Carnitine Palmitoyltransferase-1b Deficiency Aggravates Pressure Overload–Induced Cardiac Hypertrophy Caused by Lipotoxicity

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Background—Carnitine palmitoyltransferase-1 (CPT1) is a rate-limiting step of mitochondrial β-oxidation by controlling the mitochondrial uptake of long-chain acyl-CoAs. The muscle isoform, CPT1b, is the predominant isoform expressed in the heart. It has been suggested that inhibiting CPT1 activity by specific CPT1 inhibitors exerts protective effects against cardiac hypertrophy and heart failure. However, clinical and animal studies have shown mixed results, thereby creating concerns about the safety of this class of drugs. Preclinical studies using genetically modified animal models should provide a better understanding of targeting CPT1 to evaluate it as a safe and effective therapeutic approach.

Methods and Results—Heterozygous CPT1b knockout (CPT1b+/−) mice were subjected to transverse aorta constriction–induced pressure overload. These mice showed overtly normal cardiac structure/function under the basal condition. Under a severe pressure-overload condition induced by 2 weeks of transverse aorta constriction, CPT1b+/− mice were susceptible to premature death with congestive heart failure. Under a milder pressure-overload condition, CPT1b+/− mice exhibited exacerbated cardiac hypertrophy and remodeling compared with wild-type littermates. There were more pronounced impairments of cardiac contraction with greater eccentric cardiac hypertrophy in CPT1b+/− mice than in control mice. Moreover, the CPT1b+/− heart exhibited exacerbated mitochondrial abnormalities and myocardial lipid accumulation with elevated triglycerides and ceramide content, leading to greater cardiomyocyte apoptosis.

Conclusions—CPT1b deficiency can cause lipotoxicity in the heart under pathological stress, leading to exacerbation of cardiac pathology. Therefore, caution should be exercised in the clinical use of CPT1 inhibitors. (Circulation. 2012;126:1705-1716.)

Key Words: carnitine palmitoyltransferase-1b • heart failure • hypertrophy • lipotoxicity

The heart is an energy-demanding organ relying on fatty acid and glucose oxidation. Long-chain fatty acids contribute up to 70% of the energy required by an adult heart to function under normal physiological conditions (see elsewhere for review). The remaining energy needs are derived from attenuated FAO in the heart or from nonspecific confounding effects of a particular compound.

Clinical Perspective on p 1716

Carnitine palmitoyltransferase-1 (CPT1) is located within the mitochondrial outer membrane as a rate-limiting enzyme of mitochondrial β-oxidation by controlling mitochondrial entry of long-chain fatty acids. CPT1b is one of the 3 CPT1 isoforms (CPT1a, CPT1b, and CPT1c). It is expressed mainly in skeletal muscle, heart, brown adipose tissue, and testis. In adult cardiomyocytes, CPT1b is the predominant isoform and contributes ~98% of total cardiac CPT1 activity. CPT1 is a major target for metabolic therapies aiming to improve cardiac performance in patients with cardiac hypertrophy and heart failure by suppressing FAO. Small-scale
clinical trials reported beneficial effects of CPT1 inhibitors in treating heart failure patients. Specific CPT1 inhibitors such as etomoxir have been actively pursued as a therapeutic agent. On the other hand, animal studies showed mixed results, from beneficial to unchanged to harmful effects on pressure overload–induced cardiac dysfunction, hypertrophy, and progression to end-stage failure. A major hurdle is to differentiate the specific CPT1 inhibition effects from nonspecific off-target effects. Studies on animal models with genetic manipulation should help identify the CPT1 effect without potential confounding effects from a compound. The homozygous CPT1b knockout mice exhibit embryonic lethality, whereas the heterozygous CPT1b−/− mice show no apparent phenotypic change. The present study was designed to determine whether partial CPT1b deficiency in the heterozygous CPT1b knockout mice is beneficial or detrimental to cardiac structure/function under physiological and pathological conditions.

**Methods**

An expanded Methods section is available in the online-only Data Supplement.

**Experiment Animals**

Heterozygous CPT1b+/− knockout mice were generated as described previously. Wild-type (WT) littermates with CPT1b+/- genotyping were used as controls. Both male and female mice were used. Mice were kept on a 12-hour/12-hour light/dark cycle in temperature-controlled rooms and had ad libitum access to water and standard rodent diet. All experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

**CPT1 Activity Assay**

A modified mitochondrial CPT1 assay was used by measuring the rate of formation of palmitoylcarnitine from palmitoyl-CoA plus carnitine according to the method described in detail in the Methods section in the online-only Data Supplement.

**Echocardiography Measurement**

Echocardiographic measurement was performed with the high-resolution echocardiography analysis system for small animals (Vevo 770 imaging system, Visual Sonics). Mice were anesthetized by inhalation with isoflurane and O2. A 2-dimensional short-axis view and M-mode tracings of the left ventricle (LV) were obtained with a 30-MHz transducer.

**Isolated Working Mouse Heart Perusions**

Myocardial contractile function and metabolism under basal physiological conditions were determined ex vivo through isolated working heart perusions as previously described. All hearts were perfused in the working mode in a nonrecirculating manner with a preload of 8.0 mm Hg and an afterload of 55 mm Hg. Radiolabeled tracers were used to monitor glucose oxidation and palmitate oxidation, as specified in individual experiments described previously. For hypertrophied hearts subjected to pressure overload induced by transverse aorta constriction (TAC), the isolated mitochondria were used to assay FAO rate, and heart homogenates were used to assay glucose oxidation rate. Details on the TAC procedure and palmitate and glucose oxidation assays are given in the online-only Data Supplement.

