lipidomics to demonstrate that modest caloric restriction results in phospholipid depletion, membrane remodeling, and triacylglycerol (TAG) accumulation in murine myocardium. After brief periods of fasting (4 and 12 h), substantial decreases occurred in the choline and ethanolamine glycerophospholipid pools in murine myocardium (collectively, a decrease of 39 nmol of phospholipid per milligram of protein at 12 h representing ∼25% of total phospholipid mass and ∼20 cal of Gibbs free energy per gram wet weight of tissue). Remarkably, the selective loss of long-chain polyunsaturated molecular species was present in the major phospholipid classes thereby altering the physical properties of myocardial membranes. No decrease in TAG mass was present in myocardium during fasting, but rather myocardial TAG increased during 12 h of refeeding nearly 3-fold returning to baseline levels only after 24 h of refeeding. No alterations in other examined lipid classes were present during fasting. In contrast to these lipid alterations in myocardium, no decreases in phospholipid mass were present in skeletal muscle myocytes and a dramatic decrease in skeletal muscle (or skeletal muscle associated) TAG mass was prominent after 12 h of fasting. These results identify phospholipids as a rapidly mobilizable energy source during modest caloric deprivation in murine myocardium, while triacylglycerols are a major source of energy reserve in skeletal muscle. Collectively, these results demonstrate dramatic alterations in the membrane composition of mildly fasted mammalian myocardium that identify the unanticipated plasticity of myocardial phospholipids to adapt to modest chemical and physical perturbations.

The regulation of intracellular energy supply and demand is a critical and highly evolved feature of mammalian metabolism. Through integrating the metabolic processing of substrates derived from multiple specialized tissues (liver, muscle, and adipose stores) mammals have evolved a highly efficient multicomponent system to preserve energy in times of food excess and redistribute energy reserves to vital organs (e.g., heart and brain) in times of caloric deprivation (*1*−*3*). In myocardium, this is of special significance since adequate supplies of chemical energy are necessary for appropriate hemodynamic function and thus represent a sine qua non for survival of the organism as a whole. The human condition requires prolonged fasts during sleep with intervals between feedings of up to 12 h. Many studies have underscored the increased susceptibility of patients with ischemic heart disease to arrhythmogenic sudden death in the early morning hours (*4, 5*).

Traditional dogma states that the energy inherent in the C−C bonds of aliphatic chains in phospholipids cannot be accessed until exhaustive fasting has occurred (*6*). Although the roles of triacylglycerols (TAGs)[1] and glycogen as myocardial energy reserves are universally accepted, the role of phospholipids as an energy source has not been considered even though the majority of chemical Gibbs free energy present within the heart is present in the C−C bonds of phospholipids. Indeed, kinetic models of myocardial bioenergetics explicitly utilize alterations in glycogen and TAG pool sizes as the principle determinants of endogenous myocardial chemical energy reserve without consideration of the Gibbs free energy inherent in phospholipids (*7, 8*). Typically, myocardium obtains the large majority of the energy necessary for hemodynamic function by extracting lipid moieties from the coronary circulation. Under physiologic conditions, myocardium oxidizes 300−700 nmol of fatty acids per gram of dry weight per minute (*9*), which is predominantly obtained by extraction of circulating fatty

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[1] Abbreviations: ESI, electrospray ionization; FAME, fatty acid methyl ester; GroPCho, glycero-3-phosphocholine; GroPEtn, glycero-3-phosphoethanolamine; GroPGro, glycero-3-phosphoglycerol; GroPSer, glycero-3-phosphoserine; *m*:*n*, acyl chain containing *m* carbons and *n* double bonds; MS, mass spectrometry; NL, neutral loss; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; PlsEtn, plasmenylethanolamine(s); PtdCho, phosphatidylcholine(s); SM, sphingomyelin(s); TAG, triacylglycerol; T*m*:*n* TAG, tri *m*:*n* glycerol.

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acids or fatty acids transported into myocytes after lipoprotein lipase mediated hydrolysis of serum TAGs and phospholipids (see refs *10* and *11* for recent reviews). However, during cardiac ischemia, insufficient delivery of blood-borne substrate is present to support hemodynamic function and endogenous stores of energy must be utilized. Typically, energy stores thought to predominantly contribute to this reserve accessed during ischemic episodes include myocardial glycogen and TAG stores. However, calculations demonstrate that myocardium contains only a small amount of energy potentially obtainable from glycogen and TAGs to sustain hemodynamic function since the physiologic concentrations of these moieties are quite low and myocardium relies instead on a continued extraction of perfused substrates. During fasting, prior studies have demonstrated that energy is mobilized from adipocytes during TAG hydrolysis by release of aliphatic chains in adipocyte TAG pools into the serum (*12*, *13*). These free fatty acids are extracted into cardiac myocytes (or other organs) and either are utilized directly for energy production or enter cardiac myocyte TAG stores to create an energy reserve designed to protect the heart from adverse conditions during further fasting. In most circumstances, this represents an effective strategy to mobilize energy reserves to the most important functional compartments (e.g., heart and brain) for survival of the organism as a whole. However, if cardiac ischemia occurs during fasting, this results in a complex interplay of the heart being deprived of blood-born substrate (by coronary artery constriction) at a time where membrane adaptations to the fasted state have occurred. However, the types and extent of phospholipid alterations during caloric restriction in myocardium have not been previously defined. Although not typically considered, the majority of endogenous energy in cardiac myocytes in normal or fasted circumstances is present in the aliphatic chains present in myocardial phospholipids. In this study, we utilize shotgun lipidomics with multidimensional electrospray ionization mass spectrometry (ESI/MS) (*14*−*16*) to demonstrate that the majority of endogenous chemical energy mobilized during mild fasting in murine myocardium is present in phospholipids. These studies further demonstrate that fasting initiates the depletion of phospholipid and alterations in aliphatic chain composition. The consequences of myocardial ischemia in the context of these fasting-induced alterations in myocardial membranes are discussed.

MATERIALS AND METHODS

Materials. Synthetic phospholipids including 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (14:1-14:1 GroPCho), 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine (22:6-22:6 GroPCho), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine (15:0-15:0 GroPEtn), 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine (20:4-20:4 GroPEtn), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoglycerol (15:0-15:0 GroPGro), 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (14:0-14:0 GroPSer), and 1-heptadecanoyl-2-hydroxyl-*sn*-glycero-3-phosphocholine (17:0 lysoGroPCho) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Arachidic acid (20:0 FA) and triheptadecenoin (T17:1 TAG) were purchased from Nu-Chek Prep, Inc. (Elysian, MN). Deuterated (7,7,8,8-D_4) palmitic acid (d_4-16:0 FA) was purchased from Cambridge Isotope Laboratories, Inc. (Cambridge, MA). Lipid standards were quantitated by capillary GC after acid methanolysis by comparisons with an arachidic acid standard as described previously (*17*) and used as internal standards during mass spectrometric analysis. The Amplex Red cholesterol assay kit was obtained from Molecular Probe, Inc. (Eugene, OR). Starch assay kit (STA-20) and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All the solvents used for sample preparation and for mass spectrometric analysis were obtained from Burdick and Jackson (Honeywell International Inc., Burdick and Jackson, Muskegon, Michigan, USA).

