Fatty Acid Metabolic Defects and Right Ventricular Lipotoxicity in Human Pulmonary Arterial Hypertension

Running title: Brittain et al.; Fatty Acid Metabolism in PAH

Evan L. Brittain, MD, MSCI1,2; Megha Talati, PhD3; Joshua P. Fessel, MD, PhD3; He Zhu, PhD4; Niki Penner, BS3; M. Wade Calcutt, PhD5; James West, PhD3; Mitch Funke, BS3; Gregory D. Lewis, MD6,7; Robert E. Gerszten, MD6,7,8; Rizwan Hamid, MD, PhD9; Meredith E. Pugh, MD, MSCI3; Eric D. Austin, MD, MSCI10; John H. Newman, MD3; Anna R. Hemnes, MD3

1Division of Cardiovascular Medicine, Vanderbilt University School of Medicine, Nashville, TN; 2Vanderbilt Translational and Clinical Cardiovascular Center, Vanderbilt University Medical Center, Nashville, TN; 3Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University School of Medicine, Nashville, TN; 4Vanderbilt University Institute of Imaging Science, Nashville, TN; 5Dept of Biochemistry, Vanderbilt University, Nashville, TN; 6Cardiovascular Research Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA; 7Cardiology Division, Massachusetts General Hospital, Harvard Medical School, Boston, MA; 8Broad Institute of MIT and Harvard, Cambridge, MA; 9Division of Medical Genetics and Genomic Medicine, Dept of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN; 10Division of Allergy, Immunology and Pulmonary Medicine, Dept of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN

Address for Correspondence:
Evan L. Brittain MD, MSCI
Division of Cardiovascular Medicine
Vanderbilt University Medical School
2525 West End Avenue, Suite 300
Nashville, TN 37203
Tel: 615-322-4382
Fax: 615-322-0135
E-mail: evan.brittain@vanderbilt.edu

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Abstract

Background—The mechanisms of right ventricular (RV) failure in pulmonary arterial hypertension (PAH) are poorly understood. Abnormalities in fatty acid (FA) metabolism have been described in experimental models of PAH, but systemic and myocardial FA metabolism have not been studied in human PAH.

Methods and Results—We used human blood and RV tissue and non-invasive imaging to characterize multiple steps in the FA metabolic pathway in PAH subjects and controls. Circulating FFAs and long-chain acylcarnitines were elevated in PAH patients versus controls. Human RV long chain FAs were increased and long chain acylcarnitines were markedly reduced in PAH versus controls. Using proton magnetic resonance spectroscopy, in vivo myocardial triglyceride content was elevated in human PAH versus controls (1.4 ± 1.3 %TG vs. 0.22 ± 0.11 %TG, p = 0.02). Ceramide, a mediator of lipotoxicity, was increased in PAH RVs versus controls. Using an animal model of heritable PAH we demonstrated reduced fatty acid oxidation via failure of palmitoylcarnitine to stimulate oxygen consumption in the PAH RV.

Conclusions—Abnormalities in fatty acid metabolism can be detected in the blood and myocardium in human PAH and are associated with in vivo cardiac steatosis and lipotoxicity. Murine data suggests that lipotoxicity may arise from reduction in fatty acid oxidation.

Key words: pulmonary hypertension; right ventricle; lipid toxicity; magnetic resonance spectroscopy; fatty acid; Lipid and lipoprotein metabolism; pulmonary circulation and disease
Introduction

Right ventricular (RV) failure is the predominant cause of death in pulmonary arterial hypertension (PAH), but no RV-specific therapies exist because the underlying mechanisms are poorly understood. Abnormalities of glucose homeostasis and insulin resistance are well described in PAH1-4 but less is known about lipid metabolism despite the interrelated nature of glucose and lipid homeostasis. Abnormalities in fatty acid metabolism have been described in experimental models of PAH5,6 but systemic and myocardial fatty acid metabolism have not been studied in human PAH.

Given the heart’s preference for fatty acids (FA) as an energy source7, understanding FA metabolism may be particularly relevant to understanding RV adaptation to elevated afterload in PAH. We recently showed that RV failure is associated with myocardial steatosis and accumulation of the lipotoxic and pro-apoptotic mediator ceramide in human heritable PAH (HPAH) due to mutation in bone morphogenetic protein receptor type II (BMPR2)8. Others and we have also shown indirect evidence of abnormal fatty acid oxidation (FAO) in experimental models of PAH9-11. The generalizability of these abnormalities in fatty acid metabolism to idiopathic PAH (IPAH) and whether they are a systemic feature in human PAH are unknown.

We hypothesized that reduced fatty acid metabolism is ubiquitous in PAH and associated with lipotoxic cardiac steatosis in the RV. We tested this hypothesis by studying blood, RV tissue, and in vivo myocardial triglyceride imaging in patients with PAH and controls.

Methods

Study Approval

The Institutional Animal Care and Use Committee of Vanderbilt University School of Medicine
approved all animal procedures. Human studies were approved by the Institutional Review Board of Vanderbilt University School of Medicine (IRB numbers 9401, 111157, and 90782). All patients gave written informed consent.

**Study Populations**

PAH patients for this study were enrolled from the Vanderbilt Center for Pulmonary Vascular Disease. PAH patients were diagnosed by experienced clinicians according to consensus guidelines, defined as an invasively measured mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg as well as a pulmonary wedge pressure (PWP) or left ventricular end diastolic pressure ≤ 15 mmHg. PAH patient inclusion was restricted to IPAH or HPAH. Control subjects were enrolled in the prospective Vanderbilt Pulmonary Hypertension Research Cohort at Vanderbilt University Medical Center after recruitment via research network email solicitation.

**Sample Collection and Analysis**

Fasting peripheral blood samples were obtained at the time of clinic visits or at the Vanderbilt General Clinical Research Center. Plasma samples were collected into ethylenediaminetetraacetic acid (EDTA) plasma tubes. EDTA tubes were centrifuged within 45 minutes at 4000 rpm and the plasma fraction immediately aliquoted as 200μL aliquots and stored at -80°C. Plasma acylcarnitine samples were analyzed as described previously. The Hormone Assay Core of the Mouse Metabolic Phenotypic Center (MMPC) at Vanderbilt University quantified plasma free fatty acids using standard enzymatic reactions.

**RV Gene Expression Array**

RNA isolation and Microarray techniques have been described previously. All array results have been submitted to the National Center for Biotechnology Information gene expression and hybridization array data repository (GEO, http://www.ncbi.nlm.nih.gov/geo/) as series
GSE67492. Arrays were analyzed as previously described\textsuperscript{14}.

**Human Tissue Metabolite Studies**

Human PAH hearts were obtained at autopsy after informed consent from patients or their families. The right ventricular free wall was immediately dissected and flash frozen in liquid nitrogen. Right ventricular samples from unmatched donor hearts and explanted hearts from patients with non-ischemic dilated cardiomyopathy at the time of transplantation were collected in the operating room and processed and stored in the same manner. Unmatched donor hearts exhibited no gross cardiac defects and no significant abnormal findings on echocardiography. Donor hearts were excluded for transplantation due to one or more of the following reasons: intravenous drug use, abnormal T waves during stroke, possible pericarditis, known coronary artery disease, and history of smoking. All myocardial samples were kept in a -80°C freezer until used for this study. Human RV long-chain acylcarnitines, ceramides, and carnitine palmitoyltransferase I activity were quantified using standard liquid chromatography/mass spectrometric methods as described in the supplemental materials and Supplemental Tables 4 and 5.

**Histology**

Immunolocalization for ceramide was performed on paraffin-embedded sections of human RV tissue as described in the supplemental materials. Differences in samples size between experiments reflect the tissue available for testing.

