Acute Liver Carnitine Palmitoyltransferase I Overexpression Recapitulates Reduced Palmitate Oxidation of Cardiac Hypertrophy


Rationale: Muscle carnitine palmitoyltransferase I is predominant in the heart, but the liver isoform (liver carnitine palmitoyltransferase I [L-CPT1]) is elevated in hearts with low long chain fatty acid oxidation, such as fetal and hypertrophied hearts.

Objective: This work examined the effect of acute L-CPT1 expression on the regulation of palmitate oxidation and energy metabolism in intact functioning rat hearts for comparison with findings in hypertrophied hearts.

Methods and Results: L-CPT1 was expressed in vivo in rat hearts by coronary perfusion of Adv.cmv.L-CPT1 (L-CPT1, n=15) vs phosphate-buffered saline (PBS) infusion (PBS, n=7) or empty virus (empty, n=5). L-CPT1 was elevated 5-fold at 72 hours after Adv.cmv.L-CPT1 infusion (P<0.05), but muscle carnitine palmitoyltransferase I was unaffected. Despite similar tricarboxylic acid cycle rates, palmitate oxidation rates were reduced with L-CPT1 (1.12±0.29 μmol/min per gram of dry weight, mean±SE) vs PBS (1.6±0.34). Acetyl CoA production from palmitate was reduced with L-CPT1 (69±0.02%; P<0.05; PBS=79±0.01%; empty=81±0.02%), similar to what occurs in hypertrophied hearts, and with no difference in malonyl CoA content. Glucose oxidation was elevated with L-CPT1 (by 60%). Surprisingly, L-CPT1 hearts contained elevated atrial natriuretic peptide, indicating induction of hypertrophic signaling.

Conclusions: The results link L-CPT1 expression to reduced palmitate oxidation in a nondiseased adult heart, recapitulating the phenotype of reduced long chain fatty acid oxidation in cardiac hypertrophy. The implications are that L-CPT1 expression induces metabolic remodeling hypertrophic signaling and that regulatory factors beyond malonyl CoA in the heart regulate long chain fatty acid oxidation via L-CPT1. (Circ Res. 2013;112:57-65.)

Key Words: β-oxidation ■ hypertrophy ■ long chain fatty acids ■ mitochondria ■ palmitoyltransferase I
for the liver, or α, isoform of the enzyme, and 1 for the muscle, or β, isoform.1,2,7,8 These CPT1 isoforms are differentially expressed among tissues that use LCFAAs as a fuel, and both isoforms are coexpressed in heart muscle. In adult heart muscle, muscle CPT1 (M-CPT1) is the predominantly expressed isoform, with limited activity from L-CPT1.1,3-10,11 However, until recently, the actual content of L-CPT1 that is elevated in the hypertrophied heart, as opposed to transcript levels from myocardium or analysis activity in cultured neonatal cardiomyocytes, was not known.2,3,12

The liver and muscle isoforms of CPT1 have different kinetic properties, with L-CPT1 being less sensitive to malonyl CoA inhibition and displaying a higher affinity for carnitine.7,9 Yet, LCFA oxidation is lower in hearts with elevated L-CPT1 content (ie, hypertrophied adult hearts and neonatal hearts) than in normal adult hearts displaying minimal L-CPT1 activity.1,4-9,11,13,14 Elevated L-CPT1 levels in cardiomyocytes, in culture, have been explained as a potential adaptive response based on the teleological argument that L-CPT1 expression serves the functional outcome of maintaining fatty acid oxidation, albeit reduced.7 The link between increased L-CPT1 and reduced palmitate oxidation also is consistent with a reversion to fetal isoform expression of metabolic enzymes and reduced LCFA oxidation rates under conditions of limited carnitine availability in fetal and neonatal hearts.1,2,9,10,13 These seemingly incongruent findings between L-CPT1 expression and LCFA oxidation also may suggest a multifactorial level of regulation of LCFA oxidation to produce the observed metabolic phenotypes. However, no previous work has examined the direct influence of isoform shifts in CPT1 on LCFA oxidation rates in the intact adult heart in the absence of disease.

Therefore, this study examined what effect acute overexpression of L-CPT1 has on energy metabolism in the adult rat heart after in vivo delivery and expression of exogenous L-CPT1 gene. The use of the acute overexpression model enabled an examination of metabolic adaptations to L-CPT1 expression in the absence of code for L-CPT1 (Online Figure II). In other groups, anesthetized rats received transverse aortic constriction to produce pressure overload or sham surgery, as previously described.3,6,16 Transverse aortic constriction produces concentric cardiac hypertrophy and increased heart-to-body weight ratio within 10 weeks postsurgery. At 10 weeks postsurgery, experiments were performed on isolated hearts from these animals. Hearts were excised from anesthetized rats (pentobarbital, 50 mg/kg intraperitoneal) at 72 hours after intracoronary perfusion or 10 weeks after transverse aortic constriction. Excised hearts were either immediately perfused or frozen for assays.

