Carnitine Palmitoyltransferase-1b (CPT1b) Deficiency Aggravates Pressure-Overload-Induced Cardiac Hypertrophy due to Lipotoxicity

Running title: He et al.; CPT1b deficiency aggravates pressure-overload-induced cardiac hypertrophy

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Abstract:

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 CPT-1 activity by specific CPT-1 inhibitors exerts protective effects against cardiac hypertrophy and heart failure. However, clinical and animal studies have shown mixed results, thereby posting concerns on the safety of this class of drugs. Preclinical studies using genetically modified animal models should provide a better understanding of targeting CPT1 in order to evaluate it as a safe and effective therapeutic approach.

Methods and Results - Heterozygous CPT1b knockout mice (CPT1b+/−) were subjected to transverse aorta constriction (TAC)-induced pressure-overload. These mice showed overtly normal cardiac structure/function under the basal condition. Under a severe pressure-overload condition induced by two weeks of transverse aorta constriction (TAC), 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 that in wild-type littermates. There were more pronounced impairments of cardiac contraction with greater eccentric cardiac hypertrophy in CPT1b+/− than in controlled mice. Moreover, the CPT1b+/− heart exhibited exacerbated mitochondrial abnormalities and myocardial lipid accumulation with elevated triglycerides and ceramide content, leading to greater cardiomyocytes apoptosis.

Conclusions - We conclude that CPT1b deficiency can cause lipotoxicity in the heart under pathological stress, leading to exacerbation of cardiac pathology. Therefore, caution should be applied in the clinical use of CPT-1 inhibitors.

Key words: CPT1; cardiac hypertrophy; heart failure; 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 review\textsuperscript{1, 2}). The remaining energy needs are derived mainly from glucose. A failing heart usually shows impaired transcription of key enzymes involved in fatty acid metabolism.\textsuperscript{1-5} Consequently, the heart switches to utilize glucose as the main fuel. Nevertheless, whether this substrate utilization shift is adaptive or maladaptive remains controversial. Several preclinical and clinical studies showed beneficial effects by inhibiting various steps of fatty acid oxidation (FAO) in animal and human subjects with cardiac hypertrophy and heart failure.\textsuperscript{6-10} Meanwhile, others reported adverse effects of drugs that exert inhibiting effects on FAO in animal studies,\textsuperscript{11-13} raising the safety concerns of this class of drugs. Nevertheless, it remains unclear whether the adverse effects are derived from attenuated FAO in the heart or from non-specific confounding effects of a particular compound.

Carnitine palmitoyltransferase 1 (CPT1) is located within the mitochondrial outer membrane as a rate-limiting enzyme of mitochondrial \( \beta \)-oxidation by controlling mitochondrial entry of long chain fatty acids. CPT1b is one of the three CPT1 isoforms (CPT1a, b and c). It is expressed mainly in skeletal muscle, heart, brown adipose tissue, and testis.\textsuperscript{14, 15} In adult cardiomyocytes, CPT1b is the predominant isoform and contributes about 98\% of total cardiac CPT1 activity.\textsuperscript{16, 17} 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 CPT-1 inhibitors, such as etomoxir, have been actively pursued as a therapeutic agent.\textsuperscript{18, 19} On the other hand, animal studies showed mixed results, from beneficial, unchanged or harmful effects on pressure-overload induced cardiac dysfunction, hypertrophy and progression.
to end-stage failure.\textsuperscript{11,20-22} A major hurdle is to differentiate the specific CPT1 inhibition effects from non-specific 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 deficient mice show no apparent phenotypic change.\textsuperscript{23} 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\textsuperscript{+/−} knockout mice were generated as described previously.\textsuperscript{23} The wild-type (WT) littermates with CPT1b\textsuperscript{+/+} genotyping were used as controls. Both male and female mice were used. Mice were kept on a 12 h/12 h light/dark cycle in temperature-controlled rooms and had \textit{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 were 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 employed by measuring the rate of formation of palmitoylcarnitine from palmitoyl-CoA plus carnitine according to the Methods section mostly described in 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 two-dimensional short-axis view and M-mode tracings of the left ventricle were obtained with a 30 MHz transducer.

