Evidence for Intramyocardial Disruption of Lipid Metabolism and Increased Myocardial Ketone Utilization in Advanced Human Heart Failure

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Background—The failing human heart is characterized by metabolic abnormalities, but these defects remains incompletely understood. In animal models of heart failure there is a switch from a predominance of fatty acid utilization to the more oxygen-sparing carbohydrate metabolism. Recent studies have reported decreases in myocardial lipid content, but the inclusion of diabetic and nondiabetic patients obscures the distinction of adaptations to metabolic derangements from adaptations to heart failure per se.

Methods and Results—We performed both unbiased and targeted myocardial lipid surveys using liquid chromatography-mass spectroscopy in nondiabetic, lean, predominantly nonischemic, advanced heart failure patients at the time of heart transplantation or left ventricular assist device implantation. We identified significantly decreased concentrations of the majority of myocardial lipid intermediates, including long-chain acylcarnitines, the primary subset of energetic lipid substrate for mitochondrial fatty acid oxidation. We report for the first time significantly reduced levels of intermediate and anaplerotic acyl-coenzyme A (CoA) species incorporated into the Krebs cycle, whereas the myocardial concentration of acetyl-CoA was significantly increased in end-stage heart failure. In contrast, we observed an increased abundance of ketogenic β-hydroxybutyryl-CoA, in association with increased myocardial utilization of β-hydroxybutyrate. We observed a significant increase in the expression of the gene encoding succinyl-CoA:3-oxoacid-CoA transferase, the rate-limiting enzyme for myocardial oxidation of β-hydroxybutyrate and acetoacetate.

Conclusions—These findings indicate increased ketone utilization in the severely failing human heart independent of diabetes mellitus, and they support the role of ketone bodies as an alternative fuel and myocardial ketone oxidation as a key metabolic adaptation in the failing human heart. **(*Circulation*. 2016;133:706-716. DOI: 10.1161/CIRCULATIONAHA.115.017545.)**

Key words: cardiomyopathies ■ heart failure ■ ketones ■ lipids ■ metabolism

Under normal conditions, the turnover of ATP in the human heart is ≈6 kg, or the equivalent of 12 times its own weight, to perform the daily work of circulating 7 tons of blood. Heart failure (HF) is a progressive condition where the heart cannot meet this physiological demand. Despite advances in the treatment of this syndrome, our understanding of the energy metabolic mechanisms limiting cardiac pump function remains incomplete.

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Although there is consensus that HF is characterized by metabolic abnormalities, the nature of these defects remains controversial. Under normal circumstances, fatty acids (FAs) are the predominant energetic substrate for the heart, with β-oxidation providing 50% to 70% of myocardial ATP need.[1] After transport into cardiomyocytes, FAs are imported into mitochondria, via acylcarnitine intermediates, converted to acyl-coenzyme A (CoA) thioesters, then oxidized via β-oxidation or reesterified into triglycerides and stored.[1] The state of metabolism in the failing human heart has been more difficult to discern. In animal models of HF there is a quantitative switch from a predominance of FA utilization to the more oxygen-sparing carbohydrate metabolism.[2] Consistent with this altered substrate utilization, Chokshi et al[2] reported decreases in myocardial triglyceride and overall FA content but did not report specifically on changes in myocardial acylcarnitine content. In complementary studies, Gupte et al[3] reported substantially decreased medium- and short-chain acylcarnitines, including C2 acylcarnitine, a surrogate for acetyl-CoA, but did

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not report differences in the medium- and long-chain acylcarnitine species, especially those that are derived from FAs that are predominant in the human diet (palmitate and oleic acid).

Despite these observations, an integrated signature of metabolism in the nondiabetic failing human heart has not been defined. A particularly important factor confounding elucidation of the metabolic signature of human HF per se is the inclusion of metabolic comorbidities (diabetes mellitus, obesity, insulin resistance) that may affect myocardial metabolism and substrate utilization independent of HF. Accordingly, we performed both an unbiased and targeted myocardial lipid survey using liquid chromatography-mass spectroscopy in nondiabetic, advanced HF patients at the time of orthotopic heart transplantation or left ventricular assist device implantation. In these nondiabetic patients, we identified significantly decreased concentrations of the majority of myocardial lipids, including long-chain acylcarnitines, the primary subset of the energetic lipid substrate for mitochondrial FA oxidation in advanced HF. We also report for the first time significantly reduced levels of long-chain acyl-CoA species incorporated into the Krebs cycle. In contrast, we observed an increased abundance of ketogenic β-hydroxybutyryl-CoA, in association with a decrease in myocardial β-hydroxybutyrate (βOHB) and increased circulating ketones suggesting increased use of ketones as fuel in the severely failing human myocardium from nondiabetic patients. The novel observation of metabolite and gene expression signatures consistent with increased ketone oxidation in the chronically failing heart combined with alterations in the abundance of acetyl-CoA (increased) and succinyl-CoA (decreased) supports a hypothesis that the use of ketone bodies as an alternate fuel could influence carbon flux contributing to the energy metabolic derangements in HF.

