Relationship of glucose and oleate metabolism to cardiac function in lipin-1 deficient (fld) mice

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Abstract Lipin-1 is the major phosphatidate phosphatase (PAP) in the heart and a transcriptional coactivator that regulates fatty acid (FA) oxidation in the liver. As the control of FA metabolism is essential for maintaining cardiac function, we investigated whether lipin-1 deficiency affects cardiac metabolism and performance. Cardiac PAP activity in lipin-1 deficient [fatty liver dystrophy (fld)] mice was decreased by >80% compared with controls. Surprisingly, oleate oxidation and incorporation in triacylglycerol (TG), as well as glucose oxidation, were not significantly different in perfused working fld hearts. Despite this, [3H]oleate accumulation in phosphatidate and phosphatidylinositol was increased in fld hearts, reflecting the decreased PAP activity. Phosphatidate accumulation was linked to increased cardiac mammalian target of rapamycin complex 1 (mTORC1) signaling and endoplasmic reticulum (ER) stress. Transthoracic echocardiography showed decreased cardiac function in fld mice; however, cardiac dysfunction was not observed in ex vivo perfused working fld hearts. This showed that changes in systemic factors due to the global absence of lipin-1 could contribute to the decreased cardiac function in vivo. Collectively, this study shows that fld hearts exhibit unchanged oleate esterification, as well as oleate and glucose oxidation, despite the absence of lipin-1. However, lipin-1 deficiency increases the accumulation of newly synthesized phosphatidate and induces aberrant cell signaling.—Kok, B. P. C., P. C. Kienesberger, J. R. B. Dyck, and D. N. Brindley. Relationship of glucose and oleate metabolism to cardiac function in lipin-1 deficient (fld) mice. J. Lipid Res. 2012. 53: 105–118.

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The constant requirement for ATP in the beating heart is satisfied by the utilization of diverse substrates, such as fatty acids (FA), glucose, lactate, ketones, and amino acids, with FA oxidation (FAO) providing the majority (50–75%) of cardiac ATP production (1). FAs are also incorporated into triacylglycerol (TG), which is stored in lipid droplets. Cardiac TG turnover is rapid (2) and FAs released from TG can contribute up to 10–20% of ATP production (3). Plasma FA and TG levels rise in insulin resistance and diabetes (4, 5), and cardiac FA utilization can increase to supply as much as 90% of ATP (6, 7). Despite this increase in FAO, FA uptake can exceed the ability of the heart to utilize FAs, leading to excess accumulation of TG in cardiomyocytes (6–9). The compensatory changes that occur in response to excessive FA availability eventually become maladaptive, and the heart is locked in a pathological and inflexible metabolic state that contributes to cardiac dysfunction (6, 7, 10). As such, maintenance of metabolic flexibility is essential for normal cardiac function. Whereas many proteins have been identified to contribute to cardiac FA uptake and metabolism (1, 10), relatively little information is available about the role of lipin-1 in the heart. Lipin-1 is an important protein involved in regulating both TG synthesis and FAO in other organs (11).

Instead of being considered as mutually antagonistic processes, FAO and TG synthesis are now widely viewed as being companion pathways because augmenting cardiac TG synthesis increases FAO (12). Moreover, FAs released through lipolysis of endogenous TG stores contribute significantly to FAO (3, 13). Lipin-1 could play a prominent role in this regulation as it is a bifunctional protein involved in regulating both TG synthesis and FAO in the heart.

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; DG, diacylglycerol; ER, endoplasmic reticulum; FAO, fatty acid oxidation; fld, fatty liver dystrophy; HSL, hormone-sensitive lipase; LPP, lipid phosphate phosphatase; MCD, malonyl-CoA decarboxylase; mTOR, mammalian target of rapamycin; NEM, N-ethylmaleimide; PA, phosphatidate; PAP, phosphatidate phosphatase; PC, phosphatidylcholine; PDH, pyruvate dehydrogenase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PI, phosphatidylinositol; PPARα, peroxisome proliferator-activated receptor α; PS, phosphatidylserine; Tbp, TATA-binding protein; TG, triacylglycerol.

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Liver (11). First, lipin-1 functions as a phosphatidate phosphatase (PAP) through its catalytic DxDxT motif, which generates the diacylglycerol (DG) required for the synthesis of TG, phosphatidylethanolamine (PE), and phosphatidylcholine (PC) (14). There are three members in the lipin family, of which lipin-1 is the best characterized. All three lipins have PAP activity, but they are expressed in a tissue-specific manner (14). Lipin-1 appears to be the predominant lipin in the heart because of the apparent absence of cardiac PAP activity in lipin-1-deficient mice (15). These animals are known as fatty liver dystrophy (f/d) mice because they develop transient fatty livers and hypertriglyceridemia, which resolve upon weaning (16–18). F/d mice are devoid of mature adipose tissue, which is attributed to the role of lipin-1 in inducing peroxisome proliferator activated receptor γ (PPARγ) expression during adipocyte differentiation (19). The f/d mouse is also insulin resistant and is prone to developing atherosclerosis when fed a high cholesterol/cholate diet (16, 20). In addition, phosphatidate (PA) accumulation leads to demyelination in Schwann cells through aberrant ERK1/2 activation, which subsequently causes the development of peripheral neuropathy in f/d mice (21, 22).

Lipin-1 also acts as a transcriptional coactivator with peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and PPARα to upregulate the expression of proteins involved in FAO in liver (23). Consequently, f/d mice are defective in the fasting-induced hepatic expression of PPARα and its downstream targets (23). We decided to determine whether lipin-1 has a similar function in the heart. Lipin-1 consists of full-length lipin-1B and the lipin-1A splice variant, which lacks a stretch of 20 amino acids (24). Lipin-1B is the predominant isoform in the heart, and it exhibits both PAP and transcriptional coactivator activities (23, 24).

Lipin-1 expression is dynamically regulated in the liver (25, 26), heart (27), and adipose tissue (28). Lipin1 transcription and, thus, PAP activity are increased in the liver in fasting and diabetes due to the synergistic actions of glucagon (or epinephrine) through cAMP production and glucocorticoids (11). Insulin antagonizes these actions in rat and mouse hepatocytes (25). Lipin1 gene expression is regulated in neonatal rat cardiomyocytes in a way similar to that in the liver (B. P. C. Kok and D. N. Brindley, unpublished results). Lipin2 and Lipin3 expression are not regulated significantly by glucocorticoids, cAMP, or insulin in hepatocytes (25). Increased lipin-1 expression is thought to provide a reservoir of PAP activity which, together with the FA-induced translocation of lipin-1 from the cytosol to the endoplasmic reticulum (ER) (15, 29, 30), enhances the capacity for FA sequestration into TG in the face of increased FA uptake (10, 11). Furthermore, increased lipin-1 expression in fasting and diabetes could also promote FAO through its role as a transcriptional coactivator. In the fed state, lipin-1 expression is suppressed by insulin action (25), and this presumably helps to promote glucose utilization with a concurrent decline in FAO. Despite this, cardiac PAP activity has been shown to decrease in insulin-resistant rat models as well as in Type 2 diabetic patients (31, 32). The importance of lipin-1 function in metabolism was highlighted by numerous studies demonstrating associations of lipin-1 polymorphisms with different metabolic disease states (33–35).