Isolated Working Mouse Heart Perfusions

Myocardial contractile function and metabolism under basal physiological conditions were determined ex vivo through isolated working heart perfusions as previously described. For hypertrophied hearts subjected to pressure-overload induced by TAC, the isolated mitochondria were used to assay FAO rate and heart homogenates were used to assay glucose oxidation rate. The detailed methods about TAC procedure, palmitate and glucose oxidation assay were described in online-only data supplement.

Heart ceramide assay

Ceramide species were quantified by ESI-MS/MS (Applied Biosystems/MDS scieiX, Canada) as described in online-only data supplement.

Mitochondrial DNA copy number analysis

Total genomic DNA was isolated from left ventricles, processed by standard procedures using a DNA extraction kit (Wizard), and subjected to real-time qPCR analysis. Cytochrome b (Cyto b) was employed as a mitochondrial DNA (mtDNA) marker and the regulator of calcineurin 1 (Rcan1) as a nuclear DNA (nDNA) marker. The relative amount of mtDNA copy number was
assessed as an amplicon within a mitochondrial gene (Cyto b) to that of a nuclear gene (Rcan 1).

**Quantitative Real-Time RT-PCR Analyses**

Quantitative real-time RT-PCR analyses were carried out using Step one 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 “On line Table 1”.

**Western blot analyses**

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

**Statistical Analyses**

Data for two-group comparison were analyzed using Nonparametric Student's t-test, otherwise, the data were analyzed by one factor or mixed, two-factor analysis of Variance (ANOVA) using GraphPad Prism software (GraphPad Software Inc.). Survival data was 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 the P<0.05 probability level.

**Results**

**Mice with heterozygous CPT1b KO (CPT1b^{+/-}) exhibit no cardiac phenotype under basal condition**

The CPT1b^{+/-} mice were generated as described previously. While the homozygous CPT1b^{-/-} mice were embryonically lethal, the heterozygous CPT1b^{+/-} mice were overtly normal with the
same life span as compared with their wild-type (WT) littermates. The heart weight, body weight and the ratio of heart to body weight were not different between the CPT1b^+/− and their WT littermates (supplemental data, Fig.1). CPT1b^+/− mice did not exhibit detectable changes in cardiac histology and function. (supplemental data, Fig.2). Real-time PCR revealed that CPT1b transcript expression was attenuated by about 50% in CPT1b^+/− relative to WT hearts (Fig.1A). Western blots revealed that CPT1b protein content in the heart was correspondingly decreased by about 50% in CPT1b^+/− relative to WT hearts (Fig.1B). CPT1a, a dominant CPT1 isoform in neonatal hearts25, could have been re-upregulated in response to CPT1b deficiency in the heart. However, Q-PCR illustrated no change in CPT1a expression in the CPT1b deficient heart (Fig.1C). Total CPT1 activity in CPT1b^+/− hearts was substantially decreased to about 73% of those WT hearts (Fig.1D). Despite the significant CPT1b deficiency in the heart, echocardiographic assessment revealed normal anatomical structure of the heart with comparable cardiac performance in the CPT1b^+/− compared with WT hearts (Supplemental data Fig.3 and Supplemental Table 2). Isolated working heart measurements of ex vivo cardiac function revealed no difference between the CPT1b and their WT littermates (Supplemental Table 3). Both cardiac contractility (max dp/dt) and relaxation (min dp/dt) in CPT1b^+/− hearts were similar to that of the WT control. 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.