Sample Preparation. All studies were approved by the Animal Studies Committee of Washington University School of Medicine. Male mice (B6 CBAF1/J, 4 months of age) were purchased from The Jackson Laboratory (Bar Harbor, ME), housed in a full barrier facility with a 12-h light/dark cycle, and maintained on standard chow (Diet 5053; Purina Inc., St. Louis, MO) with free access to food and water. Mice subjected to caloric restriction were maintained with free access to water but no food for an experimental period ($\leq$12 h as stated in the text). Mice were sacrificed by inhalation of carbon dioxide prior to tissue collection. The hearts and livers were excised quickly and immersed in ice-cold diluted PBS buffer. After removing extraneous tissue and epicardial fat, each heart and liver was quickly dried and immediately freeze-clamped at the temperature of liquid nitrogen. Myocardial and hepatic wafers were pulverized into fine powder with a stainless steel mortar and pestle. Myocardial wafers ($\sim$25 mg) were further homogenized in 0.5 mL of ice-cold LiCl solution (50 mM) by using a Potter−Elvehjem tissue grinder for 2 min. A small volume of homogenate containing 2−5 mg of protein was transferred to a glass test tube. Methanol and chloroform (2 mL of each), as well as an additional volume of LiCl solution to make a final volume of 1.8 mL with a final LiCl concentration of 50 mM, were added to the test tube containing the heart homogenate for lipid extraction by the Bligh and Dyer procedure (18). Similarly, mouse hind limb skeletal muscle samples were collected, pulverized into fine powder at the temperature of liquid nitrogen, and further homogenized prior to a protein assay. Lipid extracts from skeletal muscle samples containing between 2 and 5 mg of protein were similarly prepared. The protein concentration of homogenates was then determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

At this point, internal standards including 14:0-14:0 GroPSer (2.0 nmol/mg of protein), 14:1-14:1 GroPCho (15 nmol/mg of protein), 15:0-15:0 GroPGro (4.2 nmol/mg of protein), 15:0-15:0 GroPEtn (18.75 nmol/mg of protein), T17:1 TAG (10 nmol/mg of protein), and d_4-16:0 FA (2 nmol/mg of protein) were added to each homogenate based on the experimentally determined protein concentrations for normalization to the protein content. Next, the extraction mixture was centrifuged at 2500 rpm for 10 min. The chloroform layer was carefully removed and saved. To the MeOH/aqueous layer of each test tube, an additional 2 mL of chloroform was added; the mixture was vortexed and centrifuged, and the chloroform layers were combined and subsequently dried under a nitrogen stream. Each residue was then resuspended in 4 mL of chloroform/methanol (1:1, v/v) and re-extracted against 1.8 mL of 20 mM LiCl aqueous solution, and the extract was dried as described

above. Each residue was resuspended in ~1 mL of chloroform and filtered with a 0.2-μm PFTE syringe filter into a 5-mL glass centrifuge tube (this step was repeated twice). The chloroform filtrate was subsequently dried under a nitrogen stream and resuspended with a volume of 500 μL/mg of protein in 1:1 (v/v) chloroform/methanol, and lipid extracts were finally flushed with nitrogen, capped, and stored at −20 °C for ESI/MS analyses (typically within 1 week). For gas chromatographic (GC) analysis of acyl moieties in myocardial lipid extracts, total myocardial lipids were extracted using a modified Bligh and Dyer procedure as described above from 50 mg of myocardial wafers in the presence of 25 μg of arachidic acid (used as an internal standard). For HPLC analysis of choline glycerophospholipid (PC) and ethanolamine glycerophospholipid (PE), molecular species in myocardial lipid extracts, total myocardial lipids were extracted using a modified Bligh and Dyer procedure as described above from ~80 mg of myocardial wafers in the presence of 15 and 19 nmol per milligram of protein of 22:6-22:6 GroPCho and 20:4-20:4 GroPEtn (used as an internal standard), respectively.

Lipid Analyses. Multidimensional ESI mass spectrometric analyses of lipids directly from lipid extracts of biological samples were performed using a triple-quadrupole mass spectrometer (ThermoFinnigan TSQ Quantum, San Jose, CA) operating under Xcalibur software as described in detail previously (*15*). Briefly, each prepared lipid solution was diluted approximately 50-fold with 1:1 (v/v) chloroform/methanol just prior to infusion for anionic lipid analyses, which contained less than 10 pmol/μL total lipids. A small amount of LiOH (50 nmol per milligram of protein) was added to the diluted lipid solution just prior to performing other lipid analyses in both negative- and positive-ion modes. The diluted lipid extract solution was directly infused into the ESI source at a flow rate of 2 μL/min with a syringe pump using an orthogonal injection. For tandem mass spectrometry, both the first and third quadrupoles served as analyzers, while the second quadrupole served as the collision cell. A collision gas pressure was set at 1.0 mTorr, and the collision energy was varied with the classes of lipids as described previously (*15*, *16*). Typically, a 1-min period of signal averaging in the profile mode was employed for each MS spectrum, and a 2-min period of signal averaging was utilized for each tandem MS spectrum.

Data analyses from multidimensional mass spectra were performed as described previously (*15*, *16*). By this technique, peak assignments can be substantiated by multiple independent mass spectrometric criteria, isobaric molecular species and regiospecificity can be discriminated, and quantitation of low abundance molecular species can be performed or refined. Accurate quantitation of the minor pseudomolecular ions can be achieved by tandem mass spectrometry by a two-stage process. First, the abundant pseudomolecular ions in a class are quantitated by comparison with internal standards in the first-dimensional mass spectra. Next, these values were used as endogenous standards for ratiometric comparisons to quantitate the low abundance individual molecular species contents from tandem mass spectra.

Miscellaneous. Capillary GC analyses of the masses of individual acyl moieties in each lipid extract after acid methanolysis were performed by comparison with an arachidic acid standard as described previously (*17*). For HPLC analysis of PC and PE molecular species, phospholipid classes were first resolved by a cation-exchange HPLC column (Partisil 10 SCX, 4.6 mm × 250 mm, Whatman) as previously described (*19*). Individual molecular species present in the PC and PE fractions were resolved by reverse-phase HPLC with an Ultrasphere ODS 5 μm 4.6 mm × 250 mm column (Beckman-Coulter) employing methanol/acetonitrile/water containing 20 mM choline chloride (90.5/2.5/7, v/v/v) as the mobile phase as previously described (*20*). Ultraviolet absorbance was monitored at 203 nm. Protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. Glycogen content of liver, skeletal muscle, and heart was determined using a starch assay kit (Sigma, St. Louis, MO) following the manufacturer's protocol. Blood glucose level was measured by using a FreeStyle Flash Blood Glucose Monitoring System (Therasense, Alameda, CA). The free cholesterol content of the samples was quantitated by an enzymatic assay using an Amplex Red cholesterol assay kit according to the instructions of the manufacturer. Data from biological samples were normalized to the protein content and all data are presented as the mean ± SEM of a minimum of four separate animals.