We, therefore, hypothesized that lipin-1 deficiency in the heart would severely inhibit TG synthesis and FAO and, thereby, cardiac function. We determined that there was cardiac dysfunction in the lipin-1-deficient mice in vivo. Surprisingly, lipin-1 deficiency did not produce major modifications in FA esterification or oxidation in isolated perfused working hearts. There was also no indication of impaired cardiac function in this ex vivo system, indicating that the dysfunction in vivo probably resulted from systemic influences on the heart due to global lipin-1 deficiency. However, there were differences in phosphatidate and phosphatidylinositol turnover and in cell signaling.

MATERIALS AND METHODS

Animal care and breeding strategy

We established a breeding colony of Balb/cByJ-Lpin1fl d/J mice from the Jackson Laboratory, Bar Harbor, ME. We used two breeding triads, the first consisting of two female fl d mice bred with one male heterozygous mouse, which produced only fl d and heterozygous offspring. The other triad contained two female heterozygous mice and one male heterozygous mouse, resulting in fl d, heterozygous, and wild-type offspring. The wild-type and heterozygous mice were both designated as the control mice. Mice were fed Lab Diet 5058 containing 9% fat by weight. Male mice were used in the experiments. The research was conducted in accordance with the policies of the Canadian Council on Animal Care, as approved by the University of Alberta Animal Policy and Welfare Committee.

Noninvasive cardiac monitoring and tissue collection

Transsthoracic echocardiography was performed on mildly anesthetized (1.5% isoflurane and 95% O3) mice using a Vevo 770 Imaging System (VisualSonics, Toronto, ON) (36). The Tei index was calculated as the sum of the isovolumic relaxation and contraction time divided by the ejection time (37). One week after echocardiographies, 11-week-old mice were fasted from 0900 to 1300 h (0600–1800 h light/dark cycle) before being euthanized by decapitation, after which cardiac tissues were collected for RT-PCR and Western blot analysis. Serum samples were also collected and assayed with the glucose-c kit (Wako Chemicals, Richmond, VA). Serum TG and unesterified FAs were measured using the TG GPO kit (Pointe Scientific, Canton, MI) and NEFA kit (Wako Chemicals), respectively. For serum lipid measurements, food was withheld from 19- to 23-week-old mice from 0900 to 1100 h.

Quantitative real-time PCR

mRNA concentrations were measured by quantitative RT-PCR relative to that of TATA-binding protein (Tbp) (25). Similar results were obtained with hypoxanthine-guanine phosphoribosyltransferase and cyclophilin A as reference genes. Primer sequences are listed in supplementary Table I.

SDS-PAGE and Western blot analysis

SDS-PAGE and Western blots were performed using antibodies as described in supplementary Table II (38). The antibody against the C terminus of lipin-1 was a gift from Dr. Thurl Harris.
(University of Virginia, Charlottesville, VA). Quantitative densitometric analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD). Antibodies against lipin-2 were raised in rabbits using the peptide sequence N′-PKGELIQTERTKNK-C′ followed by affinity purification (GenScript, Piscataway, NJ). The lipin-2 antibody was verified by comparing endogenous lipin-2 in the heart to recombinant lipin-2 protein overexpressed in MCF-7 breast cancer cells (Fig. 1B, upper panel).

Phosphatidate phosphatase enzymatic assay

PAP assays were performed at pH 6.5 and 7.4 using two methods for preparing the PA (14). The first method used a dispersion of PA with PC, which was designed to maximize the activity of PAP versus that of lipid phosphate phosphatase (LPP). Each sample was assayed in a total volume of 100 µl consisting of 100 mM Tris/maleate buffer (pH 6.5) or Tris/HCl buffer (pH 7.4) in addition to 0.6 mM dithiothreitol, 1.5 mM MgCl₂ (pH 7.4) and 5 mM MgCl₂ (pH 6.5), 2 mg/ml FA-poor BSA, protease inhibitor cocktail (Sigma-Aldrich), 30 nM microcystin-LR, 0.6 mM PA labeled with [³H]palmitate (approximately 6 × 10⁴ dpm per assay), 1 mM EDTA/EGTA, 0.4 mM PC, and 200 µM tetrahydrolipstatin to inhibit the degradation of the DG product by lipase activity (39). In the second assay, 45 mM Triton X-100 was used to disperse 5 mM PA in micelles and PC was omitted. Each sample was assayed in a total volume of 100 µl consisting of 100 mM Tris/maleate (pH 6.5) or Tris/HCl (pH 7.4) in addition to 0.6 mM dithiothreitol, 1 or 6 mM MgCl₂ (pH 7.4 and 6.5 respectively), protease inhibitor cocktail, 30 nM microcystin-LR, 1 mM PA labeled with [³H]palmitate (approximately 6 × 10⁴ dpm per assay), 9 mM Triton X-100 (from the substrate preparation), and 200 µM tetrahydrolipstatin.

Parallel measurements were performed in the absence of Mg²⁺ or in the presence of 8 mM N-ethylmaleimide to determine the contribution from LPP activity (14). For the assays where Mg²⁺ was omitted or for the assays with Mg²⁺ concentration curves, all buffers were depleted of bivalent cations with AG 50W-X8 resin Na⁺-form (40). LPP activity was determined directly by using the

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**Fig. 1.** Expression and activity of lipins in control and *fld* hearts. (A) mRNA expression of lipins in 11-week-old control (n = 7) and *fld* (n = 8) hearts. (B, upper panel) The lipin-2 antibody was verified by comparing lipin-2 in control (Lane 2) and *fld* (Lane 3) hearts to recombinant lipin-2 protein (Lane 1). Ran GTPase serves as the loading control. (B, lower panel) Representative Western blots showing protein levels of lipin-1 and lipin-2 in control and *fld* hearts with Ran GTPase as the loading control. (C) Densitometric analysis of single Western blots for lipin-1 and lipin-2 in control and *fld* hearts assayed at either pH 6.5 or 7.4 with PA/PC liposomes or PA/Triton X-100 micelles together with Mg²⁺-independent, NEM-insensitive LPP activities in control (n = 3–5) and *fld* (n = 3–5) hearts.
Triton X-100-based procedure (pH 6.5 and 7.4) in the presence of 8 mM NEM, with the exception that [3H]PA was used instead of [32P]PA (41).