Materials and Methods

Human Heart Procurement

Whole human hearts were procured from 2 separate patient groups: nonfailing brain dead organ donors with no history of HF (nonfailing [NF]) and end-stage HF transplant patients' hearts (F) that were obtained at the time of orthotopic heart transplantation. All hearts received in situ cold cardioplegia and were placed on wet ice in 4°C Krebs-Henseleit buffer. Transmural left ventricular samples, excluding epicardial fat, were snap frozen in liquid nitrogen and stored at –80°C. All study procedures were approved by the University of Pennsylvania Hospital Institutional Review Board, and prospective informed consent for research use of heart tissue was obtained from all transplant recipients and next-of-kin in the case of organ donors

Patient Cohort

The cases include n=15 patients with chronic dilated nonischemic cardiomyopathy. NF controls (n=20) were selected to match on age, no history of diabetes mellitus, and nonobese status by body mass index criteria (body mass index <30). A spectrum of donor heart nonfailing phenotypes was available and included 8 subjects with significantly increased left ventricular mass indicative of left ventricular hypertrophy (measured ex vivo at the time of tissue procurement and indexed to body surface area).[4]

The cohort consisted of patients with chronic, severe American Heart Association/American College of Cardiology stage D HF. Transmural samples of the left ventricular myocardium were obtained from the apex at the time of left ventricular assist device implantation (n=7) or the free wall at the time of heart transplantation without left ventricular assist device (n=8). Exclusion criteria for the failing cohort included diabetes mellitus, obesity (body mass index >30), myocarditis (viral, autoimmune), infiltrative or hypertrophic cardiomyopathy, hepatitis B, hepatitis C, or human immunodeficiency virus, or severe renal disease. Nonfailing control samples were derived from hearts of brain dead organ donors with no history of HF, no history of diabetes mellitus, and no history of obesity (total n=20). Subjects in the NF cohort with a left ventricular ejection fraction of <45% were excluded. The initial untargeted lipidomics for discovery were performed with myocardial samples derived from 7 end-stage failing patients and 10 nonfailing donors. The subsequent validation with targeted and untargeted lipidomics were performed on 8 additional patients with the same inclusion and exclusion criteria and 10 additional NF donor subjects.

Lipodomic Profiling

Methods are described in detail in the online-only Data Supplement. In brief, ≈50 mg (±0.1 mg) heart was extracted by a modified Folch liquid-liquid extraction and analyzed with liquid chromatography (LC)–nano electrospray–high-resolution mass spectrometry (HRMS) with minor modifications from previous methods[5,6] using [$^{2}H_{2}{}^{13}C_{2}$]-Cer-(d18:1/C18:0) (Avanti Polar Lipids, Alablaster, AL) and [$^{13}C_{4}$] palmitoylcarnitine (Sigma) as internal standards. Acyl-CoA species were extracted and analyzed by stable isotope dilution-LC-tandem mass spectrometry (SID-LC-MS/MS) as previously described[7,8] using internal standards generated from [$^{13}C_{3}{}^{15}N_{1}$] pantothenate (Isosciences, King of Prussia, PA) by *pan6*-deficient yeast culture for long-chain species[9] or in Hepa1c1c7 cells for short-chain species.[10]

RNA Isolation and cDNA Amplification

RNA was isolated from human left ventricle samples by using the Qiagen miRNeasy Mini kit (Qiagen Inc, Germantown, MD) with on-column DNase treatment. RNA quantity was measured by Broad Range RNA assay for the Qubit (Invitrogen). Two micrograms of RNA was reverse transcribed by using the ABI High Capacity cDNA Reverse Transcription Kit. The cDNA was measured by using the Single-Stranded DNA kit for the Qubit then diluted to 10 ng/μL. Four microliters of the diluted cDNA from each patient was added to a mix of TaqMan Universal Master Mix along with appropriate TaqMan assay. Probes and primers used are listed in (online-only Data Supplement Table I). All samples and reagents were mixed and dispensed using the Biomek 4000 (Beckman Coulter).

Quantitative Real-Time Polymerase Chain Reaction

Real-time analysis was performed on a 7900HT Real-time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's recommended protocol. Raw cycle threshold (Ct) values were calculated by using SDS 2.4 and RQ manager 1.2 software (Applied Biosystems) applying automatic baselines and threshold. The resulting value is the ΔCt. The difference in ΔCt (ΔΔCt) was then calculated by subtracting the average ΔCt of the endogenous control RPL5 from the ΔCt of target genes.

Ketone Body Assay

Commercially available colorimetric assay (Cayman Chemicals, item #700190, Ann Arbor, MI) were used according to the manufacturer's directions to determine total blood and myocardial β-hydroxybutyrate (βOHB). Systemic venous blood and myocardium were collected from n=24 patients (n=12 NF donors; n=12 dilated cardiomyopathy [DCM]). The assay was performed in triplicate and compared with the supplied standard. The 96-well assays contained either blood or myocardium, and 50 μL of sample was added per well to 50 μL of developer solution and incubated at 25°C in the dark. Absorbance was read at 450 nm. A protein bicinchoninic acid assay was performed to determine protein-corrected ketone body concentrations in the myocardium, whereas the serum samples were normalized by volume.