CPT-1 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 transverse aorta constriction (TAC)-induced LV pressure-overload. The CPT1b<sup>+/−</sup> mice were dramatically more susceptible to a severe pressure-overload condition induced by two weeks of TAC than their WT littermates. Majority of the CPT1b<sup>+/−</sup> mice died before the two-week term of pressure-overload with signs of heart failure (e.g., dilated heart, effluence, shortness of breath, etc.), whereas WT littermates survived (Fig. 2A). Under a milder pressure-overload condition, with a similar level of pressure gradient (Table 1), the CPT1b<sup>+/−</sup> mice showed more pronounced cardiac hypertrophy than their WT littermates. Echocardiographic assessment showed that left posterior wall thickness (LVPW) at diastole, left ventricular dimension (LVPD) volume at systole, left ventricular mass were further increased in CPT1b<sup>+/−</sup> mice. Stroke volume, cardiac output, ejection fraction (EF%) and fraction shortening (FS%) were further decreased in CPT1b<sup>+/−</sup> mice (Table 1 and Fig. 2B, C, D, & E). Heart weight to body weight and heart weight to tibia length ratios were increased in CPT1b<sup>+/−</sup> mice as compared to WT hearts (Fig. 3A & B). Furthermore, Q-PCR revealed that cardiac expression of molecular markers of cardiac hypertrophy, such as natriuretic peptide precursor A & B (Nppa and Nppb), and myosin heavy chain-β (MHC-β), were increased in CPT1b<sup>+/−</sup> mice more than their WT littermates (Fig. 3C). In addition, H&E and Trichrome Blue staining on heart sections demonstrated increased cross-sectional area of cardiomyocytes and more pronounced fibrosis in CPT1b<sup>+/−</sup> mice than in their WT littermates (Fig. 4A, B, C, & D). Transmission electron microscope assessment illustrated a dramatically increased number of swelling mitochondrial with the loss of matrix, and a reduced overall mitochondrial volume in heart sections of CPT1b<sup>+/−</sup> mice after TAC (Fig. 4E & F). Therefore, these results demonstrate that CPT1b deficiency is detrimental in hearts under mechanical stress-induced cardiac hypertrophy and heart failure.
CPT1b deficiency in heart 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. We investigated whether more pronounced cardiomyocyte apoptosis in the CPT1b deficient heart is attributed to the exacerbated pathological development. TUNEL assay on frozen heart sections revealed that an increased number of TUNEL-positive cardiac myocytes on sections from CPT1b+/- hearts (Fig. 5A & B). Western blot analysis on fractionated protein samples revealed that cytochrome c in the cytosol protein samples of CPT1b+/- hearts after TAC was markedly increased compared with that of WT controls (Fig. 5C). Therefore, these results indicate that apoptosis is more pronounced in CPT1b+/- relative to WT hearts under the TAC condition.

CPT1b+/- hearts under pressure-overload leads to myocardial lipid accumulation

Since 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. While no change could be detected under basal conditions, myocardial triglyceride concentration was markedly increased in CPT1b+/- relative to WT hearts subjected to TAC (Fig. 6A). Oil-red-O staining on frozen heart sections demonstrated numerous lipid droplets on CPT1b+/-, but not on WT heart sections (Fig. 6B). We further investigated whether myocardial ceramide content is altered with the myocardial accumulation of triglyceride in the CPT1b heart. Mass spectroscopic assessment of the total ceramide and ceramide species composition revealed that total ceramide content in CPT1b+/- hearts was substantially elevated compared with WT controlled hearts (Fig. 6C). C16, C18 and C24 were the ceramide species markedly increased in CPT1b deficient hearts (Fig. 6D).
Expression of proteins involved in fatty acid uptake is further downregulated in CPT1b+/− hearts under pressure-overload

To assess whether TAC-induced stress triggers the release of free fatty acids (FFA) from adipose tissues, we measured the serum content of FFA. As expected, serum FFA was modestly increased in both WT and CPT1b+/− mice subjected to TAC at various time points (Supplemental Figure 4). The increased serum FFA 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 (LCFA) uptake or transport were measured. We found that CD36, the heart type fatty acid binding protein (H-FABP) and fatty acid transport protein-1 (FATP-1) were unchanged under basal conditions, but decreased in CPT1b deficient heart subjected to pressure-overload (Fig.7A & B). Therefore, it is not likely that myocardial lipid accumulation in CPT1b+/− hearts is a result of an upregulation of LCFA uptake.