**Human Tissue Western Blot**

Western blots were performed on tissue homogenates according to standard protocols\textsuperscript{15}. Antibodies for carnitine palmitoyltransferase 1B1 and carnitine palmitoyltransferase 2 were purchased from Novus (product number NBP1-59576).
Transgenic Murine Model

The Rosa26-rTA2 x TetO7-Bmpr2R899X FVB/N mouse model (called BMPR2^{R899X} for brevity) has been previously described and shown to have RV lipotoxicity\textsuperscript{8,16}. Expression of the human mutant BMPR2 gene occurs only after administration of doxycycline. Transgene-negative mice were used as littermate controls and were also administered doxycycline. At the time of sacrifice, tissue was harvested and preserved in formalin, snap frozen, or immediately prepared for high resolution respirometry.

Myocardial High Resolution Respirometry

See supplemental materials

Cardiac Magnetic Resonance and Proton Magnetic Resonance Imaging Protocol

Cardiac imaging and proton spectroscopy were performed between 5/2013-5/2014 on non-fasting subjects. This technique can distinguish triglyceride that is found in the aqueous cardiomyocyte from triglyceride in adipose tissue\textsuperscript{17}. Studies were performed using a 3.0T Philips Achieva (Philips Medical Systems, The Netherlands) equipped with a package for localized cardiac spectroscopy. Imaging parameters and spectroscopy analysis are detailed in the supplemental materials

Statistics

All values are reported as mean ± standard deviation or median (interquartile range) and categorical variables as absolute value and percent unless otherwise stated. Between-group differences were calculated using Mann-Whitney U test or Chi-Square test, as appropriate. The one-way ANOVA and Student’s $t$-test were used as appropriate to compare myocardial acylcarnitines and gene expression data after log-transformation to achieve a normal distribution. To account for multiple testing using the 17 different acylcarnitines in the human plasma
samples, we used a Bonferroni corrected significance threshold of p < 0.003 (0.05/17). Correlation coefficients were calculated using Spearman’s method. Statistical analyses were performed using SPSS 22 software (SPSS Inc, Chicago, IL).

Results

Evidence of Peripherally Detectable Alterations in Fatty Acid Metabolism in PAH

Given the accumulating evidence that PAH is a systemic metabolic disease\textsuperscript{18,19}, we tested the hypothesis that peripheral blood would demonstrate evidence of altered lipid metabolism. We measured non-esterified FFAs and acylcarnitines, which shuttle fatty acids across membranes and have been associated with insulin resistance and adverse cardiovascular outcomes in other populations\textsuperscript{13,20}. Fatty acid esters of free carnitine, acylcarnitines are classified as short (2 to 4 carbons), medium (5 to 12 carbons) and long chain (defined as \geq 14 carbons). Control subjects were selected from our prospective registry to achieve a similar distribution of age, gender, and BMI as PAH subjects. Demographic and clinical data for the FFA and acylcarnitine group comparisons are found in Supplemental Table 1. The PAH patients have severe disease based on functional capacity impairment, medical treatment (50% on prostanoid therapy), and advanced RV dysfunction and remodeling. FFAs were nearly two-fold higher in 19 PAH patients compared with 22 control subjects (0.91±0.35 vs. 0.48±0.30, p < 0.001; Figure 1A). Acylcarnitines (C2-C18) were measured in 11 PAH patients and 19 healthy subjects. Because acylcarnitine values can be influenced by renal function, we selected only PAH patients with normal creatinine (0.8±0.2mg/dL). Control subjects were assumed to have normal renal function. Only the long chain acylcarnitines palmitoylcarininit (C16), stearoylcarininit (C18), oleoylcarininit (C18:1), and linoleoylcarininit (C18:2) reached the pre-specified significance
threshold, measuring 1.5-2 fold higher in PAH patients compared with healthy subjects (Figure 1B and Supplemental Table 2; \( p < 0.003 \) for all). We found no significant differences in short or medium chain acylcarnitines between PAH and controls. Total acylcarnitines were increased 1.3 fold in PAH compared with controls (\( p = 0.008 \)). Differences in long-chain acylcarnitines remained significant after adjustment for carnitine levels (Supplemental Table 2). We quantified long-chain fatty acid profiles in the controls and PAH patients with acylcarnitine measurements. The unsaturated fatty acids (C14:0, C:16:0, and C18:0) were lower in patients with PAH versus control subjects, whereas saturated fatty acids (C16:1, C18:1, C18:2) were increased in patients with PAH (Supplemental Table 3).

Isolated elevation in long-chain acylcarnitines is a feature of insulin resistance and has been attributed to incomplete FAO\(^{20,21}\), which we corroborate in data presented subsequently. Our PAH population was insulin resistant as measured by the Homeostatic Index of Insulin Resistance (HOMA-IR), but PAH patients did not differ from our controls (3.5±2.7 \( \text{vs.} \) 3.5±4.6, \( p = 0.99 \)), who were also insulin resistant. We found no association between FFAs or acylcarnitines and HOMA-IR with our relatively small sample size. We found no association between acylcarnitine levels and RV function. However, when RV function is dichotomized as normal/mild versus moderate/severe, worse RV function was association with a greater than 2 fold increase in HOMA-IR (5.1±3.3 \( \text{vs.} \) 2.3±1.0, \( p = 0.017 \)) and numerically higher FFAs (0.84±0.25 \( \text{vs.} \) 0.65±0.24, \( p = 0.14 \)). Worse New York Heart Association class was associated with elevation in the long chain acylcarnitines palmitoylcarnitine (C16), stearoylcarnitine (C18), oleoylcarnitine (C18:1), and linoleoylcarnitine (C18:2) (\( r_s = 0.54, 0.45, 0.57 \) and 0.45, respectively; \( p < 0.02 \) for all; Supplemental Figure 1). These data show that abnormalities in FA metabolism in PAH are demonstrable in plasma and may have an influence on RV function.
and functional capacity, leading us next to test the effect of these abnormalities on the myocardium directly.

**Increased Right Ventricular Long Chain Fatty Acids and Decreased Acylcarnitines in Human PAH**

To determine whether elevated peripheral FFAs in PAH were associated with increased lipid accumulation in the myocardium, we measured long-chain fatty acids (LCFA) in human RV tissue in PAH, unmatched donor hearts, and explanted hearts from patients with dilated cardiomyopathy (DCM) and RV dysfunction on echocardiography. DCM patients with RV dysfunction were included as a disease control group to separate the metabolic effects of RV dysfunction generally from PAH-specific effects. LCFAs were generally increased in PAH compared with control, particularly palmitate (C16) and palmiolate (C16:1), which were increased approximately 70% (**Figure 2A**). In contrast, linoleate (C18:2) was reduced by 31% in PAH. Linoleate must be consumed in the diet; therefore reduced levels in the PAH RV may reflect differences in consumption between groups. Values from DCM hearts were largely intermediate between PAH and controls.

The first step in the metabolism of LCFAs to undergo beta-oxidation is the conversion to acylcarnitines, which allows LCFAs to cross the mitochondrial membranes. We therefore quantified human RV long-chain acylcarnitines, which were reduced nearly 100-fold in PAH compared with both control groups (**Figure 2B**).

These data implicate a fundamental problem in the utilization of LCFAs to produce energy in the PAH RV, despite adequate delivery. Importantly, these observations closely parallel our previous findings in the BMPR2<sup>R899X</sup> murine model of PAH indicating this model strongly recapitulates the RV metabolic phenotype in human disease<sup>22</sup>. 

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Mitochondrial Fatty Acid Transport in PAH

One potential mechanism to explain LCFA excess in cardiomyocytes with suppression of long-chain acylcarnitines is failure to transport FAs into the mitochondria. Using human RV gene expression arrays, we found no difference in the major mitochondrial carnitine transporters carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), solute carrier family 22 (organic cation/carnitine transporter) member 5, or carnitine-acylcarnitine translocase in the PAH RV versus dilated cardiomyopathy or unmatched donor RVs (Supplemental Figure 2). In addition, we found similar between-group expression of long-chain acyl-coA dehydrogenase, the enzyme responsible for β-oxidation of LCFAs into acetyl-CoA (Supplemental Figure 2).