13C Enrichment Protocols for Metabolic Flux Measurements Rates of LCFA oxidation or the fraction of palmitate contributing to acetyl CoA synthesis for the TCA cycle were determined in isolated perfused rat hearts excised from rats that were anesthetized and heparinized (500 U/100 g intraperitoneal; L-CPT1, n=15; PBS sham, n=7). Similarly, hearts were perfused from rats subjected to transverse aortic constriction (n=14) or sham operation (n=11). Isolated hearts were perfused in a retrograde fashion at 37°C, as previously described, with modified Krebs-Henseleit buffer (116 mM/L NaCl, 4 mM/L KCl, 1.5 mM/L CaCl2, 1.2 mM/L MgSO4, and 1.2 mM/L NaH2PO4, equilibrated with 95% O2/5% CO2 with 0.4 mM/L unlabeled palmitate/albumin complex [3:1 molar ratio] and 5 mM/L glucose).6,16 A water-filled latex balloon in the left ventricle was set to a diastolic pressure of 5 mm Hg and provided hemodynamic recordings (Powerlab; AD Instruments). Left ventricular developed pressure and heart rate were continuously recorded. After each perfusion, hearts were frozen for biochemical analyses. For palmitate oxidation, isolated hearts were initially supplied buffer containing unlabeled palmitate/albumin and glucose for 10 minutes to ensure metabolic equilibrium and for collection of background 13C nuclear magnetic resonance (NMR) signals of naturally abundant 13C (1.1%). At the start of each enrichment protocol, the perfusate was switched to buffer (1L) containing [2,4,6,8,10,12,14,16-13C] palmitate (0.4 mM/L; Isotec) plus unlabeled glucose (5 mM/L). Perfusion with 13C-enriched media continued for 40 minutes.

For metabolic flux measurements, a subset of these hearts (L-CPT1, n=10; PBS sham, n=5) were situated in a 20-mm NMR probe within a vertical 89-mm bore 9.4-T magnet.39P and 13C NMR (2 minutes each)
measurements were acquired as described elsewhere. Energetic state was determined by \(^{31}P\) NMR detection of phosphocreatine and ATP content. 

Metabolic flux in the intact beating heart was determined during \(^1\)C palmitate delivery to the intact rat heart ex vivo using well-described methods for kinetic analysis of the progressive \(^1\)C enrichment of glutamate via NMR spectroscopy (Online Materials). Kinetic analysis provided TCA cycle flux, the interconversion rate between cytosolic glutamate and mitochondrial \(\alpha\)-ketoglutarate via the oxoglutarate–malate carrier, and \(^1\)C palmitate entry into the mitochondria as an index of CPT1 flux.

Hearts from additional experimental groups of Adv.cmv.L-CPT1–infected rats (n=7) and PBS-infused control rats (n=5) were perfused with unenriched palmitate (0.4 mmol/L) plus [1,6-\(^{13}\)C\(_2\)] glucose (5 mmol/L) to assess potential differences in glucose oxidation.

**Tissue Biochemistry**

Assays for glutamate, aspartate, citrate malate, and \(\alpha\)-ketoglutarate were determined spectrophotometrically and fluorometrically. Triacylglycerol was determined by colorimetry of processed lipid extracts (Wako Pure Chemical Industries). Malonyl CoA was assayed by high-pressure liquid chromatography with ultraviolet detection. The percent of labeled acetyl CoA entering the TCA cycle was determined from in vitro \(^1\)C NMR spectra. Enrichment of glutamate from [1,6-\(^{13}\)C\(_2\)] glucose was determined via \(^{13}\)C NMR of in vitro tissue extracts and the relative contributions of \(^{13}\)C-enriched glucose and the unlabeled (12C) endogenous pool of glycogen determined via \(^1\)H NMR of alanine enrichment.

For Western blot assay of CPT1, hearts excised from both PBS shams (n=7) and L-CPT1 heart (n=3) were perfused with ice-cold mannitol, sucrose, EGTA (MSE) media containing, 220 mM mannitol, 70 mM sucrose, 2 mM ethylene glycol-bis(aminohexylether)-tetraacetic acid (EGTA), 5 mM 3-(4-morpholinol) propyl sulfonic acid (MOPS), pH 7.4, 0.2% BSA and a protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aproitin, and sodium ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) The left ventricle was minced and homogenized in 1 mL ice-cold MSE media and centrifuged at 15 000g for 15 minutes. Rat liver was used as a positive control for CPT1 content. Total homogenate protein concentration was determined from a standard curve (Bradford assay), and equivalent samples (80–100 \(\mu\)g protein) were dissolved in Laemmli buffer and then loaded and separated on a 7.5% NuPAGE Bis-Tris gel. Gel proteins were subsequently transferred onto a nitrocellulose membrane. Western blots were performed according to the standard techniques using human heart M-CPT1 Ab and rat liver CPT1 Ab. 

Semi-quantitative densitometric analysis was performed using the Bio-Rad Universal Hood and Quantity One software. Message level for atrial natriuretic peptide (ANP) expression was determined after total RNA isolation by single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture. Myocardial contents of acetyl CoA carboxylase (ACC) 2 and phosphorylated ACC2 (P-ACC2, Ser 218) and malonyl CoA decarboxylase (MCD) were determined in the available myocardial tissue from L-CPT1 hearts (n=13), PBS shams (n=7), hypertrophied hearts (n=4), and sham-operated hearts (n=4). ACC2 and P-ACC2 were probed with corresponding antibodies, as previously described (Upstate Cell Signaling Technologies). MCD was immunoblotted with an anti-MCD antibody (H2-40 antibody, prepared in-house) as detailed elsewhere.