Nonesterified FA Assay

A commercially available colorimetric assay (Wako Diagnostics HR Series NEFA-HR, product numbers: 999-34691, 991-34891,

Table 1. Subject Characteristics

Average	Nonfailing (n=20)	Failing (CHF, n=15)	LVAD (n=7; Included in Failing)	*P* Value
Age	54.1±11.92	50.47±11.76	50±10.6	0.58
Sex	12 male 8 female	13 male 2 female	6 male 1 female	N/A
BMI, kg/m^2	25.87±3.75	25.65±5.30	29.78±6.52	0.149
Etiology	20 NF	13 DCM 2 ICM	5 DCM 2 ICM	N/A
LVEF, %	59.03±16.44	17.5±9.9	21.67±12.58	<0.005
Heart weight index, g/m^2	201.66±17.93	267.21±49.12	n/a	<0.005
BSA, m^2	1.89±0.21	1.98±0.21	2.07±0.23	0.143

The failing (CHF) group includes myocardial procurement at the time of heart transplant (n=8) and at the time of LVAD implantation (n=7). BMI indicates body mass index; BSA, body surface area; CHF, congestive heart failure DCM, dilated cardiomyopathy, ICM, ischemic cardiomyopathy; LVAD, left ventricular assist device; LVEF, left ventricular ejection fraction; N/A, not available; and NF, nonfailing.

993-35191) was used. In brief, serum from the previous 24 patients was used to characterize their nonesterified fatty acid (NEFA) content. The samples were normalized by volume and absorbance was read at 550 nm to determine NEFA in serum samples.

Statistical Analysis

A univariate statistical approach with no correction for multiple comparisons was chosen for discovery metabolomics. As Franceschi et al[11] have demonstrated, multiple comparison corrections in metabolomics do not prevent false positives completely and induce a large penalty in false negatives. Because the purpose of the discovery experiment was to enable further validation experiments (using the gold standard stable isotope dilution LC-MS), we considered the risk of type I errors, which would be then corrected by more analytically appropriate experiments to be low. Type II errors, however, would preclude the application of any validation experiments, thus resulting in an erroneous finding that would not be corrected by our experimental approach.[12] Nevertheless, to test the robustness of our results, we applied a false-discovery rate adjustment in the lipidomic analysis of the validation cohort and report these results in the online-only Data Supplement Material. Furthermore, multivariate statistical methods would be unsuited for our task because of the limited sample sizes imposed by the nature of the tissue, and the difficulty of analytic quality control in untargeted experiments versus the stringent quality control afforded by stable isotope internal standards. Analysts were blinded to heart classification during extraction, then unblinded for data analysis using SIEVE (Thermo Scientific), Metaboanalyst 2.0,[13] and GraphPad v5. To identify differential abundance in myocardial metabolite and gene expression levels, a P value of <0.05 was considered significant, as derived from the Welch unpaired t test or the nonparametric Mann-Whitney test.

Results

Global Lipidomics Reveals a Reduced Abundance of Lipids in DCM

Our discovery cohort consisted of 7 nondiabetic end-stage failing versus 10 nondiabetic, nonfailing donor hearts. Based on results from the discovery cohort, a validation set of heart tissue was analyzed by targeted and untargeted stable isotope dilution (SID) lipidomic analysis by nano electrospray ultra-performance LC-HRMS in positive ion mode with 8 failing hearts and 9 nonfailing hearts from nonobese, nondiabetic subjects (online-only Data Supplement Table II). The baseline characteristics including myocardial structure and function of the combined discovery and validation cohorts, stratified by nondiabetic failing and nonfailing status, are summarized in Table 1.

Heatmap projection of the total 4186 features detected by LC-HRMS lipid species indicated an overall decrease in FA species in the failing hearts. To interrogate the differences between failing and nonfailing hearts, differential features were sorted by an unpaired t test with the Welch correction followed by manual inspection to examine peak shape and correct automated peak integration. The remaining 63 differentially abundant features were plotted again on a heatmap, demonstrating a striking depletion of lipid species abundance in the nondiabetic, failing hearts (Figure 1).