Using a mouse model of universal expression of a human BMPR2 mutation associated with PAH (BMPR2<sup>R899X</sup>)<sup>16</sup>, we next confirmed normal carnitine transporter protein content in the PAH RV compared with control (Supplemental Figure 3A,B). Finally, we used a mass spectrometry technique to show that CPT1 activity in the human RV was similar among human PAH, DCM, and control RVs (Supplemental Figure 4). CPT1 is the rate-limiting step in acylcarnitine formation and the major point of inhibition of FAO by the glucose oxidation pathway. These data suggest that the mitochondrial transport enzymes may not play a primary role in accumulation of FAs and reduced long-chain acylcarnitines.

Failure of PAH RV Myocardium to Use A Long-chain Acylcarnitine for Oxidative Metabolism

Others and we have previously shown indirect evidence of impaired FAO in the PAH RV but reduced oxygen consumption using a fatty acid substrate has not been shown<sup>8-10,23</sup>. We next tested directly the ability of the PAH RV mitochondria to utilize a long-chain acylcarnitine as a
mitochondrial fuel source. We performed high-resolution respirometry on isolated RVs from BMPR2\textsuperscript{R899X} mice and littermate controls (n = 4 per group). Fatty acid supplementation in this system should increase oxygen consumption via mitochondrial fatty acid oxidation. We found, however, that the proportional mitochondrial oxygen consumption rate attributable to the oxidation of palmitoylcarnitine in the BMPR2\textsuperscript{R899X} RV was reduced nearly 9-fold compared to wild type RV (Figure 3), with the increase over and above Complex I-linked respiration being nearly undetectable in the BMPR2 mutant RV.

Evidence for Lipotoxicity in Human PAH RV

To determine whether FA accumulation is associated with lipotoxic lipids in the RV in PAH, we stained human tissue for ceramide, a well-described mediator of cardiac apoptosis and lipotoxicity\textsuperscript{8,24-26}. We performed immunohistochemistry analysis in 5 PAH and 8 control RVs demonstrating an increase in ceramide (113\% on average) in PAH compared with control RVs (p = 0.006; Figure 4). We validated this finding by mass spectrometry of a long-chain (C16:0) and a very long-chain (C24:1) ceramide (Figure 4). This finding suggests that lipotoxicity is a functional consequence of RV lipid accumulation in human PAH.

Detection of Elevated In Vivo Myocardial Triglyceride Content in PAH Patients

We have previously shown abundant myocardial lipid accumulation in explanted RV samples from patients with HPAH, but whether lipid accumulation occurs in the living human with PAH and whether it is present in living patients with non-end stage disease is unknown. Proton MRS is a non-invasive tool used to quantify in vivo intracellular triglyceride content that has been validated in healthy subjects and patients with obesity and/or diabetes\textsuperscript{27,28}. We performed cardiac proton MRS on 6 PAH subjects (46±19 years, 67\% female, NYHA class 1.8±0.8) and 8 healthy controls (34±8 years, 50\% female) without diabetes or known cardiopulmonary disease.
We demonstrated a high degree of intra-subject reproducibility measured on 2 controls and 1 PAH subject at < 0.05 percent triglyceride %TG. Three PAH patients were on continuous intravenous infusion of epoprostenol and 3 were on oral-only regimens. All controls had %TG levels consistent with published values (range 0.1-0.4 %TG) for healthy subjects\textsuperscript{17}. PAH patients had on average 7 fold higher %TGs compared to controls (Figure 5; 1.4 ± 1.3 %TG vs. 0.22 ± 0.11 %TG, p = 0.02). To put myocardial lipid content in context with functional status, we found a moderate inverse relationship between %TG and six-minute walk distance, however this relationship was not statistically significant (rs = -0.7, p=0.13). Of note, one PAH patient with NYHA Class I functional status and normal RV size and function had a value of 1.5%, 50% higher than average values reported from an obese, diabetic population\textsuperscript{27}. This observation suggests that myocardial lipid accumulation may occur early in the disease process.

**Discussion**

Right ventricular failure is the leading cause of death in PAH and often progresses independent of the hemodynamic response to pulmonary vasodilators.\textsuperscript{29} The lack of available RV-specific therapies largely reflects an incomplete understanding of why RV failure develops in this disease. To date, investigation into the contribution of metabolic dysregulation to RV failure in PAH has been limited to animal models\textsuperscript{19}. Here, we use plasma samples, RV tissue, and non-invasive imaging to show evidence of abnormal systemic and myocardial metabolism of fatty acids in human PAH. We demonstrate that circulating FFAs and long chain acylcarnitines are significantly elevated in PAH patients and associated with FA accumulation in the myocardium. In RV tissue from humans with PAH, we show a two-fold increase in ceramide, a lipotoxic and pro-apoptotic triglyceride metabolite\textsuperscript{1,26,30}. High resolution respirometry in a murine model of
PAH suggests that the mechanism underlying lipid accumulation is reduced FAO. Finally, applying proton MRS for the first time in this population, we demonstrate increased in vivo myocardial triglyceride accumulation in PAH.

The emphasis on human data sources in this work provides important, new evidence that animal models of PAH faithfully recapitulate human PAH RV pathophysiology. These observations lend support to the use of animal models of PAH for pre-clinical metabolic interventions. Moreover, we have identified potential therapeutic pathways of interest in humans that can be targeted by existing, well-tolerated metabolic therapies. Finally, we have established a non-invasive imaging tool to study myocardial lipid metabolism in vivo in PAH.

Clinical relevance of the present study

Our data point to two novel mechanisms of myocardial lipid accumulation in PAH: increased delivery and reduced mitochondrial capacity to use FAs to produce energy (Figure 6). In LV failure, insulin resistance—highly prevalent in PAH—and increased adrenergic tone lead to increased circulating FFAs. FFAs are then delivered to the myocardium in excess of mitochondrial oxidative capacity. As a result, lipotoxic triglyceride metabolites and reactive oxygen species accumulate causing apoptosis. Our study suggests that abnormal peripheral FA metabolism is coupled with failure of FA utilization at the mitochondrial level. Elevation of saturated long-chain FAs in the plasma in PAH suggests a possible compensatory mechanism for reduced FAO because saturated FAs are more easily oxidized (whereas unsaturated FAs are more readily used for synthesis); however, this explanation is speculative and would require further investigation. The uniform elevation of fatty acids and reduction of acylcarnitines in the RV suggests that the metabolic abnormalities are most pronounced at the tissue level.

Long-chain acylcarnitines were markedly reduced in the PAH RV despite normal
activity, gene expression, and protein content of mitochondrial transport proteins. By supplementing the RV directly with a long-chain acylcarnitine, we showed that failure of FA utilization is not simply a result of excess delivery or suppression by glucose oxidation but a primary abnormality of the mitochondria. Direct supplementation of palmitoylcarnitine to permeabilized RV tissue negates the need for CPT1b activity, which is the major point of inhibition by the glucose oxidation pathway. Therefore, when the influence of glucose oxidation is removed from the experiment, FAO remains markedly reduced. Thus paired defects in peripheral and myocardial metabolism appear to contribute to RV failure in PAH and both may be viable therapeutic targets.

This is the first study to demonstrate in vivo myocardial lipid accumulation in PAH. Proton MRS has most widely been used to study the effects of systemic metabolic diseases such as diabetes and obesity on the heart. LV steatosis has recently been observed in the setting of increased afterload (severe aortic stenosis), although mean triglyceride content in that study was about half that observed in our PAH cohort. Steatosis decreased after surgical correction of aortic stenosis raising the possibility that steatosis in our study is driven by increased afterload. However, we have previously shown absence of steatosis in a pulmonary artery banding model of isolated RV afterload indicating that pressure elevation alone is not causatory. The inverse relationship between %TG and six minute walk distance in our cohort may reflect an adverse effect of lipid accumulation on both cardiac and peripheral muscle, which we have shown in the BMPR2R899X model. Of note, we found elevated RV lipid in one PAH patient with no functional limitation and normal RV structure and function. This suggests that steatosis is present in subclinical disease and may be a cause and not just a consequence of RV failure. Studies of larger cohorts are underway to determine the relationship between myocardial
steatosis and RV afterload, RV function, and clinical outcomes. It is unclear whether RV lipid deposition might be a modifiable risk factor for right heart failure in PAH. The observation of increased ceramide in the human PAH RV suggests that lipid accumulation may have deleterious functional consequences raising its attractiveness as a therapeutic target.