**Statistical Analysis**

Intergroup statistics were analyzed using Student t test for comparison of 2 mean values and 1-way ANOVA analysis with the Tukey post hoc test for comparison of multiple group means. Statistical significance was established at 5% probability (\(P<0.05\)). All reported values are reported as averages±SEM.

**Results**

**Response of CPT1 Expression to Exogenous L-CPT1 Gene Delivery**

After adenoviral delivery of the exogenous gene for rat L-CPT1 to the in vivo heart, the level of L-CPT1 protein was increased in Adv.cmv.L-CPT1–infected hearts 5-fold over L-CPT1 content in control hearts receiving sham infusions of PBS, as displayed in Figure 1. The content of M-CPT1 remained unchanged in the presence of elevated L-CPT1 (Figure 1). The level of L-CPT1 overexpression that was achieved was similar to the approximate 4- to 5-fold increase in L-CPT1 protein that was previously reported for hypertrophied rats.

**Oxidative Rates and Metabolism of Palmitate in L-CPT1–Expressing Adult Rat Hearts**

The functional workloads, as assessed by rate–pressure products (RPP) generated by the perfused hearts, were similar (Figure 2), and thus the metabolic demand imposed by cardiac workload was the same in the sham and L-CPT1–expressing groups. Consequently, mitochondrial oxidative rates, as assessed by TCA cycle flux from dynamic-mode \(^{13}\)C NMR of the beating hearts (Figure 3A), also were similar with either L-CPT1 (12.3±1.0 \(\mu\)mol/min per gram of dry weight; mean±SE) expression or PBS infusion (15.0±1.5).

However, despite similar rates of flux within the TCA cycle, the rate of palmitate oxidation in the intact hearts was unexpectedly reduced in hearts expressing L-CPT1 in comparison with PBS-infused controls. L-CPT1 expression produced a 30% reduction in the rate of palmitate oxidation compared with hearts receiving PBS (Figure 3B), indicating lower rates of flux through CPT1 in the hearts with elevated L-CPT1.

Palmitate oxidation was reduced similarly in hypertrophied hearts and hearts with increased L-CPT1 content, as shown by reductions in the formation of acetyl CoA entering the TCA cycle from \(\beta\)-oxidation of palmitate (Figure 4). Figure 4A displays representative in vitro \(^{13}\)C NMR spectra of the 4-carbon glutamate resonance in acid extracts of myocardium from an L-CPT1–expressing heart (Figure 4A, right) and a PBS-infused control (Figure 4A, left) displaying the visual and quantitative differences between multiple structures of the resonance signals that resulted because of differences in the oxidation of \([2,4,6,8,10,12,14,16-\text{\(^{13}\)C}\text{] palmitate. From static measurements of \(^{13}\)C NMR spectra from myocardial extracts (Figure 4A), the fraction of exogenous \(^{13}\)C-enriched palmitate contributing to acetyl CoA production within the mitochondria of isolated perfused hearts was reduced by 12% in myocardium that overexpressed L-CPT1 (Figure 4B) in comparison with PBS controls. This decline mimicked the same 12% reduction observed in hypertrophy hearts vs sham-operated hearts (Figure 4C). The fractional contribution from palmitate oxidation in L-CPT1–expressing hearts (Figure 4B) were lower than that of both PBS-infused hearts and hearts receiving vector containing scrambled DNA (empty). PBS-infused controls, control empty virus infusion, and sham aortic banding all resulted in similar \(^{13}\)C fractional enrichments of acetyl CoA from \([2,4,6,8,10,12,14,16-\text{\(^{13}\)C}\text{] palmitate, with mean
values ranging from 0.77 to 0.81. The presence of virus alone was insufficient to produce the results observed with L-CPT1 delivery and expression. However, hearts expressing L-CPT1 and hypertrophied hearts displayed strikingly similar values of acetyl CoA 13C enrichment at 0.68 and 0.69, respectively.

Compensatory Increase in Glucose Oxidation

The reduced palmitate oxidation in hearts expressing L-CPT1 was compensated by increased glucose oxidation (Figure 5). From combined 13C and 1H NMR, the oxidation in the TCA cycle of both exogenous 13C-enriched and endogenous unlabeled (12C) glucose to form glutamate was elevated by 60%. No evidence of elevated oxidation of endogenous myocardial triglyceride stores was apparent, with very similar triglyceride content between L-CPT1–expressing and control hearts (L-CPT1=25.7 ± 5.1 μmol/g dry weight; PBS=25.3 ± 5.0). These data demonstrate a compensatory increase in glucose oxidation in response to reduced LCFA oxidation.

Comparisons With Hypertrophy: ANP mRNA With L-CPT1 Overexpression

Although palmitate oxidation was reduced in L-CPT1–expressing hearts to the extent of that observed in hypertrophied hearts (Figure 4), which also are known to have elevated L-CPT1,1 the L-CPT1–expressing hearts showed no evidence of energetic or pathophysiological changes. Hypertrophied hearts have a well-characterized increase in mass and a reduction in bioenergetic state.3,6,28–30 In contrast, heart weight was not elevated in L-CPT1–expressing hearts (L-CPT1=2.53 ± 0.07 g; PBS=2.60 ± 0.08 g), and the ratio of phosphocreatine to ATP content in these hearts (1.8 ± 0.2) from 31P NMR was not reduced in comparison with PBS controls (1.7 ± 0.1). Also, whereas hypertrophied myocardium has been reported to display increased anaplerotic flux of 3 carbon intermediates into the TCA cycle through malic enzyme, the otherwise normal hearts that overexpressed L-CPT1 also displayed normal ratios of anaplerotic to citrate synthase flux (L-CPT1=0.052 ± 0.009; PBS=0.068 ± 0.004). These findings are all consistent with normal contractile work and TCA cycle flux.