Mitochondrial biogenesis is decreased in CPT1b+/− hearts under pressure-overload

It has been reported that myocardial lipid accumulation may be associated with impaired mitochondrial biogenesis.29 Q-PCR revealed that the transcript levels of representative mitochondria proteins, such as mitofusin 2 (MFN 2), cytochrome b (Cyto b), cytochrome c oxidase subunit II and III (COX2 and COX3), and dynamin-related protein-1 (DRP1) were substantially attenuated (Fig. 7C). Moreover, transcription factors that are essential for mitochondrial biogenesis, such as transcription factor A, mitochondrial (TFAM), nuclear respiratory factor 1 (NRF1), NRF2 and peroxisome proliferator activated receptor γ coactivator 1α/β (PGC-1α and PGC-1β), were all decreased in CPT1b+/− hearts (Fig. 7C). Protein contents of PGC-1α and TFAM were similarly downregulated in CPT1b+/− relative to WT hearts (Fig. 7C).
7D). Mitochondria DNA copy number was also reduced in CPT1b+/− hearts (Fig. 7E). Thus, CPT1b deficiency and subsequent lipid accumulation is associated with a reduction in mitochondrial biogenesis in the heart under pressure-overload conditions.

**Fatty acid oxidation is impaired in CPT1b+/− hearts under pressure-overload**

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

**Discussion**

We investigated the effects of CPT1b deficiency on the pathological development of cardiac hypertrophy and remodeling under the left ventricular 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.30 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 exacerbate cardiac pathology.

CPT1b is the predominant CPT1 isoform expressed in the heart and 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, it remains controversial due to the 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 NF-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 non-specific side effect. It 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 due 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 prior to 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 non-enzymatic functions as a scaffold.

To our knowledge, the present study is the first study based on a gene targeted mouse
model with CPT1b deficiency. While a complete knockout of CPT1b causes embryonic lethality, the heterozygous CPT1b knockout mice are overtly normal. It is noted that in the heterozygous CPT-1b knockout heart, CPT1b expression was blunted in both transcript and protein levels. Since CPT1a is also expressed in the adult heart at low level, a compensatory upregulation of CPT1a is possible. In fact, the inverse response of increased Cpt-1b expression in livers of diet challenged CPT-1a+/− mice was reported.36 However, transcript expression of CPT1a was unchanged in the CPT1b deficient hearts. Since the total CPT1 activity was decreased by about 30% compared to 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.35,11 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 CTP1 inhibition (30% in CPT1b+/− vs 40-50% with etomoxir treatment37).

The most important finding in the present study is that the CPT1b+/− 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.18,19 The reasons for this obvious discrepancy may be derived from different degrees of CPT1 inhibition among studies, non-specific effects of CPT1 inhibitors, or the existence of certain
levels of CPT1a activity in CPT1b knockout hearts. Importantly, the current finding on the
detrimental effects of CPT1b deficiency is against the basic concept of fatty acid oxidation
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 an 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. Moreover, intramyocardial lipid accumulation is associated with contractile
dysfunction in heart tissues from patients with non-ischemic heart failure. 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. While this appears to be the case, it is insufficient to
activate PPAR signaling to increase the expression of fatty acid uptake proteins. Instead, the
expression of fatty acid uptake proteins was further decreased, possibly due to the exacerbated
cardiac pathological development. 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.\textsuperscript{40, 41} 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.\textsuperscript{42, 43} Ceramide induces apoptosis of cardiomyocyte \textit{in vitro} and \textit{in vivo}.\textsuperscript{44-47} In the PPAR\textgamma overexpression heart, myocardial lipid accumulation and increased ceramide content has been observed accompanying cardiomyocyte apoptosis.\textsuperscript{47} Therefore, the increased ceramide content in the CPT1b deficient heart subjected to TAC is likely to trigger the apoptosis signaling in the heart and aggravates the pathological development.