DG formed in these assays was extracted in 2 ml chloroform-methanol (19.5: by vol) containing 0.08% olive oil as an acylglycerol carrier (40). Activated alumina was added to remove the unreacted PA and any liberated [3H]palmitate from the chloroform phase. The chloroform phase was dried, and radioactivity was determined. PAP activity was calculated by subtracting the NEM-insensitive or Mg2+-independent LPP activity from the total activity. Each sample was assayed at three different protein concentrations (30–200 µg) to ensure a proportional response, and the conversion of PA to DG was restricted to <20%.

Quantification of organic phosphates, phosphatidate, triacylglycerol, and nonesterified fatty acids

Cardiac lipids were extracted using chloroform-methanol-2 M KCl containing 0.2 mM HCl (1:1:0.9, by vol) (42), and organic phosphate was assayed to measure phospholipid content using rac-glycerol 3-phosphate as a standard (43). Phospholipids were dried and digested with 50 µl of perchloric acid at 180°C for 30 min. The reaction mixture was cooled, and then 278 µl water, 55 µl 2.5% ammonium molybdate, and 55 µl 10% ascorbic acid were added before boiling for 15 min. Absorbance readings were quantified with a multiwell plate reader at 700 nm.

PA was measured in lipid extracts as described previously (43). Briefly, a PA standard curve (0–4 nmol) and lipids extracted from cardiac tissue were loaded half way up plastic-backed silica TLC plates (VWR International, Radnor, PA) along with PC and PA standards on the outermost lanes. The plates were developed twice in chloroform-methanol-ammonium hydroxide (65:35:7.5, by vol). The migration of PA bands was determined by cutting the outermost lanes and identifying the PA and PC standards. The TLC plates were then cut 1 cm above the migrated PA, thus removing most other phospholipids, and developed in the reverse direction in chloroform-methanol-acetic acid-acetone-water (50:20:12:10:5, by vol), followed by staining for 1 h with 0.3% Coomassie Brilliant Blue R250 in 20% methanol containing 100 mM NaCl. The TLC plates were destained in 20% methanol and scanned at 700 nm using the Odyssey Infrared Imaging scanner (LI-COR Biosciences, Lincoln, NB). PA levels were determined by comparison to the PA standard curve. TG concentrations were measured in extracts of heart using a TG GPO kit (Pointe Scientific, Canton, MI).

Perfused working heart studies and measurements of fatty acid and glucose metabolism

Hearts were perfused in working mode ex vivo (44). Left atrial preload and aortic afterload were set to 11.5 and 50 mm Hg, respectively. For the first 30 min, all hearts were perfused with Krebs-Henseleit buffer containing 118.5 mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 0.5 mM EDTA, 5 mM glucose, and 50 µU/ml insulin to deplete TGs as much as possible (3). Subsequently, hearts were perfused with the same buffer supplemented with 5 mM [U13C]glucose (16 µCi/mmole) and 1.2 mM [9,10-3H]oleate (50 µCi/mmole) conjugated to 0.45 mM BSA (fraction V, essentially fatty acid free: dialyzed) for an additional 30 min. Cardiac function and coronary effluent were measured throughout the perfusion (44, 45) and analyzed using calculations described in the supplementary data. Rates of oleate and glucose oxidation were measured by collecting H2O and 14CO2, respectively.

Rates of [3H]oleate incorporation into glycerolipids in perfused hearts were also determined. Extracted lipids (42) were loaded onto TLC plates, which were developed halfway up the plate using chloroform-methanol-acetic acid-acetone-water (50:10:10:20:5, by vol), followed by a second full-length development with hexane-diethyl ether-acetic acid (60:40:1, by vol) (46). Individual lipids were collected in scintillation vials containing 10% water and Ecolite scintillation fluid (MP Biomedicals, Solon, OH), and radioactivity was measured. A two-dimensional TLC system was used to confirm the levels of radiolabeled PC, phosphatidylserine, phosphatidylinositol (P1), sphingomyelin, and cardiolipin. TLC plates were loaded at one corner and developed with chloroform-methanol-water-NH4OH (60:40:4:0.5, by vol) and chloroform-methanol-acetic acid-acetone-water (45:15:10:20:5, by vol) in two different directions. The phospholipids were identified using standards on a separate plate as well as by ninhydrin staining for amine-containing phospholipids and Dragendorff staining for choline-containing phospholipids (47). Olate incorporation into lipids was essentially identical whether we used the one- or two-dimensional TLC system.

The two-dimensional TLC system was also used to separate individual phospholipids from the ventricles of fld and control mice. Different phospholipids were scraped off the plate and quantified using the organic phosphate assay. Glycogen content and glucose incorporation into glycogen were also measured (48, 49). Briefly, tissues were hydrolyzed at 100°C for 30 min in 40% KOH, followed by precipitation with 100% ethanol. The precipitated glycogen was washed three times with 95% ethanol and then hydrolyzed at 100°C for 3 h in 3 M HCl. After neutralization with 2 M NaOH, glucose equivalents were measured using a Glucose-c kit (Wako Chemicals).

Statistics

Results are expressed as means ± SEM. The two-tailed Student t-test or one-way ANOVA followed by Bonferroni posthoc test was used to test for significance (P < 0.05).

RESULTS

Characterization of lipins and PAP and LPP activities in fld hearts

Fld mice were identified by their physical appearance resulting from their lipodystrophy and their lower body weights (Table 1) compared with controls. We found no significant differences in serum glucose or nonesterified fatty acid levels; however, TG levels were lower in the fld mice compared with controls (Table 2). As expected, we found no significant mRNA expression of lipin-1A or lipin-1B in fld hearts (Fig. 1A). However, mRNA expression of lipin-2 and lipin-3 was increased by 40–50% in fld hearts (P < 0.03 and 0.004, respectively), whereas protein levels of lipin-2 were similar between genotypes (Fig. 1B, C). Although we could not obtain a successful Western blot for lipin-3, a recent study showed that lipin-3 levels in fld hearts are similar to those in wild-type hearts (50).