Despite these indices of normal myocardial pathology, an unexpected finding was that of significantly elevated ANP message levels by 2-fold in the L-CPT1–overexpressing rat heart (Figure 6). As previously published, mRNA levels of the marker for the hypertrophic response, ANP, are elevated in response to pressure overload in the rat heart.3,26 Although the elevation of ANP message was several-fold smaller than that observed in hypertrophied hearts after 10 weeks of aortic banding, the finding of elevated ANP expression in hearts acutely expressing L-CPT1 suggests a link between the metabolic activity of the heart, with induced switching to fetal or hypertrophic isoforms of CPT1 and initiation of hypertrophic signaling.

Acetyl CoA Carboxylase, Malonyl Decarboxylase, and Malonyl CoA Content

Malonyl CoA, understood to inhibit CPT1 activity, is produced at the outer mitochondrial membrane by ACC2 via carboxylation of acetyl CoA that is produced from oxidative metabolism. Although cytosolic ACC1 has been related to the production of malonyl CoA for LCFA synthesis and elongation, a process not as active in cardiomyocytes, ACC2 production of malonyl, in concert with MCD action, is understood to work in tandem to regulate malonyl CoA levels.

Interestingly, malonyl CoA content was similar between L-CPT1–expressing and hypertrophied hearts as with PBS-infused shams. The only measurable difference in malonyl CoA...
Phosphorylation of ACC2 reduces activity, and thus total ACC2 content and phosphorylation levels were examined with relation to the reduced LCFA oxidation observed in hearts overexpressing L-CPT1. The content and phosphorylation level of ACC2, along with MCD levels, for hearts overexpressing L-CPT1 are shown in comparison with sham hearts receiving PBS infusion (Figure 7). Values for ACC2 in L-CPT1–expressing hearts and PBS-infused controls are normalized to the total content of pyruvate carboxylase, an enzyme shown to not change in response to hypertrophy.3,6 Pyruvate carboxylase levels also were not different between hearts with L-CPT1 overexpression and PBS-infused controls (Online Materials and Online Figure I). Although total ACC2 content was unaffected by L-CPT1 overexpression (Figure 7), the level of ACC2 phosphorylated at serine 218 was reduced by 32% in the L-CPT1–expressing hearts (Figure 7). The reduced level of phosphorylation is consistent with less inhibition, and thus a greater capacity for ACC2 to catalyze the production of malonyl CoA from acetyl CoA.31–33 However, as shown in Figure 7 and as seen in other heart models and tissues that display changes in LCFA oxidation, malonyl CoA levels were not different between L-CPT1–expressing hearts and sham hearts.
Atrial natriuretic peptide (ANP) mRNA content was elevated in liver carnitine palmitoyltransferase I (L-CPT1)–expressing hearts (black bar) and hypertrophied hearts (HYP; 10-week aortic banding, white bar). Empty virus had no effect on ANP content compared with phosphate-buffered (PBS) infusion. *P<0.05 vs PBS and HYP; #P<0.05 vs PBS and L-CPT1. AU indicates arbitrary units.

Figure 6. Atrial natriuretic peptide (ANP) mRNA content was elevated in liver carnitine palmitoyltransferase I (L-CPT1)–expressing hearts (black bar) and hypertrophied hearts (HYP; 10-week aortic banding, white bar). Empty virus had no effect on ANP content compared with phosphate-buffered (PBS) infusion. *P<0.05 vs PBS and HYP; #P<0.05 vs PBS and L-CPT1. AU indicates arbitrary units.

receiving PBS infusion. Consistent with these findings are the similarity in MCD levels in the L-CPT1–expressing hearts and PBS sham-infused hearts (Figure 7), and thus the regulatory processes that lowered LCFA oxidation with increased L-CPT1 expression are yet to be identified and are likely to involve other processes beyond malonyl CoA levels.

Hypertrophied hearts resulting from transverse aortic constriction also contained similar levels of total ACC2 as surgical sham hearts (Figure 8). In contrast to L-CPT1–expressing hearts, the hypertrophied hearts displayed elevated ACC2 phosphorylation, although no direct comparison is available between the L-CPT1–expressing and hypertrophied hearts that were assayed separately. Together, the findings of ACC2 content and phosphorylation state and malonyl CoA content are consistent with a previous finding that demonstrated no directional correlation between LCFA oxidation rates and L-CPT1 content, whereas M-CPT1 content correlated more closely with LCFA oxidation rates.