Elevation of long chain acylcarnitines has also been described in obese, insulin resistant populations. This has been ascribed to increased lipid flux and incomplete FAO, both of which we demonstrated in the PAH RV. We previously showed no association between BMI and insulin resistance in PAH\(^1\), indicating that adiposity does not explain the mechanism of insulin resistance in this population. Recent evidence suggests that long chain acylcarnitines may directly impair insulin signaling, but this has not been tested in humans with PAH or experimental models. In contrast to elevated long chain acylcarnitines in plasma, we found reduced RV long chain acylcarnitines. Reverse function of the acylcarnitine transporter has been described as a mitochondrial detoxification function with active transport into the extracellular space\(^35\). It is possible active export is occurring in the myocardium, although our study did not directly test this hypothesis. More likely, elevated plasma acylcarnitines reflects altered lipid metabolism in other highly metabolically active tissue such as liver and skeletal muscle.

Previous studies have shown reduced myocardial and pulmonary vascular FAO in various experimental models of PAH. For example, we have previously shown reduced acylcarnitines and reduced expression of carnitine transporter genes in human pulmonary microvascular endothelial cells expressing a mutation in BMPR2\(^11\). The RV in the monocrotaline model of PAH demonstrates increased glycolysis and reduced glucose and fatty acid oxidation\(^10\). In the SU5416/hypoxia model, genes encoding acyl-CoA dehydrogenases are downregulated \(^9\). It is unclear whether reduced FAO in these models is adaptive or maladaptive.
When FAO is further inhibited either genetically (knock-out of malonyl-coenzyme A decarboxylase) or pharmacologically (trimetazidine, dichloroacetate) the PH phenotype and RV function improve. A clinical trial is underway to test the effects of dichloroacetate on RV function in PAH (NCT01083524). Given the inability of the PAH RV to use palmitoylcarnitine as a substrate for FAO in our study, it is unclear whether further suppression of FAO will improve myocardial energetics in humans.

Limitations

We were unable to determine the temporal relationship between plasma abnormalities and RV abnormalities because our PAH cohorts predominantly had advanced disease. Despite this limitation, our cohorts are clinically relevant because many PAH patients present with advanced disease and RV dysfunction. Our study involves relatively small sample sizes, especially the experiments involving explanted human tissue. These tissues are critical for translation of findings in experimental models, but are difficult to obtain. RV samples from our control populations were obtained at the time of transplant whereas PAH samples were obtained at autopsy. Although lipid species are unlikely to undergo significant degradation in the time to autopsy, we cannot exclude the possibility that differences in time to storage of RV samples influenced our results. We were underpowered to draw any conclusions about the relative abundance of myocardial lipid accumulation in HPAH compared with IPAH, though this is a focus of future studies. Nonetheless, we demonstrated the feasibility of this technique to assess myocardial metabolism in PAH, even in patients on continuous intravenous medications. We measured myocardial lipid accumulation in the interventricular septum because voxel size requirements of the MRS technique do not currently allow direct measurement of the RV free wall. The relative contribution of the RV and LV to the septum is a matter of dispute, but there
is evidence that the septum shares a common embryologic origin with the right ventricle\textsuperscript{39}.

Regardless, whatever metabolic abnormalities may exist in the RV, they clearly extend to the septum in our study. Finally, we used RV tissue from the BMPR2\textsuperscript{R899X} mouse to measure carnitine transporter protein and oxygen consumption. It is possible that RV metabolism in this model differs from human disease; however, we have shown that fatty acid and acylcarnitine profiles are strikingly similar in the BMPR2\textsuperscript{R899X} and human PAH RVs.

**Conclusions**

Using human blood, RV tissue, and non-invasive imaging, we demonstrate that human PAH is associated with abnormalities in systemic and myocardial FA metabolism. This study highlights specific metabolic pathways of potential therapeutic interest and establishes a tool to study their activity *in vivo*. More detailed study of FA metabolic pathways and their functional significance in human PAH is needed to draw conclusions about their potential as therapeutic targets.

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Clinical Perspective

Right ventricular (RV) failure is the predominant cause of death in patients with pulmonary arterial hypertension (PAH), but the underlying mechanisms are poorly understood. Metabolic dysfunction has recently been implicated as a mechanism of RV failure but studies of myocardial metabolism have been limited to experimental models. In this study, we used blood, tissue samples, and a non-invasive imaging technique to demonstrate that human PAH is associated with circulating and myocardial markers of abnormal fatty acid metabolism. The importance of this work for the practicing clinician is that some of the abnormalities we identified may be effectively targeted with existing and well-tolerated metabolic therapies. Further studies are needed to identify the mechanisms of reduced fatty acid oxidation in the PAH RV, but our findings may support pursuing early phase clinical trials using metabolic therapies to target insulin resistance and fatty acid oxidation. We also identified in vivo myocardial lipid accumulation in humans with PAH using proton magnetic resonance spectroscopy. This technique offers a non-invasive means of studying myocardial lipid metabolism and may be a valuable adjunct to clinical outcomes in future studies of metabolic therapies in PAH. Lastly, metabolic abnormalities in the human PAH RV recapitulate prior findings in experimental models providing support for using animal models to study RV failure in PAH.
Figure Legends:

Figure 1. Elevated Free Fatty Acids and Long-chain Acylcarnitines in PAH Patients Versus Controls. A. Ratio of fasting free fatty acids (FFA) in PAH versus age, gender, and BMI-matched control subjects. FFAs were nearly two-fold higher in 19 PAH patients compared with 22 controls (*p < 0.001). Between-group comparisons performed using the Mann-Whitney U test and data presented as mean ± standard error. B. Log-transformed long chain acylcarnitines in PAH (n = 11) versus age, gender, and BMI-matched controls (n = 19). The long-chain acylcarnitines palmitoylcarnitine (C16), stearoylcarnitine (C18), oleoylcarnitine (C18:1), and linoleylcarnitine (C18:2) were 1.5-2 fold higher in PAH patients compared to control subjects (** p < 0.003 for all). The significance threshold for this experiment was p < 0.003 (0.05/17) after Bonferroni adjustment for multiple comparisons, including all 17 measured acylcarnitines. No other acylcarnitines met the significance threshold. Raw data for all acylcarnitines are provided in Supplemental Table 2. Between-group comparisons performed using the Mann-Whitney U test and data presented as mean ± standard error.

Figure 2. Long chain fatty acid and Acylcarnitine Profiles in the Human PAH Right Ventricle. A. Long Chain Fatty Acids are Increased in Human PAH Right Ventricle. We measured long-chain fatty acids in human right ventricular tissue from PAH patients (n = 3), unmatched donor hearts (n = 5), and dilated cardiomyopathy patients (n = 2). The long chain fatty acids palmitate (C16), stearate (C18), and oleate (C18:1) were increased by 26-37% in PAH compared with control whereas linoleate (C18:2) was 50% higher in control. Values in the DCM RVs tended to be intermediate between PAH and control but linoleate was similarly elevated. ** P < 0.05 and
* P = 0.05 for comparisons between PAH and control. No statistical comparisons were made with DCM group due to availability of only 2 DCM samples. Between-group comparisons performed using the Mann-Whitney U test and data presented as mean ± standard error. B. Long Chain Acylcarnitines are Reduced in the Human PAH Right Ventricle. The long chain acylcarnitines palmitoylcarnitine (C16), stearoylcarnitine (C18), oleoylcarnitine (C18:1), and linoleylcarnitine were markedly reduced (5 to 355-fold lower) in the PAH right ventricle (n = 3) compared with unmatched donor (n = 7) and DCM (n = 6) RVs. Acylcarnitines were measured in ng/mL and log-transformed due to non-normal distribution. **P < 0.05 for all comparisons between PAH and control and ## P < 0.05 vs. DCM. There were no significant differences between DCM and control for any metabolites (p > 0.1 for all). Data presented as mean ± standard error.