One limitation of the current study is that the CPT1b deficiency pre-existed, hence we are cautious to avoid over-interpreting this result as CPT-1 inhibitors are used as treatment in heart failure patients. We cannot rule out the possibility that the initial development of the 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 current 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 CPT-1 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 left ventricular pressure-overload due to myocardial lipotoxicity.
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Conflict of Interest Disclosures: None.

References:


33. Hulsmann WC, Pescechera A, Schneijdenberg CT, Verkleij AJ. Comparison of the effects


Table 1. Echocardiography measurement in mice two weeks after TAC.

<table>
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Abbreviations: NA: not available; IVS;s and IVS; d: interventricular septal wall thickness (diastole and systole); LVID;s and LVID;d: left ventricular dimension at systole and diastole; LVPW;s and LVPW;d: left ventricular 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. *p<0.05 vs Sham, #P<0.05 vs WT TAC

Figure Legends:

Figure 1. CPT1b mRNA level and activity in CPT1b +/- mice. Mice were sacrificed at 12~14 weeks of age. RNA samples were extracted from ventricular tissues. A & C) Transcript level of CPT1b and CPT1a were determined by Q-PCR, 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 on-line supplement (n=4). E & F) Palmitate oxidation rate and glucose oxidation rate were measured in isolated working heart (n=6). *p<0.05 vs WT.

**Figure 2.** Echocardiographic parameters of the mice with pressure-overload. Mice were subjected to TAC procedures at 10~12 weeks of age. A) Kaplan-Meier survival curves for WT and CPT1b+/− mice that were subjected to severe TAC-induced pressure-overload. The survival curves were statistically different (P<0.05) by log-rank test. B, C, D & E) Mice were subjected to modest TAC at 10~12 weeks of age. Representative echocardiographic images of M-mode measurement and echocardiographic results of EF, FS, and corrected left ventricular mass to body weight (2 weeks after TAC, *p<0.05 vs sham, #p<0.05 vs WT TAC. Sham: n=9; TAC: n=11).

**Figure 3.** Transcript levels of molecular markers of pathological cardiac hypertrophy in CPT1b+/− mice with pressure-overload. Mice were subjected to a modest pressure-overload condition at 10~12 weeks of age and sacrificed two weeks after TAC. A & B) Heart (mg)/ body weight (g) ratio and heart weight (mg) to tibia length (mm), *p<0.05 vs sham (n=6), #p<0.05 vs WT TAC (n=13). C) Real-time PCR assessment of natriuretic peptide precursor A (Nppa), B (Nppb) and myosin heavy chain-β (MHC-β) transcripts, *p<0.05 vs sham, #p<0.05 vs WT TAC, (n=5).

**Figure 4.** Cardiac histology and ultrastructure in CPT1b+/− mice with pressure-overload. Mice
were subjected to TAC procedures at 10~12 weeks of age and sacrificed two weeks after TAC.

A) Representative histological images (400 x) with H&E staining on heart section. B) Relative cross-sectional areas. The mean cardiomyocyte cross-sectional area in sham 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 TEM assessment (x1100). F) Quantification results of mitochondrial volume (%) of heart sections from TEM images. *p<0.05 vs sham. #p<0.05 vs WT TAC (n=8).

**Figure 5.** Apoptosis assay of CPT1b−/− mice with pressure-overload. Mice were subjected to TAC procedures at 10~12 weeks of age and sacrificed two weeks after TAC. A) Representative image of apoptotic cardiomyocytes in a section of mice hearts. Green staining (see white arrows) indicates apoptotic cells, blue indicates nuclei (TUNEL fluorescence FITC kit, GenScript,USA). B) Quantification results of TUNEL assay (n=5). C) Western blots of cytochrome c in cytosolic and mitochondrial fractions extracted from hearts of mice. *p<0.05 vs sham. #p<0.05 vs WT TAC (n=4).