Evidence for muscle type–specific sensitivity of CPT1 activity to malonyl CoA levels also exists and may shed some insight into the current type–specific sensitivity of CPT1 activity to malonyl CoA phosphorylation, although no direct comparison is available with increased L-CPT1 content in comparison with the normal adult myocardium: a developmental condition of higher L-CPT1 content in the fetal heart; a pathological condition of elevated L-CPT1 content in hypertrophied hearts; and the experimental condition, presented here, of acute increases in L-CPT1 content in the other normal but genetically altered adult rat heart. Each of these 3 conditions that produces elevated L-CPT1 in the heart and thus shifts the isoform distribution of CPT1 from muscle toward liver enzymes are now associated with reduced fatty acid oxidation and elevated carbohydrate metabolism. Although suggested as an adaptation to maintain LCFA oxidation, L-CPT1 never has been associated with increased LCFA oxidation in cardiomyocytes. Importantly, the current study reports on actual rates of palmitate oxidation observed from online 13C NMR observations of beating hearts that are responding to an acute induction of L-CPT1 expression in the intact functioning heart.

We previously have reported the first observation of increased L-CPT1 protein expression in hypertrophied hearts. We demonstrated the inefficiency in energy metabolism as a consequence of the shift away from the high yield of ATP provided by LCFA in the absence of limited oxygen delivery and tissue Po2, as has been shown to be the case in the remodeled and hypertrophied heart. Recent findings specific to maladaptive expression of malic enzyme-1 also indicate inefficient metabolism of carbohydrates in the hypertrophied heart that were not in evidence in the current study of nonpathological hearts with an isolated elevation of L-CPT1 expression. Thus, the current findings enable focus on the link between L-CPT1 expression in heart and LCFA oxidation, without the many overlaying complexities of the diseased myocardium. Interestingly, as discussed, L-CPT1 expression induced elevated ANP levels, a marker of the hypertrophic response, suggesting a direct link to the pathogenesis of cardiac hypertrophy.

Elevated expression of L-CPT1 in hypertrophied hearts coincided with a reduced rate of LCFA oxidation. Whereas the muscle isoform, M-CPT1, is predominant in heart, the liver isoform is upregulated in hypertrophy. Interestingly, the hearts studied during brief acute overexpression of L-CPT1 after cardiac-specific introduction of the exogenous gene also showed evidence of significantly increased glucose oxidation in replacement of lost LCFA oxidation.

Triacylglyceride content was not affected by the presence of additional L-CPT1, and with no evidence of increased LCFA contributions from stored triacylglycerol present, a general
Figure 8. Protein content of total acetyl CoA carboxylase 2 (T-ACC), phosphorylated (ser 218) liver carnitine palmitoyltransferase I (P-ACC), and malonyl CoA decarboxylase (MCD) in sham-operated hearts (SHM; gray bars) and hypertrophied hearts (HYP; 10-week aortic banding, black bars). All values shown as arbitrary units (AU) normalized to glyceraldehyde dehydrogenase protein. Note elevated levels of P-ACC in HYP. *P<0.05 vs SHM.

The findings also suggest the action of alternative regulatory mechanisms for the activation of L-CPT1 vs M-CPT1 in the cardiomyocyte beyond the inhibitory effects of malonyl CoA, which are becoming increasing apparent in metabolic studies of both cardiac muscle and other tissue types. Malonyl CoA content was similar between L-CPT1-expressing and PBS-infused hearts, despite clearly reduced LCFA oxidation in hearts overexpressing L-CPT1. Yet, in confirming the well-reported reduction in LCFA oxidation in hypertrophied hearts, the current findings also show malonyl CoA to be actually lower in the hypertrophied hearts with reduced LCFA oxidation, as is consistent with limited acetyl CoA production from LCFA oxidation. Nevertheless, malonyl CoA content was similar between L-CPT1 hearts and hypertrophied hearts. The similar malonyl CoA content among these groups also is consistent with similar enzyme protein levels of malonyl decarboxylase and ACC2 between L-CPT1 hearts and PBS controls, as well as between hypertrophied hearts and surgical shams. The only distinctions appear in the level of ACC2 phosphorylation at serine 218 (P-ACC2), where L-CPT1 hearts displayed lowered P-ACC2 content compared with PBS controls and hypertrophied hearts showed elevated P-ACC2 content.

Because phosphorylation inactivates ACC2 for production of malonyl CoA, the reduced P-ACC2 content in L-CPT1 hearts may be consistent with the observed reduction in palmitate oxidation but are not consistent with the unchanged malonyl CoA content compared with controls. Hypertrophied hearts displayed lower malonyl CoA levels compared with surgical sham hearts, yet P-ACC2 content was higher than in sham-operated hearts. If malonyl CoA is the inhibitory agent to affect CPT1, then irrespective of relative P-ACC2 content, the reduced palmitate oxidation in the L-CPT1 hearts and hypertrophied hearts is not related to ACC2 phosphorylation levels. Thus, although the P-ACC2 assay results remain the only data to suggest a differential downstream regulation of L-CPT1 activity between the L-CPT1 hearts and hypertrophied hearts, malonyl CoA content in L-CPT1 hearts similar to that of the PBS controls and hypertrophied hearts strongly suggests that other factors besides total tissue malonyl CoA content regulate the observed LCFA oxidation through CPT1. This leads to questions beyond the scope of this study regarding the role of ACC1 in the cytosol in contributing to the total malonyl CoA that is measured in such studies.