We then measured PAP activity in fld and control mice under a variety of assay conditions. Optimum Mg2+ concentrations were determined for each of the two PAP assay systems at both pH 6.5 and 7.4 (Fig. 1D). Mg2+ concentrations above 2 mM inhibited cardiac PAP activity at pH 7.4 using either PA/PC mixed liposomes or PA/Triton X-100 micelles (Fig. 1D). We also verified that addition of different concentrations of Mn2+, Co2+, and Ca2+ did not reveal the presence of other PAP activities (results not shown). Earlier work (15) showed no significant PAP activity in fld.
hearts using Triton X-100-PA micelles as the substrate, and we essentially confirmed this observation at pH 6.5 or 7.4 (Fig. 1E). A Mg\(^{2+}\)-dependent, NEM-sensitive PAP activity was detected using mixed PA/PC liposomes at both pH 6.5 and 7.4 in the fdl hearts at levels approximately 15–20% of that found in the control hearts (Fig. 1E).

This value is similar to the work of Mitra et al. (50) who used \(^32\)P-labeled PA presented in Triton X-100 and measured the formation of water-soluble \(^32\)P. However, this method overestimates PAP activity unless precautions are taken to block phospholipase A-type activities, which produce labeled lysophosphatidate and glycerophosphate. These compounds can then be converted to \(^32\)P, by acid or alkaline phosphatases. All of these compounds are extracted into methanol/water rather than chloroform phase and are included in the measurement of “water-soluble” products. We avoided this complication by measuring the formation of \([\text{H}]\)DG from \([\text{H}]\)PA and blocking the degradation of DG with tetrahydrolipstatin. This latter technique provides a valid PAP assay that can be used with tissue homogenates (40).

We conclude that the absence of lipin-1 severely decreases myocardial PAP activity and that lipin-2 and lipin-3 contribute only about 15–20% of normal cardiac PAP activity under the most optimum assay conditions we could find. LPP activity, which is thought not to participate in TG synthesis (51), was similar in fdl and control hearts (Fig. 1E).

### Decreased cardiac function in fdl mice in vivo

Having demonstrated that there was a significant reduction in PAP activity in fdl mice, we next determined whether cardiac function was affected in vivo. Noninvasive transthoracic echocardiography was used to evaluate cardiac function in 19- to 23-week-old fdl and control mice. The fdl mice had significantly decreased systolic function as indicated by reduced ejection fraction and fractional shortening (Table 1). This corresponded with decreased end-diastolic left ventricular internal diameter measurements (Table 1). Stroke volume and cardiac output and measurements of pulmonary peak venous flows were also drastically decreased in fdl hearts (Table 1). We also determined

### TABLE 1. Measurements of cardiac dimensions and in vivo cardiac function from transthoracic echocardiographies of mice

| Measurement                                      | Control | fdl     |
|--------------------------------------------------|---------|---------|
| Ejection fraction (%)                            | 45 ± 1  | 39 ± 3\(^a\) |
| Fractional shortening (%)                        | 22 ± 1  | 19 ± 2\(^a\) |
| Left ventricular internal diameter–diastole (mm) | 4.26 ± 0.07 | 3.83 ± 0.09\(^b\) |
| Left ventricular internal diameter–systole (mm)  | 3.31 ± 0.07 | 3.12 ± 0.10 |
| Heart rate (beats per minute)                    | 410 ± 10 | 405 ± 15 |
| Stroke volume (µl)                               | 45 ± 4  | 28 ± 2\(^a\) |
| Cardiac output (ml/min)                          | 19 ± 1  | 12 ± 1\(^a\) |
| Interventricular septum–diastole (mm)            | 0.90 ± 0.04 | 0.85 ± 0.03 |
| Interventricular septum–systole (mm)             | 1.17 ± 0.06 | 1.05 ± 0.04 |
| Left ventricular posterior wall–diastole (mm)    | 0.89 ± 0.04 | 0.88 ± 0.04 |
| Left ventricular posterior wall–systole (mm)     | 1.14 ± 0.04 | 1.08 ± 0.03 |
| Pulmonary vein s/d ratio                         | 0.5 ± 0.1 | 0.5 ± 0.1 |
| Pulmonary vein s wave (mm/s)                      | 243 ± 39 | 138 ± 23\(^a\) |
| Pulmonary vein d wave (mm/s)                      | 445 ± 35 | 305 ± 28\(^a\) |
| Pulmonary vein a wave (mm/s)                      | 157 ± 33 | 132 ± 16 |
| Pulmonary vein a wave duration (ms)              | 24 ± 2  | 28 ± 2\(^a\) |
| Tei index [(IVRT+IVCT)/ET]                        | 0.81 ± 0.04 | 0.80 ± 0.04 |
| Isovolumic relaxation time (IVRT) (ms)           | 22 ± 1  | 26 ± 2\(^a\) |
| Isovolumic contraction time (IVCT) (ms)          | 18 ± 2  | 18 ± 1 |
| Ejection time (ET) (ms)                          | 50 ± 2  | 52 ± 2 |
| Heart weight: tibia length ratio (mg/cm)         | 74 ± 2  | 63 ± 1\(^a\) |
| Heart weight (mg)                                | 141 ± 4 | 109 ± 3\(^a\) |
| Tibia length (cm)                                | 1.91 ± 0.01 | 1.75 ± 0.03\(^a\) |
| Body weight (g)                                  | 33.2 ± 1.0 | 22.2 ± 0.4\(^a\) |

\(^a\)P < 0.05 compared with age-matched control mice and both 19- to 23-week-old control mice.

### TABLE 2. Serum profile and blood pressure measurements in 19- to 23-week-old fdl (n = 4–8) and control (n = 5–9) mice

| Measurement               | Control  | fdl      | P      |
|---------------------------|----------|----------|--------|
| Serum glucose\(^a\) (mM)  | 9.8 ± 0.5 | 9.7 ± 0.3 | 0.8    |
| Serum nonesterified FA (mM)| 0.4 ± 0.1 | 0.6 ± 0.1 | 0.3    |
| Serum TG (mM)             | 1.3 ± 0.1 | 0.9 ± 0.1 | 0.01   |
| Systolic blood pressure (mmHg) | 141 ± 5 | 135 ± 5  | 0.4    |

\(^a\)Glucose measurements were obtained from 11-week-old fdl (n = 7) and control (n = 7) mice.
cardiac function in 10-week-old fl/d and control mice and found similar results (Table 1). Heart sizes and tibia lengths of fl/d mice at 10 weeks of age were not significantly different compared with age-matched control mice (Table 1). However, these parameters were significantly lower in 19- to 23-week-old fl/d mice compared with corresponding control mice (Table 1). Interestingly, systolic blood pressure was similar between fl/d and control mice (Table 2), even though we might expect differences in vascular capacity and demand because fl/d mice are lipodystrophic and smaller in size.