**Figure 6.** Myocardial lipid accumulation of mice with pressure-overload. Mice were subjected to TAC procedures at 10~12 weeks of age and sacrificed two weeks after TAC. A) Myocardial triglyceride content in hearts (n=5). B) Representative photomicrographs depicting the histologic appearance of ventricular tissue sections stained with Oil red O. C) Ceramide species were quantified by ESI-MS/MS as described in the methods (on line supplement). D) Total ceramide was calculated from the sum of C16:0, C18:0, C20:0, C22:0, and C24:0 ceramide subspecies.
*p<0.05 vs sham. #p<0.05 vs WT TAC (n=4).

**Figure 7.** The expression of fatty acid uptake proteins and mitochondrial biogenesis in mice subjected to pressure-overload. Mice were subjected to TAC procedures at 10–12 weeks of age and sacrificed two weeks after TAC. A) QPCR assessment of CD36, FABP and 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 blots image and quantification of PGC1-α and TFAM (n=4). E) Mitochondria DNA copy number was assessed by ratio of mitochondrial gene (Cyto b) and nuclear gene (Rcan-1) as described in the methods (on line supplement). *p<0.05 vs sham. #p<0.05 vs WT TAC (n=6).

**Figure 8.** Fatty acid and glucose oxidation rates, and CPT1 activity/expression in pressure-overloaded hearts. Mice were subjected to TAC procedures at 10–12 weeks of age and sacrificed 3 days, 1 week and two weeks after TAC, respectively. A) Fatty acid oxidation rate was determined on isolated mitochondria (n=6). B) Glucose oxidation rate was determined on heart homogenate (n=6). C) CPT1 activity was assayed using isolated mitochondria (n=6). D) QPCR assessment of CPT1b, 2 weeks after TAC (n=4). E) Western blots image and quantification of CPT1b, 2 weeks after TAC. *p<0.05 vs sham. #p<0.05 vs WT TAC. âp<0.05 vs WT Sham (n=6).
Carnitine Palmitoyltransferase-1b (CPT1b) Deficiency Aggravates Pressure-Overload-Induced Cardiac Hypertrophy due to Lipotoxicity
Lan He, Teayoun Kim, Qinxiang Long, Jian Liu, Peiyong Wang, Yiqun Zhou, Yishu Ding, Jeevan Prasain, Philip A. Wood and Qinglin Yang

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Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2012/08/29/CIRCULATIONAHA.111.075978.DC1
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.\(^1,2\) 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 MgCl\(_2\), 16.7 mM KCl, 2.2 mM KCN, 40 mg/l rotenone, 0.1% BSA, and 50 AM palmitoyl CoA)\(^3\) at 37°C for 5 min. The reactions were initiated when a 2mM \(^{14}\text{C}\)-carnitine (0.1 uCi) was added. The reaction was quenched with 50ul 1.2 mM ice-cold HCl after 10 min. The formed \(^{14}\text{C}\)-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.\(^4\) 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**\(^5,6\): 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\(^7\) with reaction buffer( 75.5 mM sucrose,12.5 mM K\(_2\)HPO\(_4\),100mM KCl,1.75mM MgCl\(_2\) - 6H\(_2\)O, 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 200µM \([^{14}C]\)-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 \(^{14}\)C 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]), 400µl homogenate was used and the reaction started when 200µM \([^{14}C]\)-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 \(^{14}\)C 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.\(^8,\)\(^9\) 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.\(^10\) 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