Recently published reports indicate no direct relationship between increased malonyl CoA content and reduced LCFA oxidation in the heart. Whereas these recent studies demonstrate the absence of a link between malonyl
CoA content and LCFA oxidation were performed on hearts with a predominantly normal distribution of M-CPT1 and L-CPT1 contents, the current findings suggest that L-CPT1 may be subject to additional levels of regulation that are yet to be fully identified. Kim et al reported on a malonyl CoA–resistant level of palmitate oxidation in red vs white skeletal muscle preparations. Therefore, in light of the present findings and newly emerging considerations, we cannot rule out other factors such as posttranslational modifications invoked in the intact functioning myocardium that limit LCFA oxidation through L-CPT1 beyond malonyl CoA, although studies indicate this isoform is less responsive to malonyl CoA than M-CPT1 in culture cell preparations.

Beyond the regulation of CPT1 activity as previously described, but not yet well-characterized in the intact myocardium, the induction of increased L-CPT1 isoform content in the intact heart produced an anticipated reduction in both the rate of palmitate oxidation and the contribution of palmitate to β-oxidation for the production of tricarboxylic acid cycle intermediates within the energy yielding oxidative pathways of the mitochondria. The shift in isoform distribution between the liver and muscle isoforms present in these hearts, after acute delivery of the exogenous gene for L-CPT1, was similar to the distribution previously reported in the hypertrophied heart. Whereas L-CPT1 expression had no measurable effect on M-CPT1 content, this isoform shift in otherwise normal rat hearts produced a reduction in palmitate oxidation that recapitulated the phenotype of reduced LCFA oxidation in the hypertrophied hearts. Increased incorporation of isotope from 13C-enriched glucose into the 4-carbon position of glutamate demonstrated elevated glucose oxidation in L-CPT1–expressing hearts, indicating a shift from LCFA oxidation to glucose oxidation. However, unlike hypertrophied hearts, L-CPT1 hearts did not display a change in the metabolic fate of glycolytic end products, with a shift toward LCFA oxidation to glucose oxidation. However, unlike hypertrophied hearts, L-CPT1 hearts did not display a change in the metabolic fate of glycolytic end products, with a shift toward the anaplerotic production of malate, which has been linked to elevated malic enzyme 1 content in cardiac hypertrophy.

Rather, acute overexpression of L-CPT1 induced a reduction in palmitate oxidation, with a consequential and compensatory shift toward increased glucose oxidation to maintain energy demands.

Nevertheless, the link between L-CPT1 expression and the induction of metabolic remodeling in hypertrophied hearts has been demonstrated here to be a key component of altered LCFA oxidation. Elevated L-CPT1 is associated with provocative elevation of ANP message, a marker of hypertrophic signal induction, in the otherwise nonpathogenic myocardium. The implications of this work are that previous notions of L-CPT1 regulation are incomplete when applied to the intact functioning heart. The findings suggest that a single key metabolic shift, such as altered CPT1 isoform expression and distribution, in the transition to decompression of the pressure-overloaded heart alone can induce the shift away from fatty acid oxidation. The consequences of an acute increase in L-CPT1 expression in the rat heart, as observed here, suggest intriguing links among enzyme activity, metabolic flux, and metabolite content, and the induction of signaling pathways. The latter finding of elevated ANP message in response to increased L-CPT1 content implies the need for closer investigation into influences of metabolic signaling on myocardial remodeling.

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**Disclosures**

G.D. Lopaschuk is a major shareholder of Metabolic Modulators Research Ltd. The other authors have no conflicts to report.

**References**


L-CPT1 Expression Affects Cardiac Fat Oxidation

Lewandowski et al


Novelty and Significance

What Is Known?

- Long chain fatty acids (LCFAs) are the preferred fuel for mitochondrial ATP synthesis in the heart, but hypertrophied and failing hearts, like fetal hearts, display a metabolic phenotype of reduced long chain fatty acid oxidation.

- The rate-limiting step of LCFA oxidation is determined by transport of LCFA into the mitochondria via carnitine palmitoyltransferase I (CPT1), which exists in 2 isoforms: the adult muscle CPT1 (M-CPT1) that predominates in cardiomyocytes and the liver CPT1 (L-CPT1), which is more highly expressed in the fetal heart.

- Hypertrophied and failing hearts express higher than normal levels of the liver isoform, as do fetal hearts in comparison with normal adult hearts.

What New Information Does This Article Contribute?

- Acute overexpression of L-CPT1 in otherwise normal rat hearts, via cardiac-specific delivery of an exogenous gene, decreased LCFA oxidation.

- LCFA oxidation was reduced in hearts expressing L-CPT1, suggesting other factors influence L-CPT1 activity in intact functioning hearts.

- Increased expression of L-CPT1 and lower rate of LCFA oxidation in normal hearts were associated with increases in a marker of cardiac hypertrophy, atrial natriuretic peptide (ANP).

This study challenges dogma regarding the role of CPT1 in cardiac hypertrophy. Our previous work indicates that the reversion to a fetal gene program in cardiac hypertrophy increased myocardial content of the fetal form of CPT1, the liver isoform, coexpressed with adult M-CPT1. Mimicking the hypertrophic isoform distribution of CPT1 in nonpathogenic hearts recapitulated the lower LCFA oxidation rates that occur with cardiac hypertrophy. Importantly, the induced metabolic change elevated ANP. These findings provide novel insights into the complex interrelationship of metabolic gene expression, regulation of LCFA oxidation, and the induction of the hypertrophic response.
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DETAILED MATERIALS AND METHODS

Adenoviral delivery in vivo.