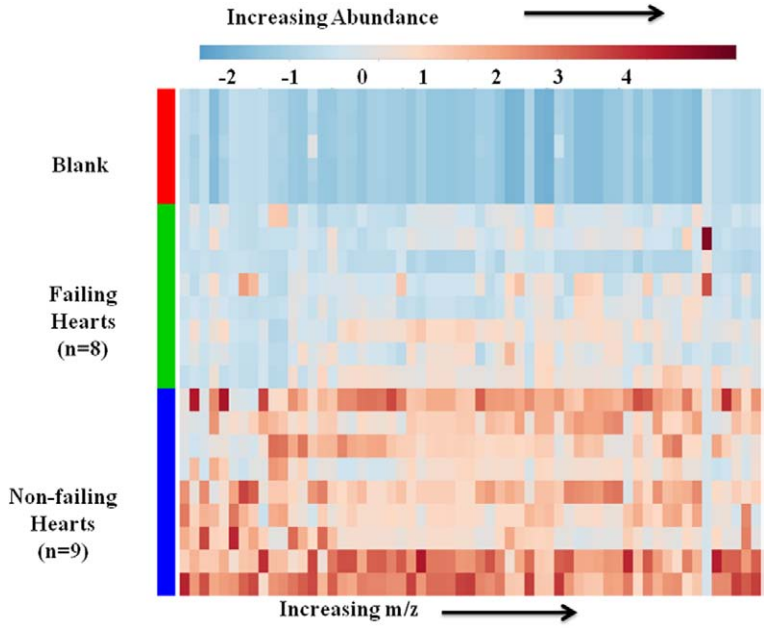

Figure 1. LC-HRMS lipidomic analysis reveals a depletion of lipid features from failing human hearts. Heatmap plot of 63 differentially abundant LC-HRMS lipid features from failing and nonfailing hearts. Differential features were sorted by P value of <0.01 using the Welch unpaired t test and manual curation for chromatographic peak shape and integration. Features that were differentially abundant between failing and nonfailing hearts were mostly depleted in the failing hearts. LC-HRMS indicates liquid chromatography–high-resolution mass spectrometry.

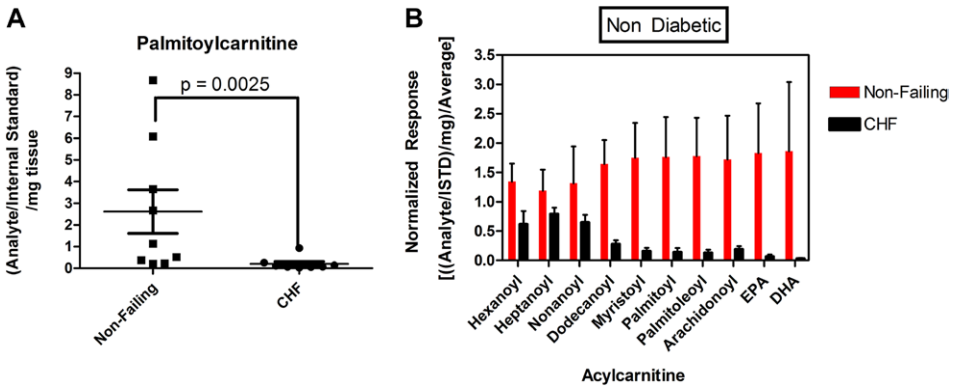

Figure 2. SID-LC-HRMS analysis indicates a reduction in acylcarnitine species in failing versus nonfailing hearts. Significant reductions, by Mann-Whitney nonparametric testing, were seen in palmitoylcarnitine (**A**) and the detected acylcarnitine profile for nonfailing (hatched) versus failing (solid black) heart tissue (**B**). CHF indicates congestive heart failure; LC-HRMS, liquid chromatography–high-resolution mass spectrometry; SID, stable isotope dilution; and STD, standard deviation.

Based on the findings of differentially abundant FA intermediates or derivatives in the discovery cohort, analysis of the acylcarnitine profile from the human heart tissues was undertaken by SID-LC-HRMS using [$^{13}C_4$]palmitoylcarnitine as a stable isotope internal standard to adjust for extraction and analysis. Assignment of chromatographic peaks to acylcarnitine species was done by an accurate mass of ≤3 ppm, retention time consistent with an acylcarnitine in the chromatographic method (<10 minutes), and MS/MS fragmentation pattern (online-only Data Supplement Figure I). The identity of the peak corresponding to palmitoylcarnitine was also confirmed by coelution with the [$^{13}C_4$]palmitoylcarnitine internal standard. SID-LC-HRMS analysis revealed a >10-fold reduction in palmitoylcarnitine in failing hearts (mean nonfailing versus failing, 2.617 versus 0.2076, P=0.0025) (Figure 2A, online-only Data Supplement Figure II). Quantitation of other acylcarnitines by LC-HRMS revealed a depletion of similar magnitude in failing hearts of acylcarnitines from hexanoylcarnitine (C6) to docosahexaenoyl carnitine (C22:6) (P<0.0001) (Figure 2B).