RESULTS

Electrospray ionization mass spectrometry (ESI/MS) of lipid extracts of myocardium from mice subjected to modest caloric deprivation (fasting for 4 h) demonstrated a substantial decrease (~20 mol %) in the content of PC (Figure 1 and Table 1). After just 4 h of fasting, 10 nmol per milligram of protein was lost from the PC pool (from control values of 70.4 ± 5.6 to 59.7 ± 4.9 nmol per milligram of protein). Moreover, this was accompanied by a substantial and selective loss of longer chain length highly unsaturated phospholipid molecular species containing 22:6 molecular species in the PC pool (Table 1). In contrast, the content of individual molecular species containing saturated or monounsaturated fatty acyl chains in other PC species was either unchanged or only minimally modified. Additional caloric restriction (fasting) for 12 h resulted in the dramatic decline of PE mass and prominent molecular species alterations in murine myocardial PE (Figure 2). Remarkably, nearly 40 mol % (i.e., ~30 nmol per milligram of protein) of the total PE mass in murine myocardium was lost after 12 h of fasting (Table 1 and Figure 2). ESI/MS analyses again demonstrated that the loss of phospholipid mass after fasting was predominantly from molecular species containing long chain length polyunsaturated fatty acyl chains (Table 1 and Figures 2 and 3). For example, the mass content of 18:0-22:6 GroPEtn decreased from 24.2 ± 4.5 in fed mice to 15.5 ± 3.1 nmol per milligram of protein in fasted mice. The mass levels of PE plasmalogen molecular species (e.g., 16:0-22:6 and 18:0-22:6 PlsEtn) decreased from 21.8 ± 2.3 to 12.6 ± 1.6 nmol per milligram of protein after fasting for 12 h. However, the ratio of diacyl PE to plasmalogen PE was not significantly different during the course of fasting and recovery (Table 1). Additional caloric restriction present from 4 to 12 h did not result in further decreases in the mass content and changes in molecular species composition in PC molecular species (panel C of Figure 1 and Table 1). Changes in the content of free cholesterol or other myocardial

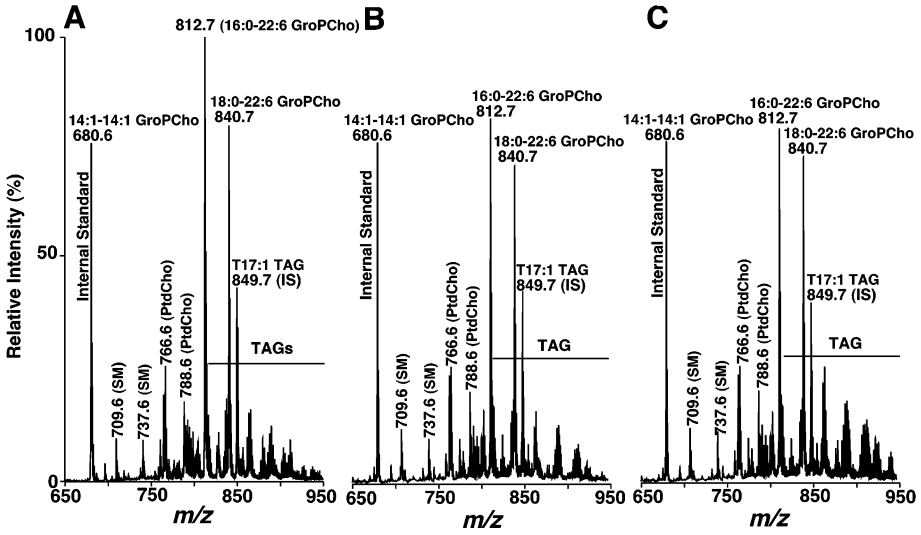

FIGURE 1: Representative positive-ion ESI mass spectra of myocardial lipid extracts of normally fed, 4-h-fasted, or 12-h-fasted mice. Myocardial lipids of normally fed (panel A), 4-h-fasted (panel B), and 12-h-fasted (panel C) mice at 4 months of age were extracted by a modified Bligh and Dyer method. Positive-ion ESI/MS of the lipid extracts in the presence of LiOH was performed as described in the Materials and Methods. All major individual molecular species as indicated were identified using 2D mass spectrometry. For comparison, the spectra in panels B and C were normalized to the internal standard peak of PtdCho in panel A. "IS" denotes the internal standard peak for TAGs. Most unlabeled ion peaks under the TAGs regions are lithium TAG adducts.

Table 1: Fasting-Induced Lipid Alterations in Mouse Myocardium[a]

class/subclass	fed	fasted (4 h)	fasted (12 h)	recovery (12 h)	recovery (24 h)
cardiolipin	0.08 ± 0.23	0.92 ± 0.15	0.86 ± 0.11	0.91 ± 0.06	0.90 ± 0.08
phosphatidylglycerol	1.76 ± 0.25	1.71 ± 0.12	1.53 ± 0.25	1.52 ± 0.10	1.68 ± 0.23
phosphatidylserine	4.98 ± 0.21	5.11 ± 0.33	5.15 ± 0.43	5.03 ± 0.44	5.04 ± 0.37
phosphatidylinositol	2.40 ± 0.18	2.26 ± 0.15	2.15 ± 0.27	2.61 ± 0.04	2.51 ± 0.33
phosphatidylcholine	70.39 ± 5.55	59.70 ± 4.88	59.95 ± 3.83	57.89 ± 3.22	69.44 ± 4.65
(16:0−22:6 PtdCho)	24.94 ± 4.07	18.08 ± 2.81	17.81 ± 2.21	15.65 ± 1.57	23.78 ± 3.56
(18:0−22:6 PtdCho)	18.08 ± 1.67	16.24 ± 2.90	17.04 ± 1.45	15.43 ± 2.00	18.22 ± 2.22
sphingomyelin	5.13 ± 0.23	4.72 ± 0.07	5.20 ± 0.33	5.52 ± 0.49	5.15 ± 0.19
ethanolamine glycerophospholipid	68.42 ± 6.59	71.68 ± 2.15	41.37 ± 7.01	72.07 ± 1.66	70.22 ± 5.45
(16:0−22:6 PtdEtn)	9.80 ± 0.57	9.25 ± 0.79	5.53 ± 0.83	8.14 ± 0.32	9.36 ± 0.88
(18:0−22:6 PtdEtn)	24.24 ± 4.47	24.38 ± 1.94	15.49 ± 3.07	24.23 ± 3.00	23.79 ± 2.85
(plasmalogen PE)	21.75 ± 2.25	22.86 ± 3.22	12.58 ± 1.59	19.55 ± 0.82	22.14 ± 3.21
(diacyl PE/plasmalogen PE)	2.17 ± 0.17	1.98 ± 0.33	2.29 ± 0.26	2.14 ± 0.33	2.22 ± 0.20
triacylglycerol	5.58 ± 0.85	4.54 ± 3.23	8.74 ± 1.88	14.62 ± 2.85	6.05 ± 1.03
free cholesterol	83.52 ± 3.46	84.01 ± 2.97	87.97 ± 4.48	88.13 ± 1.98	87.31 ± 3.79
total	243.03 ± 12.14	235.08 ± 11.86	211.72 ± 15.77	246.78 ± 9.04	248.30 ± 12.89
phospholipid/cholesterol	1.84	1.74	1.31	1.63	1.78