Figure 3. Failure of BMPR2<sup>R899X</sup> Right Ventricle to Augment Oxygen Consumption with Long-chain Acylcarnitine Supplementation. We performed high-resolution respirometry on freshly isolated RV from BMPR2<sup>R899X</sup> mice and wild type littermates under baseline conditions and in response to palmitoylcarnitine supplementation (n = 4 per group). When supplemented with palmitoylcarnitine, the wild type right ventricle (RV) augments oxygen consumption rate nearly 9-fold, whereas the PAH RV shows only a modest increase in oxygen consumption (p = 0.0003). Data presented as mean ± standard error. Between-group comparisons performed using the Mann-Whitney U test.

Figure 4. Human Right Ventricular Ceramide in PAH and Controls. A) We performed immunohistochemistry for ceramide and RVs from humans with PAH (n = 5) and control
unmatched donor hearts (n = 8). Ceramide was over two-fold higher in the PAH RVs compared with controls (p = 0.006). B) Mass spectrometric quantification confirmed increase of a long chain (C16:0) and very long chain ceramide (C24:1) in the human PAH RV (n = 5) versus controls (n = 6). Ceramides have previously been observed to cause myocardial lipotoxicity in the left ventricle, but have not been reported in the right ventricle. Data are shown as mean ± standard error. Between-group comparisons performed using the Mann-Whitney U test.

Figure 5. Human Proton Magnetic Resonance Spectroscopy in PAH and Controls. Non-fasting in vivo myocardial triglyceride content was quantified using proton magnetic resonance spectroscopy in PAH patients (n = 6) compared with control subjects (n = 8). Triglyceride content is expressed a percent of triglyceride (%TG) compared with water in a 6cm³ voxel placed in the interventricular septum. A) Representative Cardiac MRI and proton magnetic resonance spectroscopy (MRS) of a control subject demonstrates normal RV function and small lipid peak (LP). PAH patient has RV dilation and hypertrophy with a prominent lipid peak (LP). B) On average, %TG was 7 fold higher in PAH compared with control (1.4±1.3 %TG versus 0.22±0.11, P = 0.02). Intra-patient reproducibility performed on 2 controls and 1 PAH subject was within 0.05%TG. Analysis performed using the Mann-Whitney U test and data presented as mean ± SEM. LP = lipid peak; WP = water peak.

Figure 6. Possible Mechanisms of Right Ventricular Lipotoxicity in Pulmonary Arterial Hypertension. Insulin resistance and adrenergic tone contribute to increased circulating free fatty acids (FA), leading to increased delivery to the myocardium. Inside the cell, FAs can undergo beta-oxidation or be stored as triglyceride. Excess FA delivery in the presence of impaired beta-
oxidation leads to FA accumulation. Toxic triglyceride intermediates are generated, leading to cellular and ultimately organ dysfunction. Markedly reduced myocardial acylcarnitines with normal transporter function suggest either shunting of FAs away from mitochondrial transport or acylcarnitine export. Acylcarnitine export has been described from the kidney and liver\textsuperscript{31}, which may explain elevated plasma levels but decreased myocardial levels. Finally, failure of the BMPR2\textsuperscript{R899X} right ventricle to augment oxygen consumption when directly supplemented with a long-chain acylcarnitine implicates a primary abnormality of mitochondrial respiration.
Figure 2

A. Free Fatty Acids

- Palmitate (C16)
- Stearate (C18)
- Oleate (C18:1)
- Linoleate (C18:2)

B. Acylcarnitines

- Palmitoylcarnitine (C16:0)
- Stearoylcarnitine (C18)
- Oleoylcarnitine (C18:1)
- Linoleylcarnitine (C18:2)
Figure 3

ΔOxygen Flux (pmol/sec/mg tissue)

WT

BMPR2^R899X

**p = 0.0003
Figure 4
Figure 5

A. PAH Control

B. % Myocardial Triglyceride

p = 0.02
Figure 6

- Insulin Resistance
- Adrenergic Tone

↑ FA delivery

FA Transporter

↑ long-chain FA stores

Carnitine Transporters

↓↓ acylcarnitines

Impaired β-oxidation

? export

Carnitine

Mitochondria

Cardiomyocyte

Circulation

TG accumulation

↑ Ceramide, RV dysfunction
Fatty Acid Metabolic Defects and Right Ventricular Lipotoxicity in Human Pulmonary Arterial Hypertension


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Supplemental Materials

Supplemental Table I. Acylcarnitine Tandem Mass Spectrometric Detection

Supplemental Table II. Ceramide Tandem Mass Spectrometric Detection

Supplemental Table III. PAH and Control Group Characteristics for Plasma Studies

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Supplemental Figure I. Correlation of the Oleoylcarnitine and New York Heart Association Class

Supplemental Figure II. Expression of Proteins Involved in Transport and Metabolism of Long Chain Acylcarnitines

Supplemental Figure III. Mitochondrial Carnitine Palmitoyltransferase 1b and 2 in BMPR2<sup>R899X</sup> Right Ventricle

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Supplemental Methods:

- LC-MS Experimental
- Fatty Acid Quantification
- Immunohistochemistry
- High Resolution Respirometry
- Magnetic Resonance Imaging and Proton Spectroscopy
### Supplemental Table I. Acylcarnitine Tandem Mass Spectrometric Detection

<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>SRM Transition(^1)</th>
<th>Collision Energy</th>
<th>S-lens RF Amp</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>400.3 → 85.1</td>
<td>32</td>
<td>120</td>
</tr>
<tr>
<td>C16:0 ((1,2,3,4\text{-}^{13}\text{C}_4))</td>
<td>404.2 → 85.1</td>
<td>32</td>
<td>121</td>
</tr>
<tr>
<td>C18:0</td>
<td>428.3 → 85.1</td>
<td>32</td>
<td>118</td>
</tr>
<tr>
<td>C18:1</td>
<td>426.3 → 85.1</td>
<td>32</td>
<td>122</td>
</tr>
<tr>
<td>C18:2</td>
<td>424.3 → 85.1</td>
<td>32</td>
<td>126</td>
</tr>
</tbody>
</table>

\(^1\) Ar collision gas 1.0 mtorr; scan time 100 ms; Q3 scan width 0.8 m/z; Q1/Q3 peak widths at half-maximum 0.7 m/z.

### Supplemental Table II. Ceramide Tandem Mass Spectrometric Detection

<table>
<thead>
<tr>
<th>Ceramide</th>
<th>SRM Transition(^1,2)</th>
<th>Collision Energy</th>
<th>S-lens RF Amp</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>520.2 → 264.2</td>
<td>23</td>
<td>119</td>
</tr>
<tr>
<td>C24:1</td>
<td>630.2 → 264.2</td>
<td>27</td>
<td>165</td>
</tr>
</tbody>
</table>

\(^1\) Ar collision gas 1.0 mtorr; scan time 25 ms; Q3 scan width 0.5 m/z; Q1/Q3 peak widths at half-maximum 0.7 m/z.