**Metabolic gene and protein expression profiles in fl/d hearts**

To determine whether cardiac dysfunction in fl/d mice in vivo was linked to changes in FA or glucose metabolism, we analyzed the expression of genes for key metabolic regulators in control and fl/d hearts. We chose to analyze samples from 11-week-old mice to determine whether there were any changes in cardiac metabolism in the absence of differences in heart size. mRNA levels for PGC-1α and PPARα were increased in fl/d hearts (Fig. 2A), which differs from studies in liver where PPARα induction was blunted in fl/d mice (23). We further examined expression of PPARα target genes downstream of lipin-1 regulation (23). Indeed, ACOX-1 and CPT1-B gene expression were upregulated. However, gene expression of CD36 and MCAD were similar between fl/d and control hearts (Fig. 2A). mRNA expression for enzymes in the TG synthesis and lipolysis pathways were also similar between genotypes, except for small increases in mRNA expression of GPAT1 and DGAT1 (Fig. 2B) in fl/d hearts. There were no differences in mRNA expression of CD36, GLUT4, lipoprotein lipase, acetyl-CoA carboxylase (ACC), pyruvate dehydrogenase kinase 4, or malonyl-CoA carboxylase, which are known regulators of FA and glucose metabolism (Fig. 2A, C).

In addition to profiling gene expression, we determined the levels and phosphorylation states of several important regulators of cardiac metabolism and function. Expression and phosphorylation of Akt, ACC, and AMP-activated protein kinase (AMPK) were unchanged (supplementary Fig. 1). There were also no significant changes in fatty acid transport protein 1 (FATP1), GLUT4, ERK1/2, or sarcoplasmic reticulum Ca2+-ATPase (SERCA) (supplementary Fig. 1), although mRNA levels of SERCA were slightly increased (Fig. 2C). Interestingly, both adipose triglyceride lipase (ATGL) expression and hormone-sensitive lipase (HSL) phosphorylation at serine 660 were decreased (Fig. 3A, B), which indicates reduced TG hydrolysis in fl/d hearts. We also observed a decrease in long-chain acyl-CoA synthetase 1 (ACSL1) expression and in the phosphorylation of pyruvate dehydrogenase (PDH) (Fig. 3). ACSL1 deficiency has been shown to affect the acyl-CoA available for FAO (52), and reduced phosphorylation of PDH leads to its increased activity (53).

**Cardiac function and metabolism in ex vivo perfused working hearts of fl/d mice**

We next determined the effects of the changes in gene and protein expression profiles on fatty acid and glucose metabolism in perfused working hearts. We used 19- to 23-week-old fl/d mice to determine whether the smaller heart sizes would further compromise cardiac function and metabolism. We hypothesized that the absence of lipin-1 would decrease TG synthesis and FAO in fl/d hearts, leading to cardiac dysfunction. To address this, we perfused control and fl/d hearts ex vivo in the working mode. By using this system, the nutrient supply to the perfused hearts of fl/d and control mice was equalized and differences that result from the changes in hormonal balance in the lipodystrophic fl/d mice in vivo were excluded.

Hearts isolated from 19- to 23-week-old control and fl/d mice were perfused for 30 min to deplete TG stores as much as possible (3). This was followed by a 30 min perfusion with [3H]oleate and [14C]glucose to determine oleate accumulation in glycerolipids as well as oleate and glucose oxidation. Oleate was used instead of palmitate because numerous studies have shown that palmitate supplementation alone can cause lipotoxicity, which is not observed with oleate (54, 55). Additionally, oleate incorporation into TG is greater than or equal to that of palmitate (54, 56). When we analyzed functional parameters in the fl/d and control hearts, we found that there were no significant differences in contractility and function ex vivo (Table 3), even though hearts from fl/d mice were significantly smaller than those of the controls (Table 1). This was surprising because we had found cardiac dysfunction in vivo.

Interestingly, we found no significant differences between control and fl/d hearts in the rates of glucose (Fig. 4A, left and middle panels) and oleate oxidation (Fig. 5A) when expressed relative to heart dry weight or cardiac power. Glucose incorporation into glycogen was also similar between genotypes (Fig. 4A, right panel). Cardiac glycogen accumulated when both fl/d and control hearts were perfused with FA-free and glucose-containing buffer for 30 min, as expected (Fig. 4B). The perfusate was switched to oleate- and glucose-containing buffer for another 30 min, and we found that cardiac glycogen content was depleted significantly following this period of perfusion in the control, but not the fl/d, hearts (Fig. 4C).

We also determined cardiac TG content after each perfusion period (Fig. 5B). TG levels were depleted in control hearts after perfusion with FA-free and glucose-containing buffer after 30 min, as expected (Fig. 5B). However, there was no significant depletion of TG in fl/d hearts. When the perfused hearts were switched to oleate- and glucose-containing buffer for another 30 min, there was no significant change in TG levels at the end of the perfusion in either fl/d or control hearts. This demonstrated that TG turnover was at a steady state during this 30 min period (Fig. 5B). There were no significant differences in the accumulation of [3H]oleate into total glycerolipids or TG in the fl/d hearts compared with controls (Fig. 5C). However, oleate accumulation in PC, PE, and PI was increased by 1.95-, 1.9- and 3.49-fold, respectively (Fig. 5C). Consistent with reduced cardiac PAP activity, we found a 4.36-fold increase in the accumulation of oleate in PA in fl/d hearts compared with controls (Fig. 5C). Interestingly, the mass of PI and PS were increased 1.21- and 1.35-fold, respectively, in fl/d
Consequences of whole-body lipin-1 deficiency on the heart 111

hearts compared with controls (Fig. 5D). The mass of the other major phospholipids, including PA, were not significantly different between groups. The relative composition of different phospholipids in our study was similar to that reported previously (57).