[a] The results were determined by ESI/MS as described in the Materials and Methods, were expressed in nanomoles per milligram of protein, and represent $X \pm$ SE of at least four different animals.

phospholipid classes (e.g., cardiolipin, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and sphingomyelin) were not observed even after fasting for 12 h (Table 1). In contrast, TAG content in mouse myocardium after fasting for 12 h (8.7 ± 1.8 nmol per milligram of protein) nearly doubled from that present after fasting for 4 h (4.5 ± 3.2 nmol per milligram of protein) (Figure 1 and Table 1). Intriguingly, examination of TAG molecular species by 2D ESI mass spectrometry (Figure 4) demonstrated that no substantive differences in myocardial TAG molecular species in fasted and fed mice were present despite the profound alterations in choline and ethanolamine glycerophospholipid aliphatic chains that were manifest. Collectively, these results demonstrate that specific and temporally coordinated alterations in phospholipid mass and individual molecular species content occur that are utilized to cope with mild (selective loss of PC molecular species) and modest fasting (loss of both PC and PE mass with increases in TAG).

Detailed analysis of the change in the acyl moieties of mouse myocardial lipids after 12 h of fasting in comparison to those of fed mice demonstrated that a total of 57 nmol of acyl chains per milligram protein were lost (Table 2). Remarkably, over half of this loss (31.3 nmol per milligram of protein) was from molecular species containing 22:6 acyl chains. These species serve to increase the fluidity of biological membranes through packing defects induced from multiple cis double bonds resulting in a decrease in the order parameter and an increase in the rotational correlation times of molecular motion within membrane bilayers. The other major acyl chains that were lost were 16:0 and 18:0 (13.8 and 8.5 nmol per milligram of protein, respectively), which resulted largely from the loss of parent 16:0-22:6 and 18:0-22:6 molecular species. Other acyl moieties were only modestly depleted or even increased such as 16:1, 18:1, and 20:4 moieties (Table 2). These dramatic alterations were substantiated by the independent technique of capillary GC,

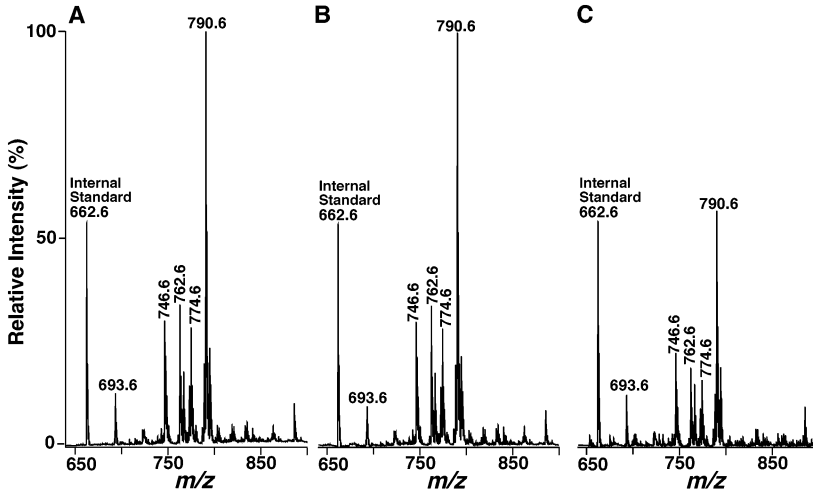

FIGURE 2: Ethanolamine glycerophospholipid analysis from the representative negative-ion ESI mass spectra of myocardial lipid extracts of normally fed, 4-h-fasted, or 12-h-fasted mice. Myocardial lipids of normally fed (panel A), 4-h-fasted (panel B), and 12-h-fasted (panel C) mice at 4 months of age were extracted by a modified Bligh and Dyer method. Negative-ion ESI/MS of the lipid extracts in the presence of LiOH was performed as described in the Materials and Methods. All major individual molecular species as indicated were identified using 2D mass spectrometry shown in Figure 3. For comparison, the spectra in panels B and C were normalized to the internal standard in the panel A.