\(^2\) \([\text{M+H}]^+\) precursor ions were not stable in the ESI source. SRM transitions in Table S2 are based on \([\text{M+H-H}_2\text{O}]^+\) precursor.
**Supplemental Table III. PAH and Control Group Characteristics for Plasma Studies**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Free Fatty Acids</th>
<th>Acylcarnitine Profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAH (n = 19)</td>
<td>Control (n = 22)</td>
</tr>
<tr>
<td><strong>Gender, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 (84)</td>
<td>16 (73)</td>
</tr>
<tr>
<td>(% female)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>48±15</td>
<td>44±10</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>31±9</td>
<td>29±7</td>
</tr>
<tr>
<td><strong>6MWD, meters</strong></td>
<td>349±101</td>
<td>560±68</td>
</tr>
<tr>
<td><strong>NYHA class, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4 (21)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>II</td>
<td>8 (42)</td>
<td>5 (45)</td>
</tr>
<tr>
<td>III/IV</td>
<td>7 (37)</td>
<td>5 (45)</td>
</tr>
<tr>
<td><strong>Medications, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3 (17)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>ERA</td>
<td>5 (28)</td>
<td>3 (27)</td>
</tr>
<tr>
<td>PDE5i</td>
<td>5 (28)</td>
<td>2 (18)</td>
</tr>
<tr>
<td>Prostanoid</td>
<td>9 (50)</td>
<td>7 (67)</td>
</tr>
<tr>
<td><strong>HgA1C (%)</strong></td>
<td>5.8±0.6</td>
<td>5.7±0.5</td>
</tr>
<tr>
<td>(n = 15)</td>
<td></td>
<td>(n = 8)</td>
</tr>
<tr>
<td><strong>BNP (pg/mL)</strong></td>
<td>253±386</td>
<td>593±1208</td>
</tr>
<tr>
<td><strong>Creatinine (mg/dL)</strong></td>
<td>0.9±0.2</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td><strong>Hemodynamics</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Right atrial pressure, mmHg 12±6 12±6
Mean PAP, mmHg 54±13 60±14
PWP, mmHg 10±5 10±5
PVR, Wood units 11±7 15±6
Cardiac Index, mL/min/m² 2.2±0.7 2.0±0.5

Echocardiography*

RV Function, n (%)  
Normal/Mild 6 (35) 2 (18)
Moderate 6 (35) 3 (27)
Severe 5 (30) 6 (67)

RV Dilation, n (%)  
Normal/Mild 2 (12) 2 (18)
Moderate 7 (41) 2 (18)
Severe 8 (47) 7 (74)

RV Hypertrophy, n (%) 15 (88) 8 (73)

BMI = body mass index, PAH = pulmonary arterial hypertension

* n = 17 for FFA cohort. Data presented as mean±standard deviation.
## Supplemental Table IV. Acylcarnitines Profiles

<table>
<thead>
<tr>
<th></th>
<th>Case/Control Ratio</th>
<th>Unadjusted t-test p value</th>
<th>Adjusted for Carnitine</th>
<th>Unadjusted t-test p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnitine</td>
<td>1.08</td>
<td>0.462</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcarnitine (C2)</td>
<td>1.55</td>
<td>0.009</td>
<td>1.37</td>
<td>0.043</td>
</tr>
<tr>
<td>Propionylcarnitine</td>
<td>0.98</td>
<td>0.701</td>
<td>0.88</td>
<td>0.272</td>
</tr>
<tr>
<td>Malonylcarnitine (C3):0b</td>
<td>2.65</td>
<td>0.026</td>
<td>4.05</td>
<td>0.114</td>
</tr>
<tr>
<td>Butyrylcarnitine (C4)</td>
<td>0.92</td>
<td>0.820</td>
<td>0.97</td>
<td>0.842</td>
</tr>
<tr>
<td>Valerylcarnitine (C5)</td>
<td>1.23</td>
<td>0.199</td>
<td>1.16</td>
<td>0.260</td>
</tr>
<tr>
<td>Hexanoylcarnitine (C6)</td>
<td>1.71</td>
<td>0.005</td>
<td>1.79</td>
<td>0.013</td>
</tr>
<tr>
<td>Heptanoylcarnitine (C7)</td>
<td>1.32</td>
<td>0.063</td>
<td>1.34</td>
<td>0.061</td>
</tr>
<tr>
<td>Octanoylcarnitine (C8)</td>
<td>1.32</td>
<td>0.143</td>
<td>1.44</td>
<td>0.10</td>
</tr>
<tr>
<td>Nonanoylcarnitine (C9)</td>
<td>0.78</td>
<td>0.464</td>
<td>0.89</td>
<td>0.538</td>
</tr>
<tr>
<td>Decanoylcarnitine (C10)</td>
<td>1.76</td>
<td>0.263</td>
<td>1.38</td>
<td>0.178</td>
</tr>
<tr>
<td>Lauroylcarnitine (C12)</td>
<td>1.93</td>
<td>0.042</td>
<td>1.59</td>
<td>0.028</td>
</tr>
<tr>
<td>Acylcarnitine</td>
<td>2007 Values</td>
<td>2008 Values</td>
<td>p-value</td>
<td>2007 Values</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>Myristoylcarnitine (C14)</td>
<td>2.18</td>
<td>0.003</td>
<td>1.85</td>
<td>0.003</td>
</tr>
<tr>
<td>Palmitoylcarnitine (C16)</td>
<td>1.58</td>
<td>&lt;0.0001</td>
<td>1.54</td>
<td>0.002</td>
</tr>
<tr>
<td>Stearoylcarnitine (C18)</td>
<td>1.55</td>
<td>0.001</td>
<td>1.54</td>
<td>0.005</td>
</tr>
<tr>
<td>Oleoylcarnitine (C18:1)</td>
<td>2.06</td>
<td>&lt;0.0001</td>
<td>2.15</td>
<td>0.001</td>
</tr>
<tr>
<td>Linoleylcarnitine (C18:2)</td>
<td>1.89</td>
<td>0.002</td>
<td>1.91</td>
<td>0.002</td>
</tr>
<tr>
<td>Total acylcarnitines</td>
<td>1.30</td>
<td>0.008</td>
<td></td>
<td></td>
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</tbody>
</table>
Supplemental Table V. Fatty Acid Profiles

<table>
<thead>
<tr>
<th></th>
<th>PAH (n = 11)</th>
<th>Control (n = 19)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>1.5±0.4</td>
<td>1.9±0.6</td>
<td>0.12</td>
</tr>
<tr>
<td>C16:0</td>
<td>23.1±2.0</td>
<td>25.6±3.0</td>
<td>0.02</td>
</tr>
<tr>
<td>C16:1</td>
<td>4.4±1.4</td>
<td>2.9±1.2</td>
<td>0.005</td>
</tr>
<tr>
<td>C18:0</td>
<td>8.1±1.4</td>
<td>11.7±3.5</td>
<td>0.003</td>
</tr>
<tr>
<td>C18:1</td>
<td>38.6±2.0</td>
<td>35.0±3.5</td>
<td>0.004</td>
</tr>
<tr>
<td>C18:2</td>
<td>19.4±1.7</td>
<td>18.8±2.9</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Data presented as mean ± standard deviation.
Elevation of all long-chain acylcarnitines was positively associated with worse New York Heart Association class. The strongest correlation with NYHA class was with oleoylcarnitine (C18:1; $r_s = 0.57, p < 0.001$). Correlation coefficients were calculated using Spearman’s method.
Supplemental Figure II. Expression of Proteins Involved in Transport and Metabolism of Long Chain Acylcarnitines

Gene expression analysis of human right ventricle from patients with pulmonary arterial hypertension (PAH) or dilated cardiomyopathy (DCM) or healthy control subjects. There were no differences across groups (n = 2 per condition) in expression of long-chain acyl-CoA dehydrogenase (ACADL) or genes involved in transport of fatty acids into the mitochondria: carnitine-acylcarnitine translocase (CACT), carnitinepalmitoyl transferase 1 or 2 (CPT1, CPT2), or solute carrier family 22 (organic cation/carnitine transporter) member 5 (SLC22A5). $P > 0.05$ for all across group comparisons by one-way ANOVA.
Supplemental Figure III. Mitochondrial Carnitine Palmitoyltransferase 1b and 2 in BMPR2^{R899X} Right Ventricle