**Fig. 2. mRNA expression for various proteins involved in FA and glucose metabolism. (A–C) mRNA expression in 11-week-old fld (n = 8–11) and control (n = 7–10) hearts was expressed relative to the housekeeping gene Tbp (TATA-binding protein). Results for fld mice were then expressed relative to the control mice. *P < 0.05 compared with controls. ACC-a, acetyl-CoA carboxylase-a; ACOX-1, acyl-CoA oxidase 1; ACS1, acyl-CoA synthetase long chain family member 1; AGPAT2, 1-acylglycerol-3-phosphate O-acyltransferase 2; CPT-1B, carnitine palmitoyltransferase-1b; DGAT1, diacylglycerol acyltransferase 1; FATP1, fatty acid transport protein 1; GPAT1, glycerol-3-phosphate acyltransferase 1; LPL, lipoprotein lipase; MCAD, acyl-CoA dehydrogenase, medium-chain; PDH, pyruvate dehydrogenase E1; PDK4, pyruvate dehydrogenase kinase 4; SERCA, sarcoplasmic reticulum Ca^2+-ATPase; Tfam, transcription factor A, mitochondrial.**

**Examination of the downstream signaling effects of aberrant phosphatidate accumulation**

PA accumulation can activate the mTORC1-p70S6 kinase-S6 ribosomal protein signaling cascade (58, 59). As such, we determined whether mTORC1 signaling was increased in the fld hearts due to aberrant PA metabolism as seen in the perfused fld hearts. There was a very marked 14-fold increase in the phosphorylation of S6 ribosomal protein in the fld hearts (Fig. 6C, E, right panel). Activation of S6 ribosomal protein occurs downstream of mTOR complex-1 (mTORC1) and p70S6 kinase. Correspondingly, p70S6 kinase phosphorylation in fld mice was significantly increased (Fig. 6B, E, left panel). Although mTOR phosphorylation was not significantly increased (Fig. 6A, E, left panel), the majority of increased S6 and p70S6 kinase phosphorylation in the fld hearts is linked to higher levels of mTOR phosphorylation (supplementary Fig. II). Several studies show that activation of the mTORC1 pathway often results in increased protein synthesis, cell enlargement, and eventually, cardiac hypertrophy (60, 61). However, the hearts of 19- to 23-week-old fld mice were significantly smaller compared with controls (Table 1). Interestingly, mTORC1 activation has also been recently implicated in the upregulation of ER stress (62, 63). Therefore, we determined the gene expression of 78 kDa glucose-regulated protein (GRP78), which is an ER chaperone induced by ER stress, and CCAAT/enhancer-binding protein homologous protein (CHOP), which is a transcription factor mediating ER stress response, and found that they were increased (Fig. 6F).  Moreover, there was an increase in the gene expression of spliced X-box binding protein 1 (XBP1), which is highly indicative of ER stress (Fig. 6F) (64). Increased ER stress signaling associated with aberrant mTORC1 activation through phosphatidate signaling could explain why the fld hearts became smaller at 19 to 23 weeks of age.
metabolism and function had not been systematically determined. We hypothesized that the dynamic regulation of lipin-1 expression in the heart would be essential for regulating cardiac FA metabolism and function in different physiological and pathological conditions. We expected that complete lipin-1 deficiency would have dramatic effects on myocardial FA esterification and oxidation, resulting in cardiac dysfunction. Indeed, *fld* mice exhibited systolic dysfunction in vivo, as determined by noninvasive echocardiography. To determine the effects of lipin-1 deficiency on cardiac metabolism, we first assessed PAP activity in *fld* hearts. We concluded from our assays that *fld* hearts have 15–20% residual PAP activity, which is explained by the expression of lipin-2 and lipin-3 in *fld* hearts.

We chose to use the isolated perfused working heart model to study the metabolic effects of the absence of lipin-1 in the heart alone. This allowed us to determine

**DISCUSSION**

Lipin-1 is a unique protein with dual functions in promoting FA esterification and FAO (11). During starvation and diabetes, the combined effects of glucocorticoids and cAMP increase *Lpin1* gene transcription and thus PAP activity in liver (11, 25). This increase, in addition to the translocation of lipin-1 to membranes stimulated by unsaturated FAs, is thought to provide a reservoir that maintains or increases the capacity for FA utilization and storage (11, 15, 65). Furthermore, PAP activity is decreased in the hearts of insulin-resistant JCR:LA corpulent rats (32), and lipin-1 expression is decreased in the ventricles of Zucker diabetic fatty rats and in Type 2 diabetic patients (31). A recent study showed that the regulation of lipin-1 expression in the heart was dependent on PGC-1α as well as ERRα and ERRγ (50). However, the role of lipin-1 in cardiac metabolism and function had not been systematically determined. We hypothesized that the dynamic regulation of lipin-1 expression in the heart would be essential for regulating cardiac FA metabolism and function in different physiological and pathological conditions. We expected that complete lipin-1 deficiency would have dramatic effects on myocardial FA esterification and oxidation, resulting in cardiac dysfunction. Indeed, *fld* mice exhibited systolic dysfunction in vivo, as determined by noninvasive echocardiography. To determine the effects of lipin-1 deficiency on cardiac metabolism, we first assessed PAP activity in *fld* hearts. We concluded from our assays that *fld* hearts have 15–20% residual PAP activity, which is explained by the expression of lipin-2 and lipin-3 in *fld* hearts.

![Image](image-url)

**Fig. 3.** Protein expression of enzymes involved in FA metabolism. (A) Representative Western blots of HSL (hormone-sensitive lipase), ACSL1 (acyl-CoA synthetase long chain family member 1) and ATGL (adipose triglyceride lipase) as well as (C) PDH (pyruvate dehydrogenase) in 11-week-old *fld* and control hearts. (B) Densitometric analysis of single Western blots for HSL, ACSL1, and ATGL in addition to (D) PDH in 11-week-old *fld* (n = 6–7) and control (n = 6–7) hearts. Total ACSL1, ATGL, PDH, and HSL were normalized to RanGTPase and then expressed relative to control values. Phospho-HSL and phospho-PDH were normalized to RanGTPase and expressed relative to total HSL and PDH, which were also normalized to RanGTPase.

*P* < 0.05 compared with controls.

**Table 3.** Cardiac function of ex vivo perfused hearts from 19- to 23-week-old *fld* (n = 5) and control (n = 5) mice over the 30 min perfusion period with radiolabeled glucose and FA

| Measurement                        | Control          | fld              | *P*  |
|------------------------------------|------------------|------------------|------|
| Heart rate (beats per min)         | 342 ± 21         | 307 ± 31         | 0.4  |
| Peak systolic pressure (mmHg)      | 57.9 ± 0.4       | 60.7 ± 1.8       | 0.2  |
| Developed pressure (mmHg)          | 12.9 ± 0.8       | 13.9 ± 2.7       | 0.7  |
| Heart rate x peak systolic pressure (x 10^-3) | 29 ± 1         | 19 ± 2           | 0.7  |
| Heart rate x developed pressure (x 10^-3) | 4.4 ± 0.4       | 4.4 ± 1.1        | 1.0  |
| Cardiac output (ml/min)            | 10 ± 1           | 8 ± 1            | 0.2  |
| Aortic outflow (ml/min)            | 7 ± 1            | 5 ± 1            | 0.2  |
| Cardiac power (mW)                 | 1.03 ± 0.08      | 0.87 ± 0.16      | 0.4  |
the consequences for cardiac metabolism and function under a workload using defined conditions in the absence of systemic factors or extraneous signals from the circulatory system. The use of perfused working hearts is also preferable to cultured cardiomyocytes for assessing the role of lipin-1 on FA and glucose metabolism because metabolism is stimulated in a physiologically appropriate manner by the need to perform mechanical work. Surprisingly, cardiac function in the perfused fld hearts was not significantly different from the control hearts even though the fld hearts were smaller. This suggests that the cardiac dysfunction we observed in fld mice in vivo may be related to the systemic changes stemming from global lipin-1 deficiency, which can be described as a combination of the absence of adipose tissue, the corresponding decrease in adipokine secretion, whole-body insulin resistance, aberrant changes in the circadian rhythm of whole-body metabolism (16, 66, 67), and higher workload in vivo compared with ex vivo. We also found decreased circulating TG levels in fld mice (Table 2), which could affect the availability of substrate for cardiac work. We only measured serum FA levels after 2 h of food deprivation in the light period, and thus, we might not have detected abnormalities in the fluctuation of plasma FA levels occurring during the diurnal cycle of the lipodystrophic fld mice, which could also negatively affect cardiac function.