Adult rats (male, Sprague Dawley, 400-450 gm) received an in vivo intracoronary perfusion of the heart with either adenovirus carrying cDNA for the rat liver isoform of the carnitine palmitoyltransferase I (Adv.cmv.L-CPT1) at 8x10^12 viral particles/ml PBS, adenovirus carrying scrambled cDNA (empty) at 8x10^12 vp/ml as a viral delivery control group, or PBS as a sham control. This open-chest cross-clamp technique, performed on anesthetized (i.p. pentobarbital, 50 mg/kg and 1% isoflurane inhalation) intubated rats, has been described in detail in our previous reports (1-3). The adenovirus expresses the liver isoform of the carnitine palmitoyltransferase I gene (10^12 viral particles/ml PBS) under a CMV promoter.

Use of the viral vector did not influence L-CPT1 expression in the absence of code for L-CPT1. L-CPT1 expression was similar in hearts that were untreated, PBS infused and supplied empty virus (See Online Figure I). As already referenced in the main text for hypertrophied hearts, for studies examining expression of ACC2, assayed relative to pyruvate carboxylase (PC), delivery and expression of exogenous L-CPT1 gene did not alter PC content (Online Figure II).

All vessels to/from the heart were cross-clamped simultaneously, and the heart was retrograde perfused in vivo for 7 min with a calcium free Tyrode solution through catheters position in the aortic root (delivery) and right ventricle (efflux). At the time of adenovirus injection, 0.4 ml of AdV.cmv.L-CPT1 was first delivered through the catheter position in the aortic root. This allowed the adenovirus to circulate down the coronary. Next, the efflux catheter positioned in the right ventricle was removed and an additional 0.5 ml/kg of adenovirus (approximately 0.2 ml) was delivered to the aortic root at 300 ± 100 mmHg peak pressure. After 90 seconds, catheters were repositioned in the right and left ventricle, and unsequestered virus was flushed from the heart with Krebs buffer containing calcium (1.5 mM). The heart rate recovered, the cross-clamp was removed, the chest was closed, and the rats recovered.

The gene delivery technique, referenced above, was reported and confirmed earlier by following the delivery and expression of Ad.cmv.LacZ in whole intact non-failing rat heart (1-3).

Pressure overload hypertrophy.

Cardiac hypertrophy from left-ventricular pressure-overload (LVH) was induced by constricting the transverse aorta (hemoclip) of three-week-old male Sprague Dawley rats, as previously described (2,4-6). This banding procedure relies on the natural growth of the animal to produce a gradually increasing degree of aortic constriction. The rats develop a concentric hypertrophy and increased heart-to-body weight ratio, which is associated, in the short term, with improvement in the systolic function of the heart (2,7-9). At 10-12 weeks post-banding, the animals enter a decompensated stage with depressed LVDP and dP/dt. In this model of left ventricular hypertrophy (LVH), no systemic activation of the sympathetic nervous system or of the renin-angiotensin-aldosterone system occurs (7). Consequently, there are no signs of cardiac lesions, peripheral arteritis, myocardial necrosis, or extensive fibrosis. The rats progress to a dilated cardiac hypertrophy with acute end-stage heart failure at 4-6 months post banding. The sham groups (SHM) underwent similar surgery without placement of the aortic-band.
**NMR spectroscopy and tissue chemistry.**

NMR measurements were performed on intact beating hearts that were situated within a 10 mm broad band NMR probe inside a 9.4 T NMR magnet interfaced to a Bruker 400 MHz AVANCE console. Sequential, proton-decoupled $^{13}$C NMR spectra were acquired (2 min each) with natural $^{13}$C abundance correction using previously reported NMR methods (2,10,11). Magnetic field homogeneity was optimized by shimming to a proton line width of 10-20 Hz. Carbon spectra were acquired at 101 MHz with bilevel broad-band decoupling and subtracted from naturally abundant endogenous $^{13}$C signal. $^{31}$P spectra were acquired at 161 MHz over 2 minute intervals at the start of each protocol, prior to acquisition of $^{13}$C enriched $^{13}$C NMR spectra.

Tissue metabolites were extracted from frozen heart tissue using 7% perchloric acid and neutralized with KOH. Tissue extracts were analyzed spectrophotometrically and fluorometrically for quantification (4,11,12). Glutamate concentration was determined with glutamate dehydrogenase and diaphorase (Roche L-Glutamic acid colorimetric kit.) α-Ketoglutarate content was measured by coupling glutamate-oxaloacetate transaminase (GOT, Roche) with malate dehydrogenase (MDH, Roche) in the presence of excess L-aspartate. Aspartate concentration was measured by coupling GOT with MDH similar to α-ketoglutarate with the exception of excess α-ketoglutarate. Citrate content was determined with citrate lyase (Roche) and MDH. *In vitro* high-resolution $^{13}$C NMR spectra of tissue extracts reconstituted I 0.5 mL of D$_2$O were collected with a 5 mm $^{13}$C probe (Bruker Instruments, Billerica, MA). Analysis was performed to determine fractional enrichment of [2-$^{13}$C] acetyl CoA (11,13,14). Isotopic enrichment of glutamate from [1,6-$^{13}$C$_2$] glucose was determined via $^{13}$C NMR of *in vitro* tissue extracts and the relative contributions of exogenous, $^{13}$C enriched glucose and the unlabeled ($^{12}$C) endogenous pool of glycogen determined via $^1$H NMR of alanine enrichment as previously described in detail (6).