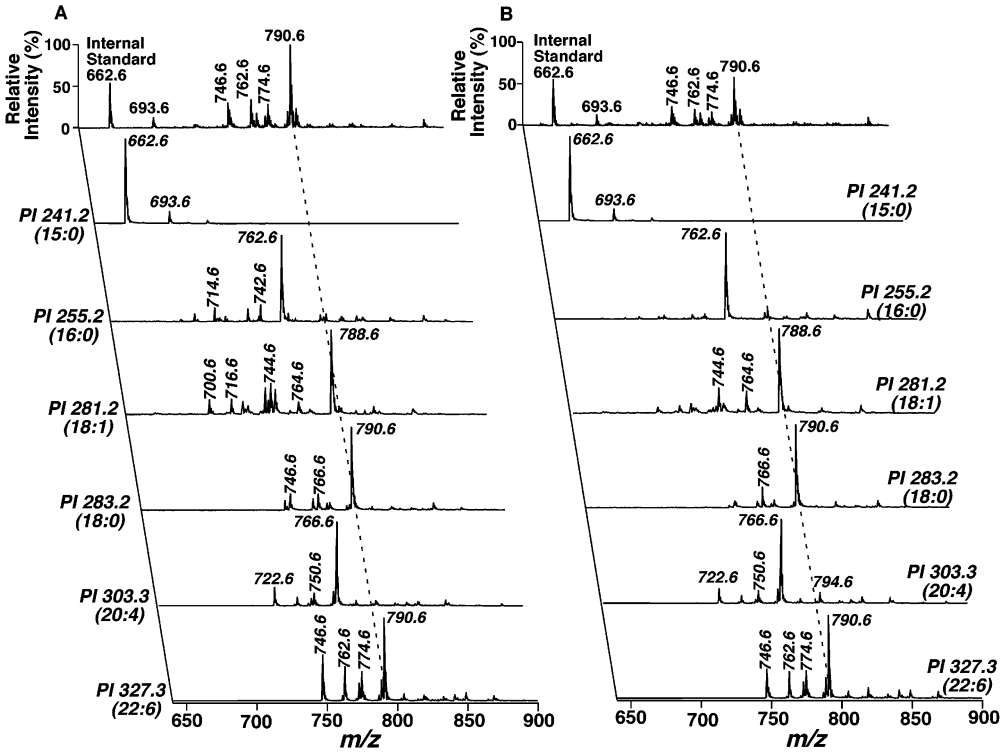

FIGURE 3: Two-dimensional ESI mass spectrometric identification of ethanolamine glycerophospholipid molecular species of the myocardial lipid extracts of normally fed or 12-h-fasted mice. Myocardial lipids of normally fed (panel A) and 12-h-fasted (panel B) mice at 4 months of age were prepared as described in the legend of Figure 1. Two-dimensional ESI mass spectrometric analyses were performed by precursor-ion scanning of all naturally occurring fatty acids in negative-ion mode in the presence of LiOH as described in the Materials and Methods. For comparison, the first-dimensional mass spectrum in panel B was normalized to the internal standard peak of ethanolamine glycerophospholipid in panel A.

which documented and confirmed losses of these aliphatic chains identified by mass spectrometry, although capillary GC obviously does not provide information on individual molecular species composition (Figure 5). These changes were further confirmed by reverse-phase HPLC analysis of PC and PE molecular species (Figure 6). After addition of internal standards (di22:6 GroPCho and di20:4 GroPEtn normalized to protein content as described in Materials and Methods) to control and 12 h fasted myocardium, ethanolamine and choline glycerophospholipids were first separated by SCX HPLC. Next, individual molecular species in each class from control or fasted myocardium were resolved by reversed-phase HPLC (Figure 6). Changes in the content of individual molecular species in each class during control or fasting can be estimated by the ratio of the major peaks to that of internal standard. It should be recognized that direct quantitative comparisons between different molecular species could not be made due to differential response factors of

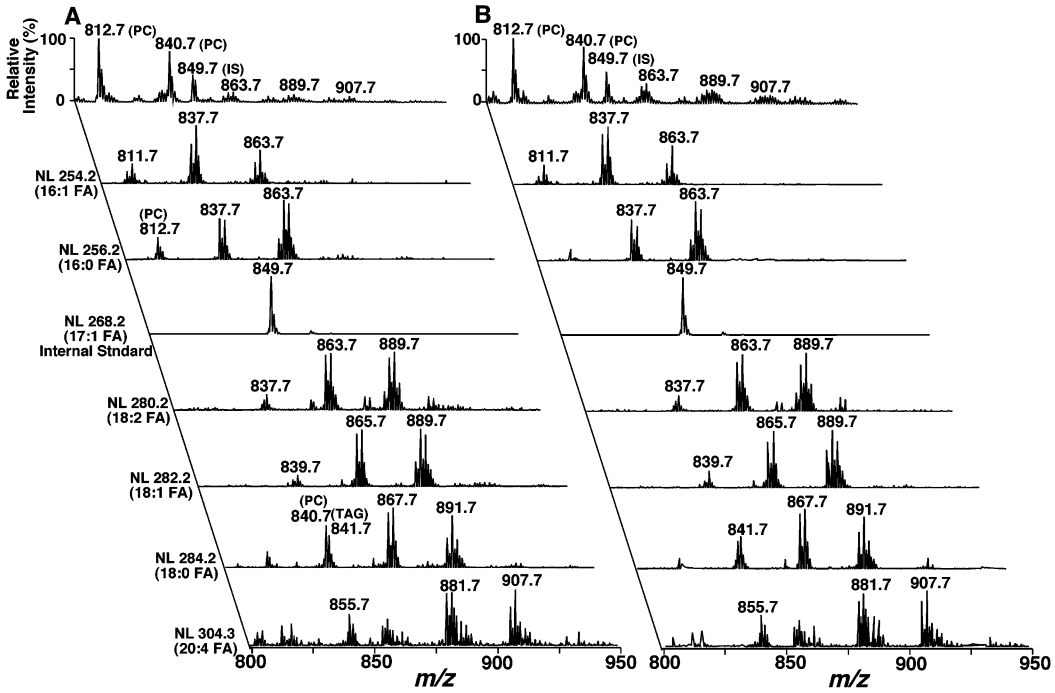

FIGURE 4: Two-dimensional ESI mass spectrometric fingerprint and quantitation of TAG molecular species of the myocardial lipid extracts of normally fed or 12-h-fasted mice. Myocardial lipids of normally fed (panel A) and 12-h-fasted (panel B) mice at 4 months of age were prepared as described in the legend of Figure 2. Two-dimensional ESI mass spectrometric analyses were performed by neutral loss scanning of all naturally occurring fatty acids in positive-ion mode in the presence of LiOH as described in the Materials and Methods.

Table 2: Changes in Acyl Moieties of Mouse Myocardial Lipids after 12-h Fasting[a]

acyl moiety	PC	PE	TAG	total
F16:0	−10.88	−5.09	2.18	−13.79
V16:0		−4.70		−4.70
F16:1			3.84	3.84
V16:1		0.75		0.75
F18:0	−0.35	−9.16		−9.51
V18:0		−2.48		−2.48
F18:1	−0.36	−2.19	5.51	2.96
V18:1		−1.69		−1.69
F18:2	−1.27		1.38	0.11
F20:4	0.65	0.81	−0.15	1.31
F22:4		−1.23		−1.23
F22:6	−8.13	−24.14		−31.27
total	−20.34	−49.12	12.76	−56.70

[a] The data were calculated from the mass content of individual molecular species that were quantitated by 2D ESI mass spectrometry as described in the section of Materials and Methods. The data were expressed in nanomoles per milligram of protein. F denotes fatty acyl moiety while V denotes vinyl ether moiety.

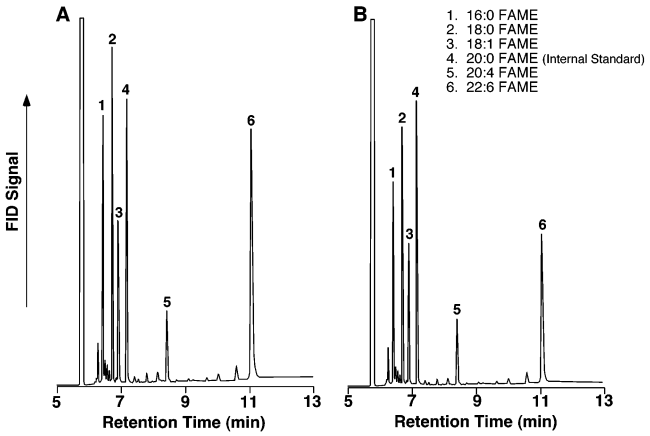

FIGURE 5: Gas chromatographs of acyl moieties in myocardial lipid extracts of normally fed and 12-h-fasted mice. Myocardial lipids of normally fed (panel A) and 12-h-fasted (panel B) mice at 4 months of age were prepared as described in the legend of Figure 2. Myocardial lipids were subjected to acid-catalyzed methanolysis and analyzed by capillary gas chromatography as described in the Materials and Methods. The identities of peaks on the chromatograph were determined by comparison of their retention times with those of authentic compounds under identical experimental conditions. FAME denotes fatty acid methyl ester from acyl-linked species.

each molecular species to UV detection at 203 nm. However, changes in the ratios of individual peaks to the internal standards during fasting are obvious and are in agreement with the results of ESI/MS (Figure 6). These losses in choline and ethanolamine phospholipid mass were also manifest when either normalized to wet weight of tissue (data not shown) or compared to most other lipid classes (e.g., phosphatidylinositol and sphingomyelin) in addition to normalization to protein content (Table 1).