We expected that fld hearts would have decreased rates of glucose oxidation because fld mice are insulin-resistant (16, 20). The fld mice had similar circulating levels of glucose compared with controls (Table 2), which was shown in a previous study (16). This latter work also found that fld mice are hyperinsulinemic. Interestingly, there was no significant decrease in glucose oxidation and no significant difference in glucose incorporation into glycogen under defined conditions ex vivo. However, glycogen depletion in the control hearts was significantly greater than in fld hearts when the perfusate was switched to glucose- and oleate-containing buffer. It is likely that upon the reintroduction of FA in the perfusate, glucose uptake and, hence, glycogen accumulation is decreased more in control hearts than in fld hearts. Glucose utilization from glycogen stores might also be greater in control hearts compared with fld hearts. Most importantly, FAO was not different in isolated perfused fld hearts compared with the controls, suggesting that cardiac lipin-1 expression is not essential for maintaining myocardial FAO. Consistent with unchanged glucose and fatty acid utilization, gene and protein expression of several key proteins involved in regulating glucose and FA metabolism were similar between fld and control hearts.

Because fasting-induced expression of PPARα and its target genes in liver was ablated by knocking down lipin-1 expression (23), we had hypothesized that lipin-1 deficiency would also limit the transcriptional regulation of PPARα in the heart. However, we found transcriptional upregulation of PPARα in the fld hearts in addition to increased gene expression of the PPARα downstream targets ACOX-1 and CPT-1, whereas mRNA expression of other PPARα target genes was unchanged. Further work needs to be done to determine how PPARα is regulated in the absence of lipin-1 in the heart. It is conceivable that lipin-2 and possibly lipin-3 compensate for the lack of lipin-1 as a PPARα coactivator, as lipin-2 also exhibits transcriptional coactivator activity (68). Moreover, Grimsey et al. established that lipin-1 and lipin-2 can be reciprocally regulated (69). However, lipin-1 is required for promoting transcriptional regulation of PPARα and its target genes in the livers of fld mice, and there does not appear to be compensation of lipin-1 transcriptional coactivator function by lipin-2 or lipin-3 in the liver (23), in spite of lipin-2 being highly expressed in hepatocytes (14, 25). Indeed, the changes in mRNA levels of PPARα, PGC-1α, and a subset of their targets could be a compensatory response in vivo to the aberrant fuel utilization of fld mice in the diurnal cycle (67). Furthermore, there was increased activation of PDH as shown by decreased phosphorylated PDH, which reflects the inability of the fld mice to utilize FAs to the same extent as control mice in the fasted state (70). Overall, our results demonstrate that the absence of lipin-1 in the heart does not overtly affect cardiac FAO and glucose metabolism when assessed ex vivo. It is likely that the
changes in cardiac function and metabolic profiling in vivo are attributable to the effects of global lipin-1 deficiency, as previously mentioned.

The initial concentrations of TG in perfused hearts of fl/l and control mice were similar. Considerable lipolysis took place in the control hearts during the 30 min incubation in glucose-containing buffer in the absence of oleate as was seen from the decrease of about 50% in TG content. However, lipolysis was decreased in hearts of fl/l mice because there was minimal TG depletion when fl/l hearts were perfused with oleate-free buffer. This conclusion is supported by the decreased levels of both ATGL expression and HSL phosphorylation in the 11-week-old fl/l hearts. When the perfused hearts were switched onto oleate- and glucose-containing buffer, we demonstrated that TG levels were constant at the start of this perfusion period compared with the end. This indicates that TG turnover was at a steady state during the perfusion with both oleate and glucose (13), unlike the initial perfusion period with glucose alone. There was no deficiency in the ability of the fl/l hearts to accumulate [3H]oleate in TG. This was surprising because we had hypothesized that TG synthesis would be compromised due to the 80% decrease in PAP activity.

By contrast, Mitra et al. found that knocking down lipin-1 by 60% in neonatal rat ventricular myocytes decreased the incorporation of [2-3H]glycerol into TG (50). However, it is very difficult to compare glycerolipid synthesis under different conditions using [2-3H]glycerol, unless the specific radioactivity in the glycerol 3-phosphate precursor pool is also measured (71). This specific activity depends on the relative activities of glycerol kinase in the fl/l and control hearts and on the rate of substrate cycling between glycerol 3-phosphate and dihydroxyacetone phosphate. This cycling involves glycerol-3-phosphate dehydrogenase, which exhibits a significant isotope effect in its use of 3H, resulting in progressive increases in the specific activity of the [2-3H]glycerol-3-phosphate precursor pool compared with that obtained using [1,3-3H] or [14C]glycerol (71). We also measured glycerolipid synthesis in neonatal rat ventricular myocytes by using [3H]oleate. E600 (100 µM), which is a general lipase inhibitor (72), was added to the incubations to prevent the hydrolysis of TG and provide a more accurate assessment of synthesis. Knocking down lipin-1 by 50% with adenovirus expressing shRNA against Lpin1 did not significantly decrease the incorporation of oleate into TG. Thus, our unpublished results with cultured cardiomyocytes confirm those from the more physiologically relevant perfused heart system. Our results show that complete depletion of lipin-1 does not significantly decrease the capacity of the heart to synthesize TG.