Quantitation of Acyl-CoA Species and Krebs Cycle Intermediates Reflects Metabolic Dysregulation in DCM

Quantification of acyl-CoAs by SID-LC-MS/MS in the myocardium from nondiabetic HF patients, in comparison with nondiabetic nonfailing subjects, revealed a significant decrease in succinyl-CoA (average 10.5 versus 17.7 pmol/mg, P=0.023), propionyl-CoA (average 0.9 versus 1.8 pmol/mg, P=0.02), and an increase in β-hydroxybutyryl (3-Hydroxybutanoyl)-CoA (average 0.57 versus 0.29 pmol/mg, P=0.015) (Table 2). This decrease in succinyl-CoA was observed while an increase in acetyl-CoA was found in end-stage failing myocardium (average 15.0 versus 7.7 pmol/mg, P=0.028). The ratio of myocardial succinyl-CoA to acetyl-CoA, a potential marker of Krebs cycle, is significantly decreased in end-stage HF (0.84 versus 1.93, P=0.003) (Figure 3A), and the absolute level of β-hydroxybutyryl-CoA, a ketogenic substrate derived when FA oxidation rates exceed the tricarboxylic acid cycle, was 2-fold higher in the failing group (Figure 3B). We also performed absolute myocardial quantitation of succinate, derived from succinyl-CoA and fumarate, derived from the oxidation of succinate-metabolites incorporated in the second phase of the Krebs cycle. A nonsignificant trend for decreased succinate (97.4 versus 55.6 ng/mg, P=0.105) and a significant decreased level of fumarate (17.4 versus 26.4 ng/mg, P=0.01) was identified in end-stage failing myocardium in comparison with nonfailing (online-only Data Supplement Figure 3). Medium- to long-chain acyl-CoAs showed no or very weak differences between failing and nonfailing subjects, with the exception of dodecanoyl-CoA (lauroyl-CoA), which was depleted in the end-stage failing heart (Table 2).

Decreased Myocardial Lipids Is Not Attributable to Reduced Peripheral Substrate Availability in HF

The myocardial utilization of substrate is correlated with peripheral availability, especially in the case of lipids and ketone bodies. If the identification of a significant decrease in energetic myocardial lipids were reflective of the peripheral

Table 2. Total Short-Chain and Medium-Chain* Acyl-CoA Profile of Nonfailing Versus Failing Hearts Quantified by SID-LC-MS/MS

	Nonfailing		Failing	
Acyl-CoA	pmol/mg	SEM	pmol/mg	SEM
CoASH	0.32	0.03	0.19	0.04
Acetyl	7.74	1.45	14.98	5.53
Propionoyl	1.76	0.20	0.89	0.18
Succinyl	17.74	2.35	10.54	2.22
Butanoyl	3.04	0.92	2.28	0.32
Malonyl	1.83	0.19	1.95	0.20
3-Hydroxybutanoyl	0.29	0.05	0.57	0.13
Hexanoyl*	0.16	0.04	0.16	0.04
Octanoyl*	0.11	0.03	0.09	0.02
Decanoyl*	0.13	0.02	0.09	0.02
Dodecanoyl*	0.12	0.03	0.06	0.01

CoA indicates coenzyme A; SEM, standard error of the mean; and SID-LC-MS/MS, stable isotope dilution-liquid chromatography-tandem mass spectrometry.