This magnitude of loss of phospholipid mass from myocardium is unprecedented. Echocardiograms of mice after 12 h of fasting demonstrated normal contractibility. Moreover, the mice recovered from the 12-h fast without difficulty, largely replenishing prefast PE mass first and subsequently returning individual molecular species composition to normal values within 24 h (Table 1). Intriguingly, TAG mass remains elevated during the first 12 h of recovery (Table 1) demonstrating a prolonged memory of the antecedent period of fasting. Total lipid mass and all individual molecular species compositions recovered to control levels after returning to normal ad libitum feeding for 24 h (Table 1).

To address the tissue specificity of fasting-induced lipid alterations in mouse myocardium, changes in skeletal muscle lipids were also examined by shotgun lipidomics employing multidimensional ESI mass spectrometry. The results demonstrated that basal TAG mass content in skeletal muscle

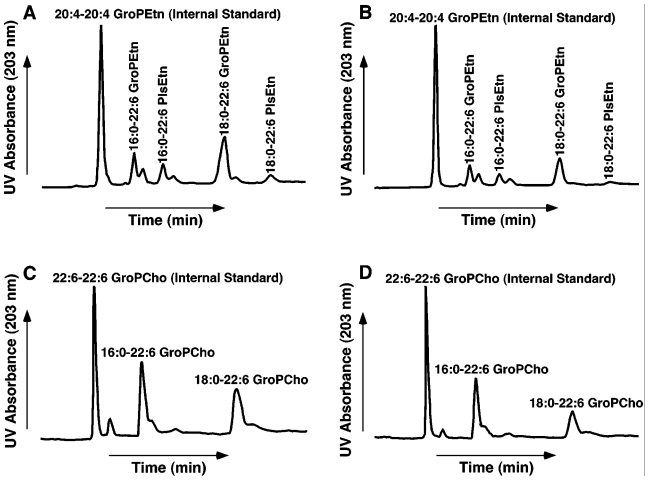

FIGURE 6: Reverse-phase HPLC of choline and ethanolamine glycerophospholipids in myocardial lipid extracts from normally fed and 12-h-fasted mice. Fractions of mouse myocardial ethanolamine glycerophospholipids (panels A and B) and choline glycerophospholipids (panels C and D) in lipid extracts of normally fed (panels A and C) and fasted (panels B and D) mouse myocardium were resolved by HPLC using a cation exchange column as described in Materials and Methods. Individual molecular species were subsequently separated by reverse-phase HPLC using an octadecyl silica column employing methanol/acetonitrile/water containing 20 mM choline chloride (90.5/2.5/7, v/v/v) as the mobile phase. Ultraviolet absorbance was monitored at 203 nm. Major peaks indicated were identified by ESI/MS.

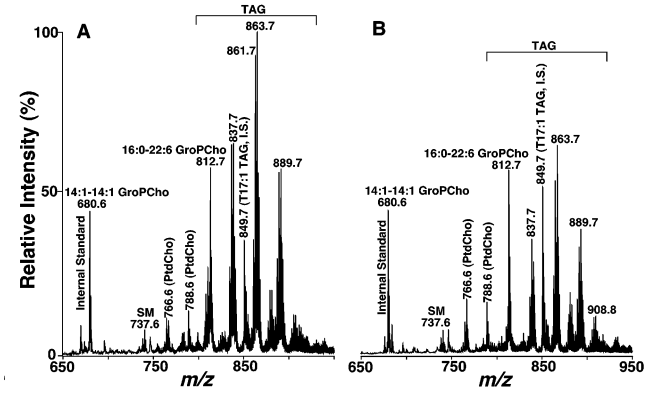

FIGURE 7: Representative positive-ion ESI mass spectra of skeletal muscle lipid extracts of normally fed or 12-h-fasted mice. Skeletal muscle lipids of normally fed (panel A) and 12-h-fasted (panel B) mice at 4 months of age were extracted by a modified Bligh and Dyer method. Positive-ion ESI/MS of the lipid extracts in the presence of LiOH was performed as described in the Materials and Methods. All major individual molecular species as indicated were identified using 2D mass spectrometry. For comparison, the spectrum in panel B was normalized to the internal standard peak of PtdCho in panel A. "IS" denotes the internal standard peak for TAGs. Most unlabeled ion peaks under the TAGs regions are lithium TAG adducts.

(71.8 ± 16.0 nmol per milligram of protein) was over 10-fold greater than that present in myocardium (compare Figure 7A to Figure 2A). In contrast to myocardial lipid alterations, the mass and molecular species content of all measured phospholipids in skeletal muscle did not change during either modest (4 h) or moderate (12 h) fasting (e.g., Figures 7 and 9). In contrast to the increased myocardial TAG mass content after fasting, the mass content of skeletal muscle TAG decreased by 54 mol % to 33.0 ± 8.0 nmol per milligram of protein after a 12 h fast (Figure 7). Examination of TAG molecular species by 2D ESI mass spectrometry demonstrated that no major differences in skeletal muscle TAG molecular species were present between fasting and fed mice although the total TAG pool size had dramatically decreased (Figure 8). These results demonstrate that in mice the predominant tissue from which aliphatic chains are mobilized during fasting is skeletal muscle while in humans, adipocytes are typically thought to be the major energy reservoir for aliphatic chains liberated for systemic distribution during fasting with more modest contributions from skeletal muscle.

Additional experiments were performed to determine the mass content of glycogen in liver, heart, and skeletal muscle, as well as the serum content of glucose, nonesterified fatty acids, and TAG in mouse plasma during these experimental intervals to characterize the severity of the 4- and 12-h fast studies. The glycogen content in liver was quickly depleted during fasting; only approximately 10% glycogen remained after 4-h fasting and nearly complete glycogen depletion was manifest at 12-h fasting (Figure 10). In contrast, glycogen content in mouse heart was only modestly decreased by fasting (Figure 10). The mass levels of TAG in plasma dramatically decreased due to fasting, that is, from 757.2 ± 88.8 nmol per milliliter of plasma in normally fed mice to 220.4 ± 29.3 and 127.8 ± 13.5 nmol/mL after 4- and 12-h fasting, respectively. The blood glucose content was only modestly changed after 12-h fasting (i.e., 87.3 ± 4.8 mg/mL after 12-h fasting compared to 111.0 ± 4.6 and 127.8 ± 13.4 mg/mL with fed and after 4-h fasting, respectively). There were no significant differences in nonesterified fatty acids in plasma between the fed and fasting mice (125.0 ± 12.4 and 128.0 ± 14.3 nmol per milligram of protein, respectively).