This result can possibly be explained by the residual PAP activity, attributed to lipin-2 and/or lipin-3, in fl/l hearts. Although fl/l hearts express relatively high phosphatidate phosphatase activity from their LPPs, these enzymes probably do not participate in glycerolipid synthesis, as their active sites are facing the extracellular space or the

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**Fig. 5.** Oleate oxidation and accumulation in glycerolipids in fl/l and control hearts. (A) Oleate oxidation is expressed per milligram dry heart weight per minute or normalized per second per milliwatt in perfused 19- to 23-week-old fl/l (n = 5) and control (n = 5) hearts. (B) TG content in nonperfused fl/l (n = 7) and control (n = 5) hearts or fl/l (n = 5) and control (n = 5) hearts after 30 min perfusion with glucose-containing and FA-free buffer, as well as after 30 min perfusion with glucose-containing and FA-free buffer, followed by another 30 min with glucose- and oleate-containing buffer. (C) [3H]oleate accumulation in glycerolipids extracted from ex vivo perfused fl/l (n = 5) and control (n = 5) hearts. (D) Phospholipid content in fl/l (n = 10) and control (n = 9) hearts. †P < 0.05 compared with the nonperfused control group; *P < 0.05 compared with controls.
Consequences of whole-body lipin-1 deficiency on the heart

We also detected increased [3H]oleate accumulation in PA in the fld hearts without observing an increase in total PA mass. The latter analysis of PA mass was performed after completely separating PA from PS and PI by sequential chromatography on silica gel using a basic solvent system followed by an acidic system (43). Presumably, the labeling experiment in perfused hearts identified the pool of PA that is formed de novo by lipin activity, whereas the total PA pool reflects the balance of a variety of enzymes, including phospholipase D and diacylglycerol kinase, that contribute to PA turnover. Interestingly, another group has shown that there are increases in total PA levels in fld hearts as analyzed by LC/mass spectrometry (50). However, no details of the full molecular species of the PA are

Lumenal sides of internal membranes (51, 73). By contrast, glycerolipid synthesis occurs on the cytosolic surface of internal organelles. It has been hypothesized that cytosolic PAP activity provides a reservoir of activity that can be recruited to the ER in response to the FA load (29, 74). Presumably, the reservoir of lipin-2 and lipin-3 activity in fld hearts is sufficient to provide the capacity for relatively normal rates of TG accumulation. In addition, the accumulation of oleate in the major phospholipids, PC and PE, was not compromised in fld hearts; in fact, there was increased labeling of PC and PE with [3H]oleate in fld hearts. This observation might be due to decreased fatty acid remodeling of phospholipids, which occurs rapidly in the heart (56, 75).

Fig. 6. mTOR-p70S6 kinase signaling in fld and control hearts. Representative Western blots of (A) mTOR, (B) p70S6 kinase, and (C) S6 ribosomal protein in 11-week-old fld and control hearts. Densitometric analysis of single Western blots for (D) total and (E) phosphorylated mTOR, p70S6 kinase, and S6 ribosomal protein in 11-week-old fld (n = 6–7) and control (n = 6–7) hearts. Total mTOR, p70S6 kinase, and S6 ribosomal protein were normalized to Ran GTPase and then expressed relative to control values. Phospho-S6, phospho-p70S6 kinase, and phospho-mTOR were normalized to Ran GTPase and expressed relative to total S6, p70S6 kinase, and mTOR, which were also normalized to Ran GTPase. *P < 0.05 compared with controls. (F) mRNA expression of proteins involved in ER stress response. mRNA expression in 11-week-old fld (n = 8–11) and control (n = 7–10) hearts was expressed relative to Tbp (TATA-binding protein). Results for fld mice were then expressed relative to control mice. *P < 0.05 compared with controls. CHOP, CCAAT/Enhancer-binding protein homologous protein; GRP78, 78 kDa glucose-regulated protein; XBP-1, X-box binding protein 1.
given, and it is unclear whether the authors achieved efficient separation of PA from the other phospholipids, as shown in previous studies (76, 77). This separation would be essential because we have demonstrated an increase in PS and PI mass in the fld hearts.

Our group demonstrated that decreasing PAP activity diverts PA metabolism to CDP-diacylglycerol and acidic phospholipid production (78). This could contribute to the increased labeling of PI and the increase in PI mass that we observed in fld hearts. The accumulation of PA, PS, and PI in the fld hearts could lead to aberrant cell signaling. For example, PA accumulation in peripheral nerves of fld mice increases ERK1/2 activation, leading to demyelination (22). We did not observe significant increases in ERK1/2 phosphorylation in the fld hearts. However, PA can also activate mTORC1, leading to the downstream activation of p70S6 kinase (58, 59), which phosphorylates and activates S6 ribosomal protein to promote cell growth (58, 59). In fact, activation of mTORC1-p70S6 kinase leads to cardiac hypertrophy (79). We now show that there is an increase in signaling downstream of mTOR, i.e., increased p70S6 kinase phosphorylation in combination with a 14-fold increase in S6 ribosomal protein activation. However, there was no evidence of cardiac hypertrophy in 11-week-old mice, which is the age at which we first observed cardiac dysfunction. Instead, the 19- to 23-week-old fld mice have significantly smaller hearts in the absence of defective FAO. Although the mTORC1 signaling pathway is classically linked to cell growth, recent studies have shown that mTORC1 activation can also result in the unfolded protein response and ER stress (62, 80). There was increased GRP78 and CHOP gene expression as well as Xbp1 splicing in the fld mice, which could be due to mTORC1 activation (63). The increased ER stress response in fld mice could explain why the hearts became smaller at 19 to 23 weeks of age. Alternatively, mTORC1 has been implicated in regulating metabolism. Aberrant activation of mTORC1 increases glycolysis and the oxidative arm of the pentose phosphate pathway (81). Although we did not see differences in glucose oxidation, we found increased PDH activation. Other studies on fld mice show changes in energy partitioning in vivo, which could aberrantly affect cardiac function (66, 67).

The present work provides a comprehensive assessment of the effects of lipin-1 deficiency in fld mice on the work output of the heart in vivo and ex vivo relative to the use of fatty acids and glucose as fuels. Lipin-1 deficiency led to cardiac dysfunction in fld mice as measured in vivo, probably because of systemic factors stemming from global lipin-1 deficiency, such as lipodystrophy, modified hormonal regulation, and fuel availability. When these factors were equalized in the perfused working heart system, there were no significant differences in work output or the use of oleate and glucose for oxidative metabolism. We conclude that TG accumulation in fld hearts is similar to that in control hearts because of residual PAP activity resulting from lipin-2/3 and reduced TG hydrolysis. Despite this, fld hearts displayed increased oleate accumulation in PA, which could be linked to the mTORC1-p70S6 kinase signaling axis. This does not compensate for the development of significantly smaller hearts in the fld mice. Instead, aberrant mTORC1 activation could be associated with the development of ER stress. This work provides novel information contributing to the understanding of lipin-1 in the regulation of glycerolipid synthesis, energy partitioning, and signaling in the heart.

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