A) Carnitinepalmitoyl transferase 1B1 (CPT1B1) content is not different in BMPR2^{R899X} mouse RV compared with littermate control. B) Similar Carnitinepalmitoyl transferase 2 (CPT2) content in BMPR2^{R899X} mouse RV compared with littermate control. CPT1B1 and CPT2 content were measured by Western and densitometry normalized to β actin. Blot is a portion of a representative blot that was run in duplicate on three animals per group. There was no statistical difference by Student’s t-test between the two groups.
Carnitine palmitoyltransferase I activity in human right ventricle from patients with pulmonary arterial hypertension (PAH), dilated cardiomyopathy (DCM), or healthy control subjects (n = 5 per group). CPT1 is the rate-limiting enzyme in the formation of acylcarnitines. Activity is indexed to protein loaded for each sample. There were no differences across groups (Kruskal Wallis p = 0.93) or directly comparing PAH with control (Mann-Whitney U test p = 0.84). Data are shown as mean ± standard error.
Supplemental Methods

LC-MS Experimental

Materials

Palmitoyl (C16:0) and oleoyl (C18:1) L-carnitines were purchased from Avanti Polar Lipids (Alabaster, AL). Stearoyl (C18:0) L-carnitine chloride and linoleoyl (C18:2) L-carnitine were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Internal standard 1,2,3,4-$^{13}$C$_4$ palmitoyl-L-carnitine chloride (Aldrich, 99 atom % $^{13}$C), [U-$^{13}$C]-palmitoyl CoA lithium salt (Aldrich, 99 atom % $^{13}$C), and formic acid (Fluka, MS grade, 98%) was purchased from Sigma-Aldrich (St. Louis, MO). Malonyl coenzyme A lithium salt (≥90% purity) was purchased from Santa Cruz Biotechnology (Dallas, TX). Double charcoal stripped human plasma (K$_3$ EDTA, 0.2 μm filtered) was purchased from BioreclamationIVT (Hicksville, NY). HPLC-grade water (J.T. Baker) and acetonitrile (J.T. Baker) were purchased from VWR (Radnor, PA).

Instrumentation

Acylcarnitine and ceramide quantification. Sample analyses were carried out using a Waters Acquity UPLC system (Waters, Milford, MA), made up of a binary solvent manager, refrigerated sample manager, and a heated column manager. Tandem mass spectrometric detection was performed using a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an Ion Max API source, a HESI-II probe, and a 50 μm ID stainless steel high voltage capillary. The instrument was tuned and calibrated every four to six weeks over a mass range of m/z 182 to m/z 997 with a mixture of tyrosine peptides using the manufacturer's autotune procedure. Data
acquisition and analysis were carried out using Xcalibur v.2.1.0, Vantage v.2.3.0 and LCQuan v.2.7.0 software (Thermo).

**CPT-1 activity assay.** UPLC-high resolution mass spectrometry was performed using an Acquity UPLC system (Waters, Milford MA) and an LTQ Orbitrap mass spectrometer equipped with an Ion Max API source, a standard ESI probe, and a 50 mm ID stainless steel high voltage capillary (Thermo, San Jose CA). The Orbitrap was calibrated on the day of analysis over a mass range of $m/z$ 195 to $m/z$ 1822 with a solution of caffeine, MRFA, and Ultramark 1621 using the manufacturer’s recommended calibration procedure.

*Liquid chromatographic conditions*

**Acylcarnitine quantification (including CPT-1 activity assay).** A Kinetix C18 analytical column (2.1 mm x 100 mm, 1.7 mm particle size, Phenomenex, Torrance, CA) was used for all chromatographic separations. The column and autosampler tray temperatures were maintained at 50 °C and 5 °C respectively. Mobile phases were made up of 0.2% HCOOH in (A) H$_2$O/CH$_3$CN/CH$_3$OH (3:1:1) and (B) CH$_3$CN/iPrOH (1:1). Gradient conditions were as follows: 0–1 min, B = 0 %; 1–8 min, B = 0–95 %; 8–10 min, B = 95 %; 10–10.5 min, B = 95–0 %; 10.5–15 min, B = 0 %. The flow rate was maintained at 300 µL/min; a software-controlled divert valve was used to transfer eluent from 0–2.0 min and from 10.5–15 min of each chromatographic run to waste. The total chromatographic run time was 15 min. The sample injection volume (partial loop) was 10 µL. The autosampler injection valve and syringe needle were flushed sequentially
with mobile phase B (0.5 mL) and mobile phase A (1.0 mL) between sample injections. To minimize complications resulting from the gradual buildup of hydrophobic tissue components, the column was washed overnight with a solution containing tert-butyl methyl ether (MTBE) after each batch of ~100 samples were injected (CH$_3$CN/i-PrOH/MTBE/H$_2$O, 4:3:2:1).

**Ceramide quantification.** A Kinetix pentafluorophenyl analytical column (2.1 mm x 100 mm, 1.7 µm particle size, Phenomenex, Torrance, CA) was used for all chromatographic separations. The autosampler tray temperature was maintained at 5 °C; the column compartment was not thermostatted. Mobile phases were made up of 0.2% HCOOH in (A) H$_2$O/CH$_3$CN/CH$_3$OH (3:2:2) and (B) CH$_3$CN/iPrOH (1:1). Gradient conditions were as follows: 0–1 min, B = 25 %; 1–11 min, B = 25–100 %; 11–13 min, B = 100 %; 13–14 min, B = 100–25 %; 14–18 min, B = 25 %. The flow rate was maintained at 300 µL/min; the total chromatographic run time was 18 min. The sample injection volume (partial loop) was 10 µL.

**Mass spectrometry**

**Acylcarnitine and ceramide quantification.** The triple quadrupole mass spectrometer was operated in positive ion mode. Quantitation was based on multiple reaction monitoring detection (see Supplemental Tables 4 and 5 for MRM transitions). The following optimized source parameters were used for the detection of analyte and internal standards. Acylcarnitines: N$_2$ sheath gas 30 psi; N$_2$ auxiliary gas 15 psi; spray voltage 4.0 kV; capillary temperature 300 °C; declustering voltage 10 V. Ceramides: N$_2$
sheath gas 60 psi; \( N_2 \) auxiliary gas 5 psi; spray voltage 3.5 kV; HESI probe temperature 386 °C; capillary temperature 300 °C; declustering voltage 5 V. Calibration curves were constructed for acylcarnitines C16:0, C18:0, C18:1, and C18:2 and ceramides C16:0 and C24:1 by plotting peak area ratios (analyte / internal standard) against analyte concentrations for a series of ten calibrants, ranging in concentration from 10 ng/mL to 20 \( \mu \)g/mL. A weighting factor of \( 1/C_i^2 \) was applied in the linear least-squares regression analysis to maintain homogeneity of variance across the concentration range (% error ≤ 20% for at least four out of every five standards).

**CPT-1 activity assay.** The Orbitrap mass spectrometer was operated in positive ionization mode; electrospray source parameters were as follows: \( N_2 \) sheath gas, 33; \( N_2 \) aux gas, 40; spray voltage, 5.0 kV; capillary temp, 300 °C; tube lens voltage, 110 V at \( m/z \) 650; capillary voltage, 32 V; skimmer offset, 0. Full scan spectra of 100-1200 \( m/z \) were acquired in FTMS mode at a resolving power of 30,000 FWHM with the following AGC parameters: FT target, \( 2 \times 10^5 \); 1 microscan; maximum inject time, 200 ms. Selected ion chromatograms were extracted from the full scan data based on the accurate masses (+/- 10 ppm) of the propyl esters of \(^{13}\text{C}_{16}\) palmitoyl-L-carnitine (\( m/z \) 458.44276) and internal standard \(^{13}\text{C}_4\) palmitoyl-L-carnitine (446.40250). Data acquisition and analysis were carried out using Xcalibur v.2.0.7, LTQ Orbitrap v.2.5.5, and LCQuan v.2.7.0 software (Thermo). Response ratios \((^{13}\text{C}_{16}/^{13}\text{C}_4)\) were used to approximate enzyme activity using the following expression:

\[
\text{rate of product formation} = \frac{\text{response ratio} \times [\text{int std}]}{[\text{protein}] \times \text{reaction time}}
\]
Sample Processing