DISCUSSION

The results of the present study demonstrate that the lipid content and molecular species composition of murine myocardium can be dramatically modified by modest caloric restriction. The observed alterations in lipid content and molecular species composition are completely reversible after 24 h of refeeding. The return to basal conditions took longer than anticipated, since increases in TAG content were still manifest even after 12 h of refeeding. These results demonstrate that myocardium has a "metabolic memory" being influenced by its nutritional history and modulating lipid levels reflecting its metabolic past even after substantial periods of refeeding had occurred. Both the net loss of phospholipid mass and the remodeling of phospholipid molecular species demonstrated tissue specificity since neither skeletal muscle phospholipid content nor molecular species composition were altered during these brief intervals of fasting. Similarly, TAG content decreased dramatically in skeletal muscle, while myocardial TAG content mildly increased during brief fasting and became further elevated after refeeding for 12 h. These 4 and 12 h fasts were of only modest severity as mice typically survive caloric deprivation for 48 h without lethal effects (*12*, *21*, *22*). Furthermore, the relative metabolic stress levels to myocardium induced by this brief duration of fasting were mild as assessed by conventional criteria. In large part, alterations in traditional endogenous metabolic reservoirs (i.e., glycogen and TAG) were restricted to noncardiac tissues (liver and skeletal muscle), while cardiac alterations were predominated by changes in phospholipid mass and molecular species com-

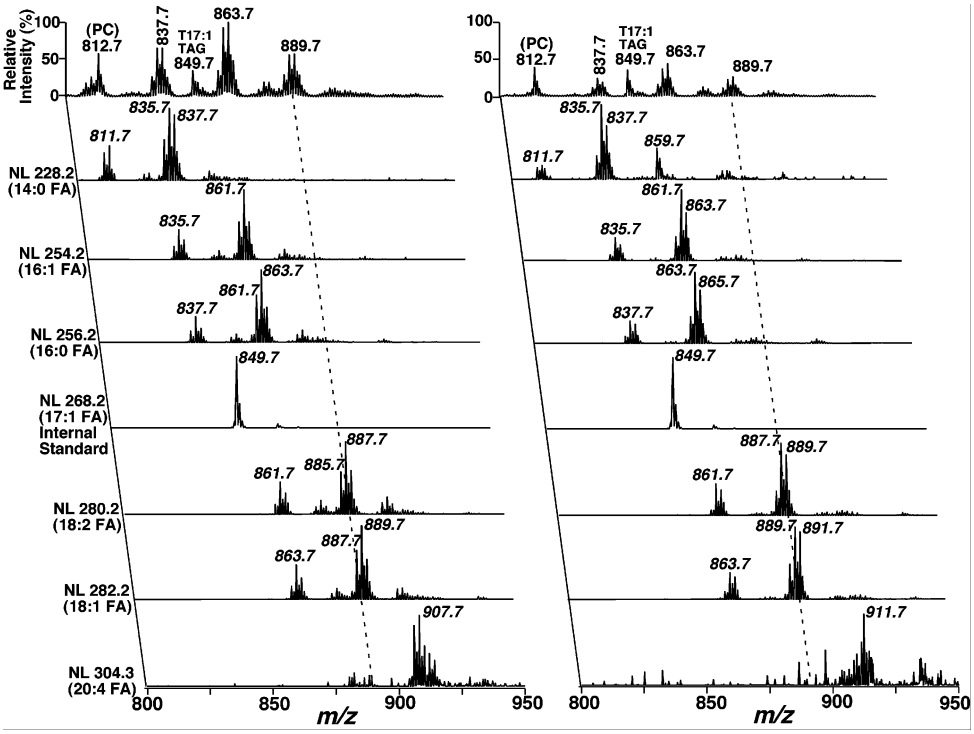

FIGURE 8: Two-dimensional ESI mass spectrometric fingerprint and quantitation of triacylglycerol molecular species of the skeletal muscle lipid extracts of normally fed or 12-h-fasted mice. Muscle lipids of normally fed (panel A) and 12-h-fasted (panel B) mice at 4 months of age were prepared as described in the legend of Figure 6. Two-dimensional ESI mass spectrometric analyses were performed by neutral loss scanning of all naturally occurring fatty acids in positive-ion mode in the presence of LiOH as described in the Materials and Methods.

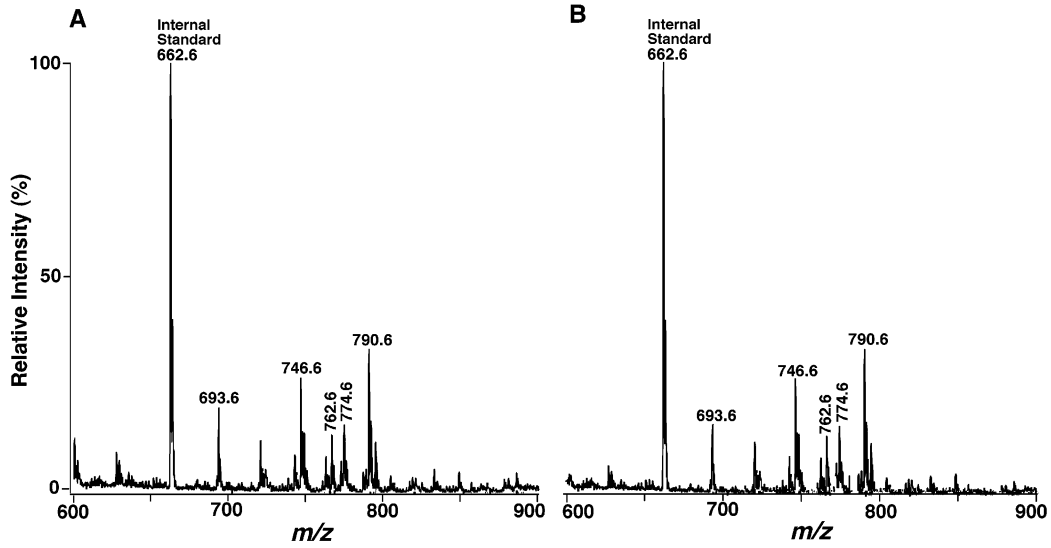

FIGURE 9: Ethanolamine glycerophospholipid analysis from the representative negative-ion ESI mass spectra of skeletal muscle lipid extracts of normally fed or 12-h-fasted mice. Skeletal muscle lipids of normally fed (panel A) and 12-h-fasted (panel B) mice at 4 months of age were extracted by a modified Bligh and Dyer method. Negative-ion ESI/MS of the lipid extracts in the presence of LiOH was performed as described in the Materials and Methods. All major individual molecular species as indicated were identified using 2D mass spectrometry similar to Figure 3.

position. These results identify phospholipids in myocardial membranes as a dynamic metabolic entity that have profound responses to organismal nutritional status and its concomitant metabolic and hormonal signals. Collectively, these results demonstrate that during fasting myocardium can access the aliphatic chains present in its phospholipid pools. A very minor portion of this net mass shift in aliphatic chain composition results in the net transfer of aliphatic groups to TAG stores (either directly or indirectly), while the majority of the aliphatic chains lost from phospholipid pools can potentially be utilized as substrates for energy production after phospholipase-mediated release of fatty acid from the targeted phospholipid pools.