**$^{13}$C enrichment kinetics and metabolic flux.**

A set of nine differential equations describes the concentration history of the $^{13}$C in each metabolite and developed in our laboratory was modified to include the additional, rate-determining components of long chain fatty acid uptake into the mitochondria. With a single 9x1 vector $q$ to represent the fractional enrichment of each compartment as a function of time, the model is described in matrix form as

$$\frac{d}{dt}q = M_{TCA} \cdot q + U_{Acetyl-CoA}$$

where $M_{TCA}$ is a 9x9 matrix characteristic of the TCA cycle, its elements are determined by the TCA cycle flux ($V_{TCA}$), the interconversion rates between the TCA cycle intermediate and glutamate or aspartate ($F_1$ and $F_2$), the level of anaplerosis ($y$), and the concentrations of each metabolite. The input vector, $U_{Acetyl-CoA}$, is governed by the fraction of $^{13}$C enriched acetyl-CoA entering the TCA cycle through citrate synthase ($F_C$). The only non-zero element in $U_{Acetyl-CoA}$ corresponds to the labeling of the 4-carbon position of citrate since [2-$^{13}$C] acetyl-CoA enters the TCA cycle through citrate synthase to enrich the 4-carbon position of citrate (3, 14-15). The nine differential equation in series are:

$$\frac{d}{dt}CIT4 = \frac{V_{TCA}}{[CIT]} \cdot (F_C - CIT4)$$
\[
\frac{d}{dt} \frac{\alpha KG}{\alpha KG} = \frac{V_{TCA}}{[\alpha KG]} \cdot CIT4 - \frac{V_{TCA}}{[\alpha KG]} \cdot \frac{F_1}{[\alpha KG]} \cdot \frac{\alpha KG}{\alpha KG} + \frac{F_1}{[\alpha KG]} \cdot \frac{GLU}{GLU} \\
\frac{d}{dt} \frac{GLU}{GLU} = \frac{F_1}{[GLU]} \cdot (\alpha KG - GLU) \\
\frac{d}{dt} \frac{CIT}{CIT} = \frac{V_{TCA}}{CIT} \cdot (OAA2 - CIT2) \\
\frac{d}{dt} \frac{\alpha KG}{\alpha KG} = \frac{V_{TCA}}{[\alpha KG]} \cdot CIT2 - \frac{V_{TCA}}{[\alpha KG]} \cdot \frac{F_1}{[\alpha KG]} \cdot \frac{\alpha KG}{\alpha KG} + \frac{F_1}{[\alpha KG]} \cdot \frac{GLU}{GLU} \\
\frac{d}{dt} \frac{GLU}{GLU} = \frac{F_1}{[GLU]} \cdot (\alpha KG - GLU) \\
\frac{d}{dt} \frac{MAL}{MAL} = \frac{V_{TCA}}{MAL} \cdot \left(\frac{1}{2} \cdot \alpha KG + \frac{1}{2} \cdot \alpha KG4 - (1 + y) \cdot MAL2\right) \\
\frac{d}{dt} \frac{OAA}{OAA} = \frac{V_{TCA}}{OAA} \cdot MAL2 - \frac{V_{TCA}}{OAA} \cdot \frac{F_2}{[OAA]} \cdot OAA2 + \frac{F_2}{[OAA]} \cdot ASP2 \\
\frac{d}{dt} \frac{ASP}{ASP} = \frac{F_2}{[ASP]} \cdot (OAA2 - ASP2)
\]

Where CIT, \(\alpha KG\), GLU, MAL, OAA, and ASP denote the metabolites citrate, \(\alpha\)-ketoglutarate, glutamate, malate, oxaloacetate, and aspartate, respectively, with the corresponding number of the \(\text{^{13}}\)C enriched carbon position indicated. Where CIT4 is the fractional enrichment level of \(\text{^{13}}\)C at the 4-carbon position of citrate; (i.e., CIT4=[(4-\(\text{^{13}}\)C)CIT]/[CIT]). The equation describing malate enrichment includes anaplerotic and cataplerotic effects (4, 11, 14-15). \(F_1\) and \(F_2\) are fluxes for interconversion via both transamination and membrane transport, between \(\alpha\)-ketoglutarate and glutamate, and between aspartate and oxaloacetate, respectively. Under the current experimental conditions of limited aspartate and alanine, \(F_1 = F_2\) (3,11,14-15).

The rate of palmitate oxidation (R) was calculated, under these precise experimental and isotopic enrichment conditions, as the product of \(V_{TCA}\) and acetyl CoA enrichment from \(\text{^{13}}\)C palmitate \((F_c)\) divided by 8 to account for the 8 acetyl groups produced from the 16 carbon palmitate \((V_{TCA} \times F_c/8)\).
References

Online Figure I. L-CPT1 expression, relative to Na⁺ K⁺-ATPase, was not altered by viral vector. L-CPT1 content was similar with PBS infused hearts, hearts receiving empty virus, and untreated hearts.
Online Figure II. Pyruvate carboxylase content in hearts was not affected by delivery and expression of the exogenous L-CPT1 gene compared to PBS infused control hearts. These data further validate the use of PC as a reference for ACC2 and MCD content data shown in Figure 7.