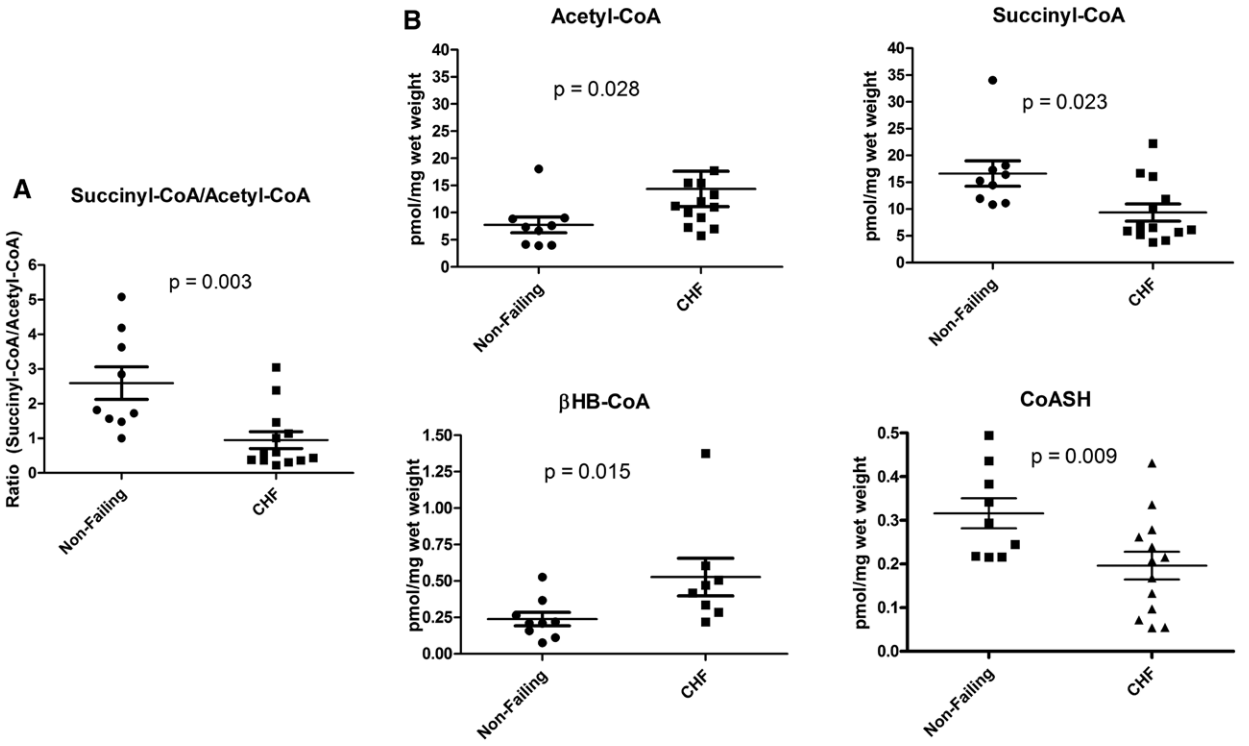

Figure 3. Acyl-CoA quantitation by SID-LC-MS/MS analysis demonstrates metabolic alterations. **A,** The ratio of succinyl-CoA/acetyl-CoA is significantly decreased in failing myocardium. **B,** A significant change in failing hearts of short-chain acyl-CoA species, including the ketogenic 3-hydroxybutanoyl-CoA (βHB-CoA), is observed in comparison with nonfailing hearts. A Mann-Whitney nonparametric test was performed for each of these comparisons. CHF indicates congestive heart failure; CoA, coenzyme A; and SID-LC-MS/MS, stable isotope dilution-liquid chromatography-tandem mass spectrometry.

milieu, a decreased concentration of plasma free FAs would be present in the patients with end-stage HF. However, we have identified a significant increase in the plasma concentration of NEFAs in nondiabetic patients with advanced HF at the time of cardiac transplantation in comparison with the nonfailing subjects (Figure 4: mean 1.03 mmol/L versus 0.56 mmol/L, P=0.016 by unpaired t test with the Welch correction).

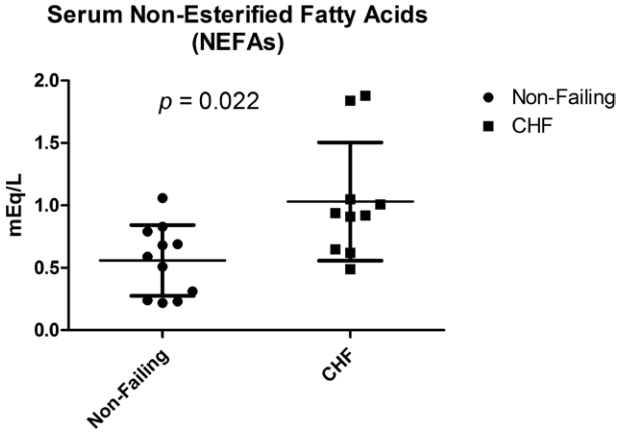

Figure 4. Nonesterified fatty acids (NEFAs) in nondiabetic patients. NEFA in nonfailing and nondiabetic DCM patients demonstrate significantly increased circulating free fatty acids in the advanced heart failure cohort (Mann-Whitney nonparametric test). CHF indicates congestive heart failure; and DCM, dilated cardiomyopathy.

Downregulation of Lipid Storage and Carnitine Transport Genes in Advanced HF

The selected panel for quantification with real-time polymerase chain reaction included genes implicated in a broad range of lipid metabolism: transport, storage, and energetics. Online-only Data Supplement Table III is the list of all genes assayed; fold change is expressed as the myocardial expression in failing relative to nonfailing. All genes assayed were relatively abundantly expressed in myocardium in comparison with the housekeeping gene RPL5. Differential expression was plotted against RPL5 and significance was determined by using the Welch corrected unpaired t test and false-discovery rate <P=0.05 (Figure 5). Among the genes significantly decreased in the DCM myocardium were the high-affinity carnitine transporter, SLC22A5 (P=0.003), perilipin 2 (P<0.0001), PLA2G2A (P=0.0003), and the phospholipid transport protein PTLP (P=0.001). In comparison with nonfailing, we observed no significant differences in the myocardial expression of key regulators of FA β-oxidation, carnitine palmitoyltransferases (CPT1a, CPT1b, CPT2), or the peroxisome proliferator–activated receptor alpha (PPARα) (online-only Data Supplement Table III).

Evidence for Utilization of Ketone Bodies as an Alternative Fuel in Advanced HF

We measured paired serum and myocardial βOHB in subjects with DCM and nonfailing donor subjects. There was an increased concentration of serum ketone bodies present in the DCM group (mean, 145 μmol/L in DCM versus 19 μmol/L

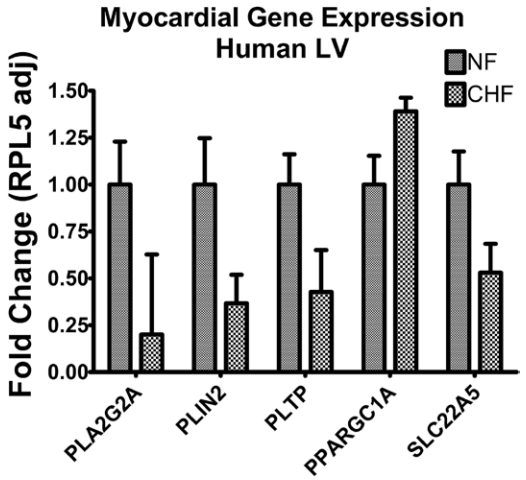

Figure 5. Myocardial gene expression. Adjusted for the housekeeping gene RPL5, myocardial gene expression is reported as a fold change in 21 failing samples relative to transcript abundance in 21 nonfailing samples. The transcripts with differential gene expression by parametric (*t* test with the Welch correction) testing include phospholipase A2 (PLA2G2A), perilipin 2 (PLIN2), phospholipid transfer protein (PLTP), peroxisome proliferator-activated receptor γ coactivator (PGC-1α Fold Change 1.49, *P*=0.0008, Solute Carrier Family 22 (organic cation/carnitine transporter) member 5 (SLC22A5). CHF indicates congestive heart failure; LV, left ventricle; and NF, nonfailing.

in the NF group, *P*=0.02), but myocardial ketone bodies were decreased in the DCM patients (mean, 110 μmol/L in DCM versus 179 μmol/L in NF, *P*=0.005, Figure 6). The ratio of serum to myocardial ketone bodies, an index of the utilization of βOHB in the heart, is significantly increased in patients with end-stage HF (ratio in failing=1.48 versus 0.22 in NF, *P*=0.019).

To determine whether the gene regulatory program driving the utilization of ketone bodies is activated in HF, we examined the myocardial expression of genes implicated in the processing of exogenous ketone bodies to acetyl-CoA (Figure 7). We identified increased expression of 3-D-hydroxybutyrate dehydrogenases (BDH1 and BDH2), enzymes involved in the conversion of ketone bodies back into acetyl-CoA for combustion and energy production via the Krebs cycle. We also identified a significant increase in the levels of mRNA encoding the rate-limiting enzyme of ketone body metabolism, 3-oxoacid-CoA-transferase (OXCT1). Conversely, hydroxymethylglutaryl-CoA synthase, the rate-limiting enzyme for primarily hepatic production of ketones from acetyl-CoA (ketogenesis), is significantly downregulated in the myocardium of DCM.

Expression of SCOT (OXCT1) Is Correlated With Myocardial Succinyl-CoA in DCM

Recognizing that OXCT1 (also known as SCOT) is essential for conversion of acetoacetyl-CoA into 2 acetyl-CoA molecules that can enter the tricarboxylic acid cycle for oxidation and ATP production,[14] we examined the relationship between SCOT and myocardial succinyl-CoA. This analysis identified a very significant positive correlation only in the DCM group (Figure 8). There was no correlation observed in the nonfailing nondiabetic subjects.

Myocardial Lipid Signatures of Pathological Hypertrophy Versus Failure

In a subset of 8 nonfailing (left ventricular ejection fraction>55%) subjects with myocardial hypertrophy by indexed heart weight to body surface criteria, we investigated whether the changes in lipid species and the expression of ketolytic oxidation genes that were observed in end-stage failing myocardium would also be present in the hearts with left ventricular hypertrophy (LVH). In this comparison, we observed no lipid species concordance between the LVH and DCM groups. The only significant difference in the abundance of myocardial lipid species, in comparison with the nonhypertrophied group, was identified in the end-stage failing group of patients. No difference in any of the medium- and long-chain acylcarnitines was present between the hypertrophied and nonhypertrophied nonfailing hearts. In fact, our principal component analysis of the 3 groups—failing, LVH, and nonfailing—depicts the separation in myocardial lipid abundance that is driven by a marked decrease in the end-stage failing group only (see Heatmap and principal component analysis plot in online-only Data Supplement Figures IV and V, respectively). An unsupervised cluster analysis (online-only Data Supplement Figure VI) of all the probed lipid species (4186) correctly separated the clinical phenotypes into 2 groups: end-stage failing patients versus nonfailing hearts

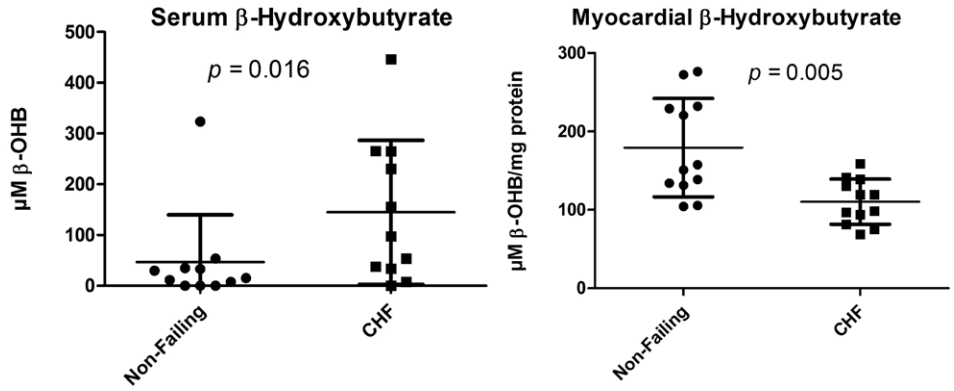

Figure 6. Paired serum and myocardial measurements of β-hydroxybutyrate. There is a marked increase in systemic blood in DCM patients, whereas there is a marked decrease of β-hydroxybutyrate within the failing myocardium from the paired data, implicating a process of increased myocardial ketone utilization. The *P* values reflect a Mann-Whitney nonparametric test. CHF indicates congestive heart failure; DCM, dilated cardiomyopathy; and βOHB, β-hydroxybutyrate.

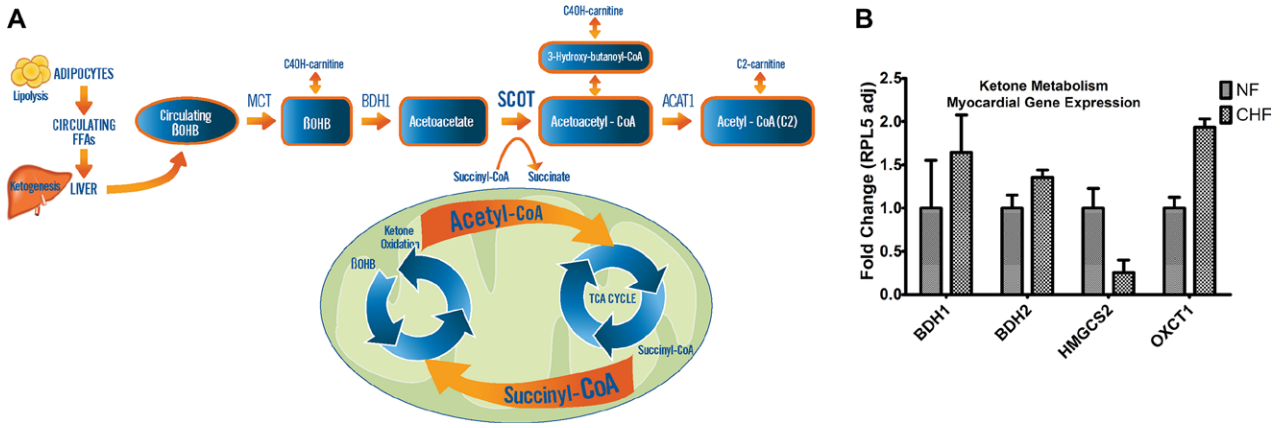

Figure 7. Increased expression of the genes implicated in ketone oxidation was identified, including β-hydroxybutyrate dehydrogenase type 1 and 2 (BDH1 and BDH2, *P*=0.01 for both) and 3-oxoacid-CoA transferase 1 (OXCT1) also known as succinyl-CoA:3-oxoacid CoA transferase (SCOT, *P*=0.0006) in advanced heart failure (n=21 samples), relative to nonfailing (n=21 samples). Note that the enzyme involved in ketogenesis, 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) is decreased in the myocardium of end-stage failing patients (*P*=0.006). **Top,** The illustration demonstrates the proposed link between the decreased pool of succinyl-CoA that is necessary for Krebs (TCA) cycling and the presence of ketone oxidation as the rate limiting enzyme OXCT1 (also known as SCOT) requires succinyl-CoA as a CoA donor for acetoacetate to yield acetoacetyl-CoA. Increased myocardial ketone oxidation could also explain the increased pool of acetyl-CoA that was identified in end-stage failing myocardium, especially with the disruption in the Krebs cycle. ACAT1 indicates acetyl-CoA acetyltransferase 1; CHF, congestive heart failure; CoA, coenzyme A; C4OH-carnitine, hydroxybutyrylcarnitine; MCT, monocarboxylate transporter; NF, nonfailing; βOHB, β-hydroxybutyrate; and TCA, tricarboxylic acid.

from nondiabetic donors with and without LVH. Subject 1305, who was not insulin requiring but did have a history of using oral glycemic agents, was the only subject who was not classified correctly. Similar to what we observed with the abundance of lipid species, the expression of myocardial genes encoding the enzymes for ketone oxidation were not significantly different in LVH (online-only Data Supplement Figure VII) with the differentially increased expression only observed in end-stage failing myocardium.

Discussion

In this case control study of nondiabetic advanced HF, we report a significantly decreased abundance in end-stage failing myocardium of medium- to long-chain acylcarnitines, the primary subset of energetic lipid substrate for mitochondrial FA oxidation. Despite an increased level of acetyl-CoA in the myocardium, we observed significantly reduced levels of second-span Krebs cycle intermediates, including succinyl-CoA, fumarate, and the anaplerotic propionyl-CoA. In contrast, we identified signals suggesting increased use of ketones as fuel in nondiabetic failing myocardium: an increased abundance of ketogenic βhydroxybutyryl-CoA and a decreased level of myocardial βOHB despite increased circulating levels of ketones. Finally, it appears that these metabolic characteristics emerge in the severely failing hearts, but not in nonfailing donor hearts with significant pathological LVH. Thus, these