Caloric restriction was accompanied by a massive remodeling of aliphatic chain composition manifest by a selective loss of polyunsaturated long chain length molecular species in myocardium. Such changes alter the physical dimensions, dynamics, and organization of myocardial membranes (*23*, *24*). We speculate that these alterations represent a programmed response to nutritional deprivation designed to maximize energy efficiency at times of nutritional stress. Based on multiple past molecular dynamics studies, these

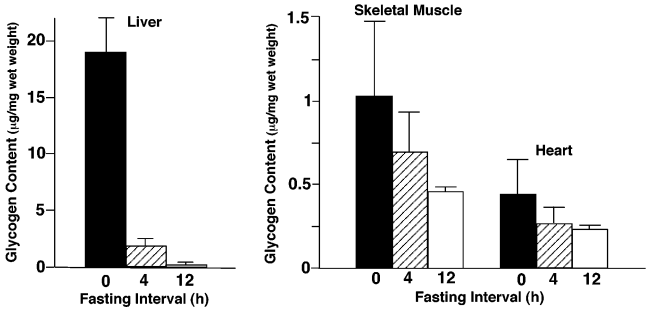

FIGURE 10: Glycogen content in liver, skeletal muscle, and heart of normally fed, 4-h-fasted, and 12-h-fasted mice. Tissue glycogen content was determined using a starch assay kit (Sigma, St. Louis, MO) following the manufacturer's protocol. The results represent means ± SD of at least four separate animals.

changes in aliphatic chain composition are anticipated to result in an increase in the order parameter and a decrease in the rotational correlation time by removing the multiple cis unsaturated centers, which introduce packing defects in biological membranes. Similarly, the thickness and the amount of membrane area are anticipated to be decreased by these modifications based on traditional chemical principles. In myocardium, at least 80% of the overall lipid mass is present within the mitochondrial compartment (*17, 20*). In mitochondria, the overwhelming majority of lipid mass is present in the inner membrane compartment. The loss of 30 mol % total heart phospholipid after 12 h of fasting thus necessarily reflects loss of at least a portion of the cristae phospholipid pool. This is further underscored by the entirely normal electrical characteristics, hemodynamic function, and electromechanical coupling of hearts present at this modest level of caloric restriction since dramatic losses of lipid in other cardiac myocytic membrane compartments (e.g., sarcolemma or sarcoplasmic reticulum) would be anticipated to be accompanied by severe and potentially dire electrical and mechanical alterations (*25, 26*). The unchanged myocardial cholesterol content during fasting and recovery supports this hypothesis.

Alterations in the chemiosmotic potential in the inner mitochondrial membranes underlie the capacity of mitochondria to regulate the generation, coupling, and chemical efficiency of ATP production (*27, 28*). Through alterations in the physical properties of the membrane (e.g., the decreased aliphatic chain length and altered molecular dynamics), the efficiency of energy generation and the kinetics of individual steps in mitochondrial chemical energy production are likely altered. For example, if the transmembrane potential is unchanged, then the electric field across the membrane is altered by membrane thinning rendering a host of physiologic adaptations possible including possibly changes in the kinetics of the electron flow along electron transport chain in the inner membrane. We speculate that the observed changes may have evolved as part of a protective mechanism against caloric deprivation.

This study underscores the power of shotgun lipidomics to identify specific alterations in molecular species composition directly from chloroform extracts of myocardium. Moreover, through intrasource separation and direct infusion, the process of signal averaging allows markedly increased reproducibility and accuracy in comparison to on-line chromatographic approaches in which quantitation is done during the waxing and waning of chromatographic peaks containing multiple different components present in different concentrations in each part of the peak. Prior work by multiple groups indicates that direct comparisons of peak intensities in the dilute concentration regime are reliable indicators of the relative proportions and absolute amounts of individual molecular species present after appropriate consideration of ^{13}C isotope composition (*29*−*39*). Further refinement and confirmation of data from primary ion spectra are also possible by 2D mass spectrometry employing neutral loss or precursor ion scanning to provide detailed information about molecular species content and allow refinement and confirmation of data (*15*). Thus, through utilization of multidimensional mass spectrometry, the relative changes between two states can be readily identified.

It seems obvious that the observed alterations reflect either increased rates of degradation (i.e., increased phospholipase activity) or decreased rates of synthesis (or combinations of both). The observed decrease in phospholipid net mass, specific decreases in polyunsaturated 22:6 containing phospholipids, and loss of specific classes of lipids suggest that accelerated phospholipase activity was the prominent mechanism. Furthermore, prior studies of myocardial lipid synthesis and remodeling demonstrate that the endogenous synthetic rate of heart lipids is far slower than the rate needed to explain the amount of phospholipid depletion found in this study based on decreases in the synthetic rate alone (*40*). Accordingly, these results suggest that accelerated phospholipid hydrolysis contributes to and results in the majority of decreased phospholipid mass and altered molecular species in nutritionally deprived myocardium. Central to this theme is the role that lipids play in shaping the electrophysiologic alterations of the action potential (*25, 26, 41*) and its alterations in ischemic zones. Thus, we speculate that increased incidence of sudden death from ischemia-induced electrophysiologic dysfunction during the early morning hours may be related to preexisting myocardial phospholipid alterations during a 12-h fast humans routinely are subject to in addition to hormonal influences engendered by feed−fasting and sleep−wake cycles (*42, 43*).

Classic texts either state that phospholipid contents do not change until near terminal starvation or do not consider phospholipids as a substantive source of chemical energy in mammals until near lethal starvation (*6*). The present results demonstrate dramatic reversible tissue-specific alterations in the phospholipid content and molecular species composition during modest caloric deprivation in murine myocardium. The encoded record of the nutritional history of myocardium is maintained by its signature phospholipid profile, which likely plays a role in the diverse responses previously observed to cardiac ischemia in different metabolic contexts (e.g., preconditioning). Thus, these results represent a paradigm shift in the traditional dogma of energy supply and demand in myocardium and underscore the role of phospholipases as a dynamic response element in myocardial bioenergetics.

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