Acylcarnitine quantification. Milligram quantities of acylcarnitine standards were weighed out in aluminum weigh boats using a UMT2 microbalance (Mettler Toledo, Columbus, OH), dissolved in an appropriate volume of methanol to produce primary stock solutions at a concentration of 1–3 mg/mL, and stored in the dark at -20 °C. Working stocks were prepared by serial dilution of primary stocks in CH$_3$OH/H$_2$O (2:1) and stored in the dark at 2–8 °C for up to 4 weeks before use. To avoid complications due to the abundance of endogenous acylcarnitines in most tissue types, charcoal stripped plasma was used as a surrogate matrix for calibrating the instrument response. Plasma was stored at -20 °C in 2-mL polypropylene tubes and allowed to thaw on ice before processing. Aliquots of thawed plasma (50 µL) were transferred to clean microcentrifuge tubes and spiked with the appropriate working stocks of acylcarnitines (2.5 µL) and internal standard 1,2,3,4-$^{13}$C$_4$ palmitoyl-L-carnitine (2.5 µL). Tissue homogenates (50 µL) were spiked with internal standard only. Spiked samples was lightly vortexed, allowed to stand at room temperature for 15–20 min, and deproteinized with 150 µL cold acetonitrile. Precipitated proteins were removed by centrifugation (10,000 x g, 10 min, 5 °C). The clear supernatant (~200 µL) of each sample was transferred to a clean Eppendorf tube and evaporated under a gentle stream of N$_2$ gas. The residue was reconstituted in 100 µL of H$_2$O/CH$_3$OH (3:1), vigorously vortexed, and transferred to 200-µL silanized autosampler vials equipped with Teflon-lined bonded rubber septa.
**Ceramide quantification.** Milligram quantities of ceramide standards (in powder form) were weighed out in aluminum weigh boats using a UMT2 microbalance (Mettler Toledo, Columbus, OH), dissolved in an appropriate volume of EtOH/CHCl$_3$ (3:1) to produce primary stock solutions at a concentration of 0.1–0.2 mg/mL, and stored in the dark at -20 °C. Working stocks were prepared by serial dilution of primary stocks in ethanol and stored in the dark at 2–8 °C for up to 4 weeks before use. PBS was used as a surrogate matrix for calibrating the instrument response. Calibration samples (25 µL PBS) were spiked with the appropriate working stocks of C16:0 and C24:1 ceramides (2.5 µL) and internal standard C12:0 ceramide (2.5 mL). Tissue homogenates (25 µL) were spiked with internal standard only. Spiked samples was lightly vortexed, allowed to stand at room temperature for 15–20 min, and deproteinized with 75 µL cold ethanol. Precipitated proteins were removed by centrifugation (10,000 x g, 10 min, 5 °C). The clear supernatant (~ 80 µL) of each sample was transferred to a 200-µL silanized autosampler vial equipped with a Teflon-lined bonded rubber septa.

**CPT-1 activity assay.** To assess CPT-1 enzyme activity, tissue samples were homogenized to a final protein concentration of 100 µg/mL and incubated in the presence of carnitine and the non-physiological CPT-1 substrate [U-$^{13}$C]-palmitoyl CoA as previously described$^1$. Potassium cyanide was added to the enzyme reactions to minimize degradation products by inhibiting CACT, CPT-II, and other enzymes involved in the β- oxidation pathway. Incubations were done for 10 min in an Eppendorf Thermomixer at 37 °C and terminated by the addition of cold acetonitrile. Following centrifugation, supernatants were transferred to clean tubes, spiked with internal...
standard $^{13}$C$_4$-palmitoyl carnitine, and evaporated completely to dryness under a gentle stream of nitrogen gas. Residues were reconstituted in 100 µL of freshly prepared 1-propanol/acetyl chloride (4:1) and maintained at 65 °C for 15 min to convert the acyl carnitine free acids to their corresponding propyl esters. Derivatized samples were allowed to cool to room temperature and evaporated to dryness under a stream of nitrogen gas. The final residues were reconstituted in 100 µl of H$_2$O:CH$_3$OH (1:1, v/v) and transferred to autosampler vials equipped with low-volume inserts and Teflon-lined rubber septa. The major reaction product [U-$^{13}$C] palmitoyl carnitine was quantified by LC-high resolution MS (vide supra). When enzyme reactions were carried out in the presence of the CPT-1 specific inhibitor malonyl CoA, the rates of product formation were reduced by 80-90%.

**Fatty Acid Quantification**

Fatty acids were extracted using the method of Folch-Lees $^2$, and individual lipid classes were separated by thin layer chromatography. Free fatty acids were scraped from the plates and methylated using BF$_3$ /methanol as described by Morrison and Smith $^3$. The methylated fatty acids were analyzed by gas chromatography using an Agilent 7890A gas chromatograph equipped with flame ionization detectors and a capillary column (SP2380, 0.25 mm x 30 m, 0.25 µm film, Supelco, Bellefonte, PA). Fatty acid methyl esters were identified by comparing the retention times to those of known standards. Inclusion of an internal standard (C15:0) permitted quantitation of the total amount of unesterified fatty acid in the samples.
**Immunohistochemistry**

Briefly, the sections underwent either deparaffinization or fixation with cold acetone, and were blocked with 5% bovine serum albumin. The sections were incubated overnight with primary antibody (ceramide antibody, dilution 1:50, MID 15B4, Enzo Life Sciences, Farmingdale, NY) at 4°C and then treated with biotin labeled secondary antibody (IgM, clone 11/41, 13-5790-82, eBioscience, San Diego, CA) and visualized using Vectastain ABC kit (PK-4001, Burlingame, CA). The slides were then counterstained, dehydrated mounted using Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI) for light microscopic examination. Semiquantitative analysis for ceramide localization was performed in a blinded fashion using Image J64 software.

**High Resolution Respirometry**

At the time of sacrifice, the right ventricular free wall was rapidly dissected and placed into ice-cold respiration buffer (MiR05, prepared as previously described⁴) containing an additional 2 mM EGTA to quantitatively chelate extracellular calcium. Tissue was then permeabilized as previously described ⁴. Briefly, RV tissue was mechanically separated under a dissecting scope into fiber bundles, then transferred to ice-cold buffer containing additional 2 mM EGTA and 50 ug/mL saponin and incubated at 4C for 30 minutes. Tissue was then washed twice in buffer plus 2 mM EGTA at 4C for 15-20 minutes. Pieces of tissue were blotted to dryness, quickly weighed, and 2-4 mg tissue placed into the pre-equilibrated chambers of an O2k Oxygraph (Oroboros Instruments, Innsbruck) containing buffer without additional EGTA. Chambers were closed, and oxygen consumption rate was recorded with sequential additions of glutamate (10 mM), malate
(2 mM), and palmitoylcarnitine (50 µM). Data were recorded as the mass specific oxygen flux (pmol/min/mg tissue).

*Magnetic Resonance Imaging and Proton Spectroscopy*

Imaging parameters were: echo time, 1.56 milliseconds; repetition time, 3.1 milliseconds; flip angle 40 degrees; slice thickness, 8mm with a gap of 2mm; field of view, 350 x 350mm; and reconstructed matrix size, 352 x 352. Electrocardiographic and respiratory gating were used at end-systole and end-expiration to minimize motion artifact. A 2x1x3cm³ voxel was placed over the interventricular septum to avoid contamination from epicardial fat. Water (suppressed and unsuppressed) spectra were collected to serve as an internal reference for chemical shift offset and fat concentration⁵. PRESS spectra (echo time, 30 milliseconds) with and without water suppression were performed to quantify fat measurements⁶. Peak amplitudes of water and fat were measured using an on-line spectral processing software (Spectroview; Philips Medical Systems, The Netherlands). Myocardial triglyceride content was expressed as a percentage relative to water based on the formula: signal amplitude of triglyceride divided by signal amplitude of water x 100⁵.
REFERENCES