**Heart Ceramide Assay**

Ceramide species were quantified by electrospray ionization–mass spectrometry/mass spectrometry (ESI-MS/MS, Applied Biosystems/MDS SCIEX, Canada) as described in the online-only Data Supplement.

**Mitochondrial DNA Copy Number Analysis**

Total genomic DNA was isolated from Lvs, processed by standard procedures with a DNA extraction kit (Wizard), and subjected to real-time quantitative polymerase chain reaction (PCR) analysis. Cytochrome b (Cyto b) was used as a mitochondrial DNA (mtDNA) marker, and the regulator of calcineurin 1 (Rcan1) was used as a nuclear DNA marker. The relative copy number of mtDNA was assessed by the ampliton within a mitochondrial gene (Cyto b) to that of a nuclear gene (Rcan 1).

**Quantitative Real-Time Reverse Transcriptase–PCR Analyses**

Quantitative real-time reverse transcriptase–PCR analyses were carried out with the Step 1 real-time PCR system (Applied Biosystems). Results from each gene/primer pair were normalized to β-actin and compared across conditions. The sequences of the primers are listed in Table I in the online-only Data Supplement.

**Western Blot Analyses**

Western blots were conducted with commercially available antibodies. The immunoblotting images were captured with KODAK image Station 4000R (Carestream Health Inc) by developing the membranes in Supersignal West substrates (Thermo Scientific) and analyzed with KODAK IM software (version 4.5.1).

**Statistical Analyses**

Data for 2-group comparisons were analyzed with the nonparametric Student t test; otherwise, data were analyzed by 1-factor or mixed, 2-factor ANOVA using GraphPad Prism software (GraphPad Software Inc). Survival data were analyzed with the Kaplan-Meier method using GraphPad Prism software. Values of quantitative results were expressed as mean±SEM. Differences between groups and treatments were regarded as significant at P<0.05.

**Results**

**Mice With Heterozygous CPT1b Knockout (CPT1b+/−) Exhibit No Cardiac Phenotype Under the Basal Condition**

Although the homozygous CPT1b−/− mice were embryonically lethal, the heterozygous CPT1b+/− mice were overtly normal with the same lifespan as their WT littermates. Heart weight, body weight, and ratio of heart to body weight were not different between the CPT1b+/− mice and their WT littermates (Figure I in the online-only Data Supplement). CPT1b+/− mice did not exhibit detectable changes in cardiac histology and function (Figure II in the online-only Data Supplement). Real-time PCR revealed that CPT1b transcript expression was attenuated by 50% in CPT1b+/− relative to WT hearts (Figure 1A). Western blots revealed that CPT1b protein content in the heart was correspondingly decreased by 50% in CPT1b+/− relative to WT hearts (Figure 1B). CPT1a, a dominant CPT1 isoform in neonatal hearts, could have been upregulated in response to CPT1b deficiency in the heart. However, quantitative PCR illustrated no change in
CPT1 expression in the CPT1b-deficient heart (Figure 1C). Total CPT1 activity in CPT1b−/− hearts was substantially decreased to 73% of that in WT hearts (Figure 1D). Despite the significant CPT1b deficiency in the heart, echocardiographic assessment revealed normal anatomic structure of the heart with comparable cardiac performance in the CPT1b−/− and WT hearts (Figure III and Table II in the online-only Data Supplement). Isolated working heart measurements of ex vivo cardiac function revealed no difference between the CPT1b mice and their WT littermates (Table III in the online-only Data Supplement). Both cardiac contractility (dP/dtmax) and relaxation (dP/dtmin) in CPT1b−/− hearts were similar to those in WT controls. Interestingly, the rates of FAO and glucose oxidation were not changed in CPT1b−/− hearts. Therefore, these results indicate that a modest CPT1b deficiency in the heart is not sufficient to cause cardiac dysfunction under a basal physiological condition.

CPT1 Deficiency Aggravates Cardiac Hypertrophy and Dysfunction Induced by Pressure Overload

The absence of phenotypic changes in the CPT1b−/− heart provides an ideal animal model to determine whether a partially reduced CPT1 activity can prevent cardiac pathological development under pathological stress conditions. Adult mice were subjected to TAC-induced LV pressure overload. The CPT1b−/− mice were dramatically more susceptible to a severe pressure-overload condition induced by 3 weeks of TAC than their WT littermates. The majority of the CPT1b−/− mice died before the 2-week term of pressure overload with signs of heart failure (dilated heart, effluence, shortness of breath, etc), whereas WT littermates survived (Figure 2A). Under a milder pressure-overload condition, with a similar level of pressure gradient (the Table), the CPT1b−/− mice showed more pronounced cardiac hypertrophy than their WT littermates. Echocardiographic assessment showed that left posterior wall thickness at diastole, LV dimension volume at systole, and LV mass were further increased in CPT1b−/− mice. Stroke volume, cardiac output, ejection fraction, and fractional shortening were further decreased in CPT1b−/− mice (the Table and Figure 2B–2E). Ratios of heart weight to body weight and heart weight to tibia length were increased in CPT1b−/− mice compared with WT hearts (Figure 3A and 3B). Furthermore, quantitative PCR revealed that cardiac expression of molecular markers of cardiac hypertrophy such as natriuretic peptide precursor A and B (Nppa and Nppb) and myosin heavy chain-2B was increased more in CPT1b−/− mice than in their WT littermates (Figure 3C). In addition, hematoxylin and eosin and trichrome blue staining on heart sections demonstrated increased cross-sectional area of cardiomyocytes and more pronounced fibrosis in CPT1b−/− mice than in their WT littermates (Figure 3C). Therefore, these results demonstrate that CPT1b deficiency is detrimental in hearts under mechanical stress–induced cardiac hypertrophy and heart failure.

Figure 1. Carnitine palmitoyltransferase-1b (CPT1b) mRNA level and activity in CPT1b−/− mice. Mice were euthanized at 12 to 14 weeks of age. RNA samples were extracted from ventricular tissues. A and C, Transcript levels of CPT1b and CPT1a were determined by quantitative polymerase chain reaction; results from each gene/primer pair were normalized to β-actin (n=4). B, Protein expression was determined by Western blot (n=4). D, CPT1 activity was measured using isolated mitochondria according to the method described in the online-only Data Supplement (n=4). E and F, Palmitate oxidation rate and glucose oxidation rate were measured in isolated working heart (n=6). *P<0.05 vs wild-type (WT).
CPT1b Deficiency in Hearts Under Pressure Overload Leads to Increased Cardiomyocyte Apoptosis

Apoptosis has been shown to be one of the major pathological events involved in the development of cardiac hypertrophy and heart failure induced by pressure overload.26–28 We investigated whether more pronounced cardiomyocyte apoptosis in the CPT1b-deficient heart is attributed to the exacerbated pathological development.

Table. Echocardiography Measurement in Mice 2 Weeks After Transverse Aorta Constriction

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT (Sham n=9)</th>
<th>TAC (n=11)</th>
<th>CPT1b+/− (Sham n=9)</th>
<th>TAC (n=11)</th>
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<tr>
<td>Pressure gradient, mm Hg</td>
<td>NA</td>
<td>54.91±6.28</td>
<td>NA</td>
<td>52.02±7.94</td>
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<td>IVSd, mm</td>
<td>0.81±0.17</td>
<td>1.21±0.13*</td>
<td>0.75±0.08</td>
<td>1.24±0.16*</td>
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<tr>
<td>IVSs, mm</td>
<td>1.13±0.08</td>
<td>1.39±0.23*</td>
<td>1.33±0.05</td>
<td>1.42±0.17*</td>
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<td>LVIDd, mm</td>
<td>3.79±0.36</td>
<td>4.02±0.24</td>
<td>3.82±0.35</td>
<td>3.90±0.26</td>
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<tr>
<td>LVIDs, mm</td>
<td>2.71±0.40</td>
<td>3.04±0.28*</td>
<td>2.65±0.30</td>
<td>3.23±0.27*</td>
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<tr>
<td>LVPWd, mm</td>
<td>0.72±0.18</td>
<td>1.09±0.19*</td>
<td>0.75±0.16</td>
<td>1.31±0.26†</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>1.03±0.19</td>
<td>1.41±0.11*</td>
<td>1.04±0.15</td>
<td>1.52±0.29†</td>
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<tr>
<td>LVIDd VOL, µL</td>
<td>65.27±12.03</td>
<td>76.15±12.02*</td>
<td>65.28±11.06</td>
<td>70.89±10.92</td>
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<tr>
<td>LVIDs VOL, µL</td>
<td>25.18±7.26</td>
<td>33.40±5.88*</td>
<td>23.14±6.56</td>
<td>41.61±9.06†</td>
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<tr>
<td>Stroke volume, µL</td>
<td>40.11±7.24</td>
<td>41.88±8.54</td>
<td>42.14±5.95</td>
<td>29.28±7.70†</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>17.64±2.87</td>
<td>17.40±2.97</td>
<td>18.62±2.95</td>
<td>13.65±4.94†</td>
</tr>
</tbody>
</table>

WT indicates wild type; IVSd, diastolic interventricular septal wall thickness; IVSs, systolic interventricular septal wall thickness; LVIDd, diastolic left ventricular dimension; LVIDs, systolic left ventricular dimension; LVPWd, diastolic left ventricular posterior wall thickness; LVPWs, systolic left ventricular posterior wall thickness; LVIDd VOL, diastolic left ventricular dimension estimated left ventricular volume; LVIDs VOL, systolic left ventricular dimension estimated left ventricular volume.

*P<0.05 vs sham.
†P<0.05 vs WT TAC.
(TUNEL) assay on frozen heart sections revealed an increased number of TUNEL-positive cardiac myocytes on sections from CPT1b<sup>−/−</sup> hearts (Figure 5A and 5B). Western blot analysis on fractionated protein samples revealed that cytochrome c in the cytosol protein samples of CPT1b<sup>−/−</sup> hearts after TAC was markedly increased compared with that of WT controls (Figure 5C). Therefore, these results indicate that apoptosis is more pronounced in CPT1b<sup>−/−</sup> relative to WT hearts under the TAC condition.

**Pressure Overload in CPT1b<sup>−/−</sup> Hearts Leads to Myocardial Lipid Accumulation**

Because CPT1b is a key enzyme for the entry of long-chain fatty acids into the mitochondria, we investigated whether CPT1b deficiency in the heart leads to myocardial triglyceride accumulation. Although no change could be detected under basal conditions, myocardial triglyceride concentration was markedly increased in CPT1b<sup>−/−</sup> relative to WT hearts subjected to TAC (Figure 6A). Oil Red O staining on frozen heart sections demonstrated numerous lipid droplets on CPT1b<sup>−/−</sup> but not on WT heart sections (Figure 6B). We further investigated whether myocardial ceramide content is altered with the myocardial accumulation of triglycerides in the CPT1b heart. Mass spectroscopic assessment of the total ceramide and ceramide species composition revealed that total ceramide content in CPT1b<sup>−/−</sup> hearts was substantially increased compared with WT controls (Figure 6C). C16, C18, and C24 were the ceramide species markedly increased in CPT1b-deficient hearts (Figure 6D).

**Expression of Proteins Involved in Fatty Acid Uptake Is Further Downregulated in CPT1b<sup>−/−</sup> Hearts Under Pressure Overload**

To assess whether TAC-induced stress triggers the release of free fatty acids from adipose tissues, we measured the serum content of free fatty acids. As expected, serum free fatty acids were modestly increased in both WT and CPT1b<sup>−/−</sup> mice subjected to TAC at various time points (Figure IV in the online-only Data Supplement). The increased serum free fatty acids under TAC condition may contribute to myocardial lipid accumulation in CPT1b-deficient hearts by enhancing fatty acid uptake through peroxisomal proliferator-activated receptor (PPAR) α and PPARγ activation. The expression of PPAR target genes involved in long-chain fatty acid uptake or transport was measured. We found that CD36, the heart-type fatty acid–binding protein, and fatty acid transport protein-1 were unchanged under basal conditions but decreased in CPT1b-deficient heart subjected to pressure overload (Figure 7A and 7B). Therefore, it is not likely that myocardial lipid accumulation in CPT1b<sup>−/−</sup> hearts is a result of an upregulation of long-chain fatty acid uptake.

**Mitochondrial Biogenesis Is Decreased in CPT1b<sup>−/−</sup> Hearts Under Pressure Overload**

It has been reported that myocardial lipid accumulation may be associated with impaired mitochondrial biogenesis. Quantitative PCR revealed that the transcript levels of representative mitochondrial proteins such as mitochondrial c oxidase subunit II and III, and dynamin-related protein-1 were substantially attenuated (Figure 7C). Moreover, transcription factors that are essential for mitochondrial biogenesis such as transcription factor A, mitochondrial coactivator 1α (PGC-1α), nuclear respiratory factors 1 and 2, and peroxisome proliferator-activated receptor-γ coactivator 1α/β (PGC-1α and PGC-1β) transcripts were all decreased in CPT1b<sup>−/−</sup> hearts (Figure 7C). Protein contents of PGC-1α and TFAM were similarly downregulated in CPT1b<sup>−/−</sup> relative to WT hearts (Figure 7D). Mitochondria DNA copy number was also reduced in CPT1b<sup>−/−</sup> hearts (Figure 7E). Thus, CPT1b deficiency and subsequent lipid accumulation are associated with a reduction in mitochondrial biogenesis in the heart under pressure-overload conditions.
Figure 4. Cardiac histology and ultrastructure in CPT1b−/− mice with pressure overload. Mice were subjected to transverse aorta constriction (TAC) procedures at 10 to 12 weeks of age and euthanized 2 weeks after TAC. A, Representative histological images (×400) with hematoxylin and eosin staining on a heart section. B, Relative cross-sectional areas. The mean cardiomyocyte cross-sectional area in sham wild-type (WT) mice was set as 1. *P<0.05 vs sham; #P<0.05 vs WT TAC (n=6). C, Representative images of heart sections stained with Trichrome blue. D, Relative fibrosis areas. The whole section area was set as 100%. E, Representative images of left ventricular transmission electron microscope (TEM) assessment (×1100). F, Quantification results of mitochondrial volume (%) of heart sections from transmission electron microscope images. *P<0.05 vs sham; #P<0.05 vs WT TAC (n=8).
Fatty Acid Oxidation Is Impaired in CPT1b−/− Hearts Under Pressure Overload

To investigate how CPT1b deficiency affects myocardial substrate use under the TAC-induced pressure-overload condition, we assessed the rates of FAO and glucose oxidation in cardiac samples 3 days, 1 week, and 2 weeks after TAC. Interestingly, mitochondrial FAO was increased in WT but not in CPT1b−/− hearts at all 3 time points after TAC (Figure 8A). On the other hand, the glucose oxidation rate in cardiac homogenates was not changed at the earlier time points but was increased in CPT1b−/− compared with WT hearts at 2 weeks after TAC (Figure 8B). CPT1 activity assay showed that mitochondrial CPT1 activity was modestly but significantly increased at all 3 time points in WT hearts with TAC. However, CPT1 activity was increased at 3 days but was decreased at 1 and 2 weeks after TAC in CPT1b−/− hearts compared with sham hearts for the same time points (Figure 8C). Similarly, CPT1b expression remained 50% lower in both transcript and protein levels in CPT1b−/− hearts than in WT hearts (Figure 8E and 8F).

Discussion

We investigated the effects of CPT1b deficiency on the pathological development of cardiac hypertrophy and remodeling under the LV pressure-overload condition in mice. We previously demonstrated cardiac hypertrophy in mice with other FAO enzyme deficiencies, which is most predominant in homozygous long-chain acyl-CoA dehydrogenase deficiency. The most important finding based on the genetic mouse model of CPT1b deficiency is that CPT1b deficiency causes lipotoxicity in the heart under the pressure-overload condition and leads to exacerbated cardiac pathology.

CPT1b, the predominant CPT1 isoform expressed in the heart, plays an essential role in myocardial FAO. Repressing myocardial FAO has been proposed as a therapeutic target to improve cardiac efficiency in the failing heart. Inhibitors of CPT1 have already been developed and tested in preclinical animal studies and small clinical trials. However, they remain controversial because of mixed results from animal studies. Schwarzer et al showed that etomoxir failed to reverse pressure overload–induced heart failure in vivo. Strikingly, the most studied compound, etomoxir, has been shown to exert adverse effects that eventually lead to cardiac hypertrophy. It was proposed that etomoxir treatment may induce cardiac hypertrophy via increased cellular oxidative stress and nuclear factor-κB activation. Wolkowicz et al showed that another CPT1 inhibitor, 2-tetradecylglycidic acid, induces myocardial hypertrophy via the AT1 receptor. It is clear that the potential adverse effects of CPT1 inhibitors may not be just a nonspecific side effect. They may be associated with the irreversible inhibition of CPT1 activity in the heart. The present study provides evidence to support that partial CPT1b deficiency in a mouse model of CPT1 knockout is detrimental to cardiac structure/function owing to pressure overload–induced LV systolic dysfunction. CPT1b−/− mice showed a more pronounced systolic dysfunction but remained in concentric hypertrophy. It is likely that most CPT1b−/− hearts were still at the stage before the transition to dilated cardiomyopathy. Therefore, we could not rule out the possibility that CPT1 inhibition might exert beneficial effects only in dilated cardiomyopathy. Additionally, it is possible that inhibitors and the gene knockout will exhibit distinct functions when the corresponding proteins have nonenzymatic functions as a scaffold.
To the best of our knowledge, the present study is the first study based on a gene-targeted mouse model with CPT1b deficiency. Although a complete knockout of CPT1b causes embryonic lethality, the heterozygous CPT1b knockout mice are overtly normal. It is noted that in the heterozygous CPT1b knockout heart, CPT1b expression was blunted in both transcript and protein levels. Because CPT1a is also expressed in the adult heart at a low level, a compensatory upregulation of CPT1a is possible. In fact, the inverse response of increased CPT1b expression in livers of diet-challenged CPT1a/H11001/H11002 mice was reported. However, transcript expression of CPT1a was unchanged in the CPT1b-deficient hearts. Because the total CPT1 activity was decreased by ~30% compared with WT littermates and CPT1 activity is upregulated during at least the early stage of cardiac hypertrophy, the depression of CPT1 activity in the CPT1b-deficient heart appears to be the key determinant for the detrimental response to pressure overload–induced cardiac pathology. The partial CPT1b deficiency in the heart seems insufficient to affect basal cardiac performance and cardiac metabolism. This result does not support previous observations that partial inhibition of CPT1 activity by CPT1 inhibitors such as etomoxir leads to cardiac dysfunction and hypertrophy under physiological conditions. Therefore, it is likely that the detrimental cardiac effects of etomoxir treatment under normal physiological conditions may be associated with effects unrelated to CPT1 inhibition or more severe cardiac CPT1 inhibition (30% in CPT1b/H11001/H11002 versus 40%–50% with etomoxir treatment).

The most important finding in the present study is that the CPT1b/H11001/H11002 mice were much more susceptible to pressure overload–induced pathological cardiac hypertrophy, suggesting that partial CPT1b deficiency is detrimental with pathological development under mechanical stress conditions. This result is in sharp contrast to those using CPT1 inhibitors in human studies. The reasons for this obvious discrepancy may be derived from different degrees of CPT1 inhibition among studies, nonspecific effects of CPT1 inhibitors, or the existence of certain levels of CPT1a activity in CPT1b knockout hearts. Importantly, the present finding on the detrimental effects of CPT1b deficiency is against the basic concept of FAO inhibition as a therapeutic approach in treating heart failure patients. A specific site and specific quantity of the inhibition could be crucial. Moreover, our results demonstrated that mitochondrial FAO and CPT1b activity were upregulated in mitochondria samples from
hearts with pressure overload–induced hypertrophy. It is likely that increased CPT1 activity is crucial to maintain mitochondrial FAO among the remaining mitochondria during the development of pathological cardiac hypertrophy. However, the suppressed mitochondrial biogenesis and function in the heart may potentially impair overall myocardial FAO.

Lipotoxicity in addition to the pressure overload–associated systolic dysfunction appears to be the cause of the detrimental effect of CPT1b deficiency under pathological conditions. Patients with inborn errors of FAO typically manifest cardiomyopathy with diminished systolic function.37 Moreover, intramyocardial lipid accumulation is associated with contractile dysfunction in heart tissues from patients with nonischemic heart failure.38 The reduction of mitochondrial biogenesis should be the consequence of the progression of cardiac pathology and cardiomyocyte apoptosis. The increased sympathetic activity in response to hemodynamic overload might lead to increased lipolysis. Although this appears to be the case, it is insufficient to activate peroxisome proliferator-activated receptor–coactivator 1α (PGC1α) and transcription factor A, mitochondrial (TFAM) signaling to increase the expression of fatty acid uptake proteins. Instead, the expression of fatty acid uptake proteins was further decreased, possibly because of the exacerbated cardiac pathological development.

Figure 7. The expression of fatty acid uptake proteins and mitochondrial biogenesis in mice subjected to pressure overload. Mice were subjected to transverse aorta constriction (TAC) procedures at 10 to 12 weeks of age and euthanized 2 weeks after TAC. A, Quantitative polymerase chain reaction (qPCR) assessment of CD36, FABP, and fatty acid transport protein (FATP) transcripts (n=4). B, Western blot images and quantification of CD36 and FATP (n=4). C, qPCR assessment of mitochondrial biogenesis genes transcripts (n=4). D, Western blot images and quantification of peroxisomal proliferator-activated receptor–coactivator 1α (PGC1α) and transcription factor A, mitochondrial (TFAM; n=4). E, Mitochondrial DNA copy number was assessed by the ratio of mitochondrial gene (Cyto b) to nuclear gene (Rcan-1) as described in Methods in the online-only Data Supplement. *P<0.05 vs sham; #P<0.05 vs wild-type (WT) TAC (n=6).
Therefore, it is plausible that the increased myocardial triglyceride content in CPT1b−/− hearts is due to the relative reduction of mitochondrial FAO and mitochondrial biogenesis. As a result, myocardial lipid accumulates and feeds the ceramide synthesis. Our observation is in agreement with the previous report that oxfenicine induced myocardial lipid accumulation in rats.39,40 Myocardial lipid accumulation and the elevation of ceramide content in the heart should be the mechanisms underlying the detrimental effects during the development of pathological cardiac hypertrophy in response to the pressure-overload condition. The cytotoxic effect of ceramide in cardiomyocytes has been well established.41,42 Ceramide induces apoptosis of cardiomyocyte in vitro and in vivo.43–46 In the peroxisome proliferator-activated receptor-γ overexpression heart, myocardial lipid accumulation and increased ceramide content have been observed to accompany cardiomyocyte apoptosis.46 Therefore, the increased ceramide content in the CPT1b-deficient heart subjected to TAC is likely to trigger apoptosis signaling in the heart and to aggravate the pathological development.

One limitation of the present study is that the CPT1b deficiency preexisted; hence, we are cautious to avoid over-interpreting this result because CPT1 inhibitors are used as treatment in heart failure patients. We cannot rule out the possibility that the initial development of pressure overload–induced cardiac hypertrophy is exceptionally susceptible to CPT1b deficiency. A conditional gene-targeting model of CPT1b could provide more in-depth insights into the effects of CPT1b deficiency in the heart during the various stages of cardiac hypertrophy and heart failure. Despite this limitation, the present finding clearly demonstrates the necessity to further evaluate the use of CPT1 inhibitors as a therapeutic approach to treat patients with cardiac hypertrophy and even heart failure.

The present study demonstrates in a genetic mouse model that partial CPT1 deficiency is not sufficient to cause cardiac dysfunction under the normal physiological conditions. However, it is detrimental in animals subjected to TAC-induced LV pressure overload. CPT1b deficiency exacerbates the cardiac pathological development induced by LV pressure overload caused by myocardial lipotoxicity.

Acknowledgments
We thank the technical services of the UAB Metabolism Core Laboratory and the Targeted Metabolomics and Proteomics Laboratory. We also thank Kevin Yang for proofreading and editing the manuscript.

Sources of Funding
This work was supported by grants from National Institutes of Health (1R01 HL085499 and 1R01 HL084456 to Dr. Yang, 1R01 RR02599 to Dr. Wood, and T32 HL007457 to Dr. Kim). The technical services of the UAB Metabolism Core Laboratory were supported by grants.
P30DK56336 and UL1RR025777. The Targeted Metabolomics and Proteomics Laboratory was supported partly by P30DK079337 and P30AR50948.

Disclosures

None.

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**CLINICAL PERSPECTIVE**

The present study based on a mouse model with heterozygous carnitine palmitoyltransferase-1b (CPT1b) deficiency demonstrated that CPT1b deficiency is detrimental to the heart under left ventricular pressure-overload conditions. This finding contrasts with the common view that fatty acid oxidation (FAO) depression may be beneficial for the heart in the progression of cardiac hypertrophy and heart failure. It is recognized that myocardial FAO provides the majority of cardiac energy in a normal heart. However, the more oxygen-consuming FAO becomes a burden for the heart under hypertrophy and heart failure conditions. Consequently, the heart switches to use glucose as the main fuel with better energy efficiency. Therefore, compounds of FAO inhibitors targeting various FAO steps for the treatment of cardiac hypertrophy and heart failure have been developed and tested. Some beneficial effects have been shown in small clinical trials, especially for CPT1 inhibitors. However, this therapy is also associated with adverse effects. These untoward effects limit clinical utility and highlight the need for more preclinical studies. A major hurdle for the clinical use of this class of drugs is the lack of understanding of the potential detrimental effects of FAO deficiency in the heart under pathological conditions. Therefore, the present studies based on a genetic mouse model should provide definitive insights into the potential problems with the clinical use of this class of drugs to treat cardiac hypertrophy and heart failure.
Carnitine Palmitoyltransferase-1b Deficiency Aggravates Pressure Overload–Induced Cardiac Hypertrophy Caused by Lipotoxicity
Lan He, Teayoun Kim, Qinqiang Long, Jian Liu, Peiyong Wang, Yiqun Zhou, Yishu Ding, Jeevan Prasain, Philip A. Wood and Qinglin Yang

*Circulation*. 2012;126:1705-1716; originally published online August 29, 2012; doi: 10.1161/CIRCULATIONAHA.111.075978

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Method details:

**CPT1 activity assay:** A modified mitochondrial CPT1 assay was employed by measuring the rate of formation of palmitoylcarnitine from palmitoyl-CoA plus carnitine as described previously. Quantified mitochondria (100 µg protein) were incubated with the reaction buffer (containing 117 mM Tris-HCl, 0.28 mM reduced glutathione, 4.4 mM ATP, 4.4 mM MgCl2, 16.7 mM KCl, 2.2 mM KCN, 40 mg/l rotenone, 0.1% BSA, and 50 AM palmitoyl CoA) at 37°C for 5 min. The reactions were initiated when a 2mM [14C]-carnitine (0.1 uCi) was added. The reaction was quenched with 50ul 1.2 mM ice-cold HCl after 10 min. The formed [14C]-palmitoylcarnitine was extracted with water-saturated butanol and determined by liquid scintillation counting.

**Pressure-overload induced hypertrophy:** The surgical procedure of transverse aorta constriction (TAC) has been previously described. A 27-gauge needle was used to generate the severe pressure-overload condition; a 26-gauge needle was used for medium pressure-overload condition. Mice were sacrificed 2 weeks after TAC or sham operation.

**Palmitate and glucose oxidation assay of the heart subjected to TAC:** Freshly isolated heart ventricles were minced and homogenized in ice-cold buffer (250 mM sucrose, 10 mM Tris-HCl, 2 mM EDTA, and 1 mM ATP [pH 7.4]), mitochondria were isolated, quantified (100 µg protein) and used for palmitate oxidation assay in a sealed system with reaction buffer (75.5 mM sucrose, 12.5 mM K2HPO4, 100 mM KCl, 1.75mM MgCl₂ - 6H₂O, 1.75mM L-carnitine, 0.125mM L(-) malic acid, 1.75mM...
DTT, 0.07 mM NAD⁺, 2 mM ATP, 10 mM Tris-HCl, 0.07 mM Coenzyme A). The reaction started when 200 µM [¹⁴C]-palmitate-15% BSA (1:6) complex (0.04 µCi/reaction mixture) was added and stopped by 3.5 M perchloric acid after 30 min, 37°C incubation. CO₂-trapping medium (NaOH, 0.1 M) for ¹⁴C radioactivity was measured by liquid scintillation to calculate palmitate oxidation rate.

For glucose oxidation, fresh heart ventricles were homogenized in ice-cold buffer (5 mM KCl, 2 mM Tris-HCl, 0.5 mM Tris base, 0.25 mM MgCl₂ - 6H₂O, 0.05 mM EDTA, and 0.05 mM ATP [pH 7.4]), 400 µl homogenate was used and the reaction started when 200 µM [¹⁴C]-glucose (0.1 µCi/reaction mixture) was added. The reaction buffer and condition are same as above. CO₂-trapping medium (NaOH, 0.1 M) for ¹⁴C radioactivity was measured by liquid scintillation to calculate glucose oxidation and quantified by weight of heart tissue for homogenate.

**Heart ceramide assay**: Ceramide species were quantified by ESI-MS/MS (Applied Biosystems/MDS sciex, Canada) as described previously.³⁸, ³⁹ Heart tissue homogenates, in parallel with standard solutions, were spiked with 50 ng C₁₇:0 ceramide as internal standard and were extracted lipid according to the protocol.⁴⁰ After the extracted solution was evaporated to dryness and reconstituted, the samples were analyzed by mass spectrometry. The analysis was performed in positive ion mode electrospray ion (ESI-MS) source and precursor ion scans m/z 264 and 282 (ceramides). Ceramide subspecies were quantified by taking the ratios of the integrated intensity for each subspecies to the intensity of C₁₇:0. Total ceramide was calculated from the sum of C₁₆:0, C₁₈:0, C₂₀:0, C₂₂:0, and C₂₄:0 ceramide subspecies.
Supplementary references


### Table 1: Primers set for quantitative Real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' to 3')</th>
<th>Reverse (5' to 3')</th>
</tr>
</thead>
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<tr>
<td>β-actin</td>
<td>CTGTCCCTGTATGCTCTTG</td>
<td>ATGTCAGCACGATTTCC</td>
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<td>CPT1b</td>
<td>TTCAACACTACAGCCATCCC</td>
<td>GCCCTCATAGAGCCAGACC</td>
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<tr>
<td>CPT1a</td>
<td>CTCAGTGAGCCGACCTTTCA</td>
<td>GCCTCTCTGTTGCACAGGACAA</td>
</tr>
<tr>
<td>Nppa</td>
<td>ACCGAAGATAAAGAGCAAGGAG</td>
<td>TCGTGATAGATGAAGGAGGAA</td>
</tr>
<tr>
<td>Nppb</td>
<td>TCGTGATAGATGAAGGAGGAG</td>
<td>TGCTCTGGAGAAGACTGGCTAGGAC</td>
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<tr>
<td>MHC-β</td>
<td>TTTCGTCCTGGTCTTCTG</td>
<td>CAAAGGTGTCGTCCTGAGGAT</td>
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<tr>
<td>Mitofusion 2</td>
<td>AAGTCCGGGAAAGCTGAAAGT</td>
<td>TCTCGTTATGGAAACCAAACC</td>
</tr>
<tr>
<td>COX2</td>
<td>TCTCGGTTATGGAAACCAAACC</td>
<td>GCACAGGAGAGAAGGAAAC</td>
</tr>
<tr>
<td>Cyto b</td>
<td>GCAGGATTCTCTGAGCGTTC</td>
<td>AGGGCTTTGATTTATGTGTTTC</td>
</tr>
<tr>
<td>NRF1</td>
<td>GCCGGAACACACGCTAGATAGA</td>
<td>AGCAGCCGATGCGCATCTGA</td>
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<tr>
<td>NRF2</td>
<td>GAAGTGTTAACCAGGAGATAC</td>
<td>CTTGCTGGTATCCGAGGACCATCG</td>
</tr>
<tr>
<td>DRP1</td>
<td>AAACCTTCCATCATACATACAT</td>
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<td>PGC1-α</td>
<td>TGTTCGCCATACCATATCC</td>
<td>TCCGCTTTCTGCTGCTTT</td>
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<td>PGC1-β</td>
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<td>FABP</td>
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<td>CTGCACTGAGGTGAGGACT</td>
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<tr>
<td>FATP</td>
<td>CAGCGGATGGCGGAGGTTGA</td>
<td>AGAAGCGGCTGCGGAGAACT</td>
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<tr>
<td>Rcan 1</td>
<td>TTCTGCTTCTCCCACTGCGTC</td>
<td>GAAACATCAACACATTTGCTCC</td>
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</table>

### Table 2. Echocardiography measurement in mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>CPT1b+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS;d (mm)</td>
<td>0.79±0.17</td>
<td>0.74±0.07</td>
</tr>
<tr>
<td>IVS;s (mm)</td>
<td>1.19±0.22</td>
<td>1.13±0.14</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>3.63±0.39</td>
<td>3.70±0.24</td>
</tr>
<tr>
<td>LVID;s (mm)</td>
<td>2.44±0.33</td>
<td>2.55±0.26</td>
</tr>
<tr>
<td>LV PW;d (mm)</td>
<td>0.78±0.23</td>
<td>0.75±0.14</td>
</tr>
<tr>
<td>LV PW;s (mm)</td>
<td>1.11±0.21</td>
<td>1.04±0.77</td>
</tr>
<tr>
<td>LVID VOL;d(µL)</td>
<td>60.44±13.85</td>
<td>65.28±9.14</td>
</tr>
<tr>
<td>LVID VOL;s(µL)</td>
<td>20.41±5.59</td>
<td>22.72±3.89</td>
</tr>
<tr>
<td>Stroke Volume (µL)</td>
<td>40.03±9.57</td>
<td>42.50±6.64</td>
</tr>
<tr>
<td>Cardiac Output (ml/min)</td>
<td>16.45±5.15</td>
<td>19.72±3.21</td>
</tr>
<tr>
<td>EF(%)</td>
<td>62.11±6.75</td>
<td>59.80±4.83</td>
</tr>
<tr>
<td>FS(%)</td>
<td>32.93±4.82</td>
<td>31.26±3.34</td>
</tr>
<tr>
<td>LV Mass</td>
<td>98.56±15.32</td>
<td>94.26±12.57</td>
</tr>
</tbody>
</table>

Abbreviations: IVS;s and IVS; d: interventricular septal wall thickness (diastole and systole); LVID;s and LVID;d: left ventricular dimension at systole and diastole; LV PW;s
and LVPW;d: posterior wall thickness at systole and diastole; LVID VOL;s and LVID VOL;d: left ventricular dimension estimated left ventricular volume at systole and diastole; EF%: ejection fraction; FS%: fractional shortening. LV Mass: left ventricular mass.

**On line Table 3. Hemodynamic measurement of isolated working heart**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>CPT1b+/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>413.72±83.77</td>
<td>405.24±77.43</td>
</tr>
<tr>
<td>LVPsys(mmHg)</td>
<td>107.16±8.60</td>
<td>114.74±10.90</td>
</tr>
<tr>
<td>LVEDP(mmHg)</td>
<td>7.07±1.51</td>
<td>7.00±0.83</td>
</tr>
<tr>
<td>dLVPdmax(mmHg/s)</td>
<td>6084.57±805.19</td>
<td>6557.24±1385.26</td>
</tr>
<tr>
<td>-dLVPdtmin(mmHg/s)</td>
<td>-6730.96±480.65</td>
<td>-6757.19±1244.36</td>
</tr>
<tr>
<td>CI</td>
<td>123.47±12.23</td>
<td>119.70±22.55</td>
</tr>
<tr>
<td>RT50(ms)</td>
<td>25.35±2.91</td>
<td>27.07±3.85</td>
</tr>
</tbody>
</table>

Abbreviations: Heart rate (HR), systolic left ventricular pressure (LVPsys), left ventricular end-diastolic pressure (LVEDP), cardiac output index (CI), left ventricular maximal and minimal dP/dt indicate rates of cardiac contractility and relaxation, 50% relaxation time (RT50). n=6.

**Supplemental Figure Legends**

**Supplemental Figure 1. Body weight, heart weight and heart body weight ratio.**
Mice were sacrificed at their 12~14 weeks of age (n=5).

**Supplemental Figure 2. Representative histological images (400 X) with H&E staining on heart section.** Mice were sacrificed at 12~14 weeks of age.

**Supplemental Figure 3. Representative echocardiographic images of M-mode measurement.** Measurements were performed on mice at their 12~14 weeks of age.
Supplemental Figure 4. Serum Free fatty acid content in mice subjected to TAC at different time points. Mice were subjected to TAC procedures at 10~12 weeks of age and sacrificed 3 days, 1 week, two weeks after TAC, respectively. A commercial kit (NFFA-HR Assay kit, Wako) was used. *p<0.05 vs sham (n= 6).
Supplemental Figure 1

A. 

B. 

C. 

Body weight (g) 

Heart weight (g) 

Heart /Body weight (mg/g) 

WT  CPT1b^{+/−}  WT  CPT1b^{+/−}  WT  CPT1b^{+/−}
Supplemental Figure 2

WT

CPT1b +/-
Supplemental Figure 3

WT

CPT1b +/-
Supplemental Figure 4

![Bar chart showing serum free fatty acids (mEq/L) for different groups and time points. The chart compares WT Sham, WT TAC, CPT1b+/− Sham, and CPT1b+/− TAC groups across TAC 3d, TAC 1w, and TAC 2w].

**WT Sham**

**WT TAC**

**CPT1b+/− Sham**

**CPT1b+/− TAC**