### On line 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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<td>β-actin</td>
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<td>ATGTACGCACGACATTTCC</td>
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<td>CPT1b</td>
<td>TTCAACACTACACGCATCCC</td>
<td>GCCCTCATAGACCGCAGACC</td>
</tr>
<tr>
<td>CPT1a</td>
<td>CTCAGTGCCAGCGACTCTTCA</td>
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<td>Nppa</td>
<td>ACCGAAGATAACAGCAAGGAGG</td>
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</tr>
<tr>
<td>Nppb</td>
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<td>COX2</td>
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<tr>
<td>COX3</td>
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<td>AGGGCTTGATTTATGTTTTC</td>
</tr>
<tr>
<td>Cyto b</td>
<td>TTCCCATTTCCTTCCCGAGAC</td>
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</tr>
<tr>
<td>NRF1</td>
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<td>NRF2</td>
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<td>DRP1</td>
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<td>PGC1-α</td>
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<td>PGC1-β</td>
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<td>FABP</td>
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<td>FATP</td>
<td>CCGCGGGTGCGCGGTGAGGTGA</td>
<td>AGAAGCGGTGGCGGGAGAACT</td>
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<tr>
<td>Rcan 1</td>
<td>TTCTGCTTTCGGACATCGTG</td>
<td>GAAACATCAACCGATTTGCTCC</td>
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### On line Table 2. Echocardiography measurement in mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT ± standard deviation</th>
<th>CPT1b+/− ± standard deviation</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>
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<tr>
<td>LVPW;d (mm)</td>
<td>0.78±0.23</td>
<td>0.75±0.14</td>
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<tr>
<td>LVPW;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>
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<tr>
<td>LVID VOL;s(µL)</td>
<td>20.41±5.59</td>
<td>22.72±3.89</td>
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<tr>
<td>Stroke Volume (µL)</td>
<td>40.03±9.57</td>
<td>42.50±6.64</td>
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<tr>
<td>Cardiac Output (mL/min)</td>
<td>16.45±5.15</td>
<td>19.72±3.21</td>
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<tr>
<td>EF(%)</td>
<td>62.11±6.75</td>
<td>59.50±4.83</td>
</tr>
<tr>
<td>FS(%)</td>
<td>32.93±4.82</td>
<td>31.26±3.34</td>
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<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; LVPW;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>
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<tr>
<th>Parameters</th>
<th>WT</th>
<th>CPT1b+/−</th>
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<tbody>
<tr>
<td>HR</td>
<td>413.72±83.77</td>
<td>405.24±77.43</td>
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<tr>
<td>LVPsys (mmHg)</td>
<td>107.16±8.60</td>
<td>114.74±10.90</td>
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<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>-dLVPdmin (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>
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<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)
Supplemental Figure 2

WT

CPT1b +/-
Supplemental Figure 3

WT

CPT1b +/-
Supplemental Figure 4

![Supplemental Figure 4](image-url)

**Figure Legend:**

- **WT Sham**
- **WT TAC**
- **CPT1b<sup>+/−</sup> Sham**
- **CPT1b<sup>+/−</sup> TAC**

**Abbreviations:**

- TAC: Transverse Aortic Constriction
- WT: Wild Type
- CPT1b: Carnitine Palmitoyl Transferase 1b

**Graph:**

- The graph shows the serum free fatty acids (mEq/L) over time (TAC 3d, TAC 1w, TAC 2w).
- Bars indicate the mean ± standard error of the mean (SEM).
- Asterisks (*) denote statistically significant differences between groups.

**Analysis:**

- WT Sham and WT TAC groups show a trend of increasing serum free fatty acids over time.
- CPT1b<sup>+/−</sup> Sham and CPT1b<sup>+/−</sup> TAC groups exhibit a different pattern, with a significant increase in serum free fatty acids in the CPT1b<sup>+/−</sup> TAC group compared to controls.

**Conclusion:**

The results suggest that TAC and the absence of CPT1b have differential effects on serum free fatty acids, with TAC leading to increased free fatty acids in both WT and CPT1b<sup>+/−</sup> groups, but with a more pronounced effect in the CPT1b<sup>+/−</sup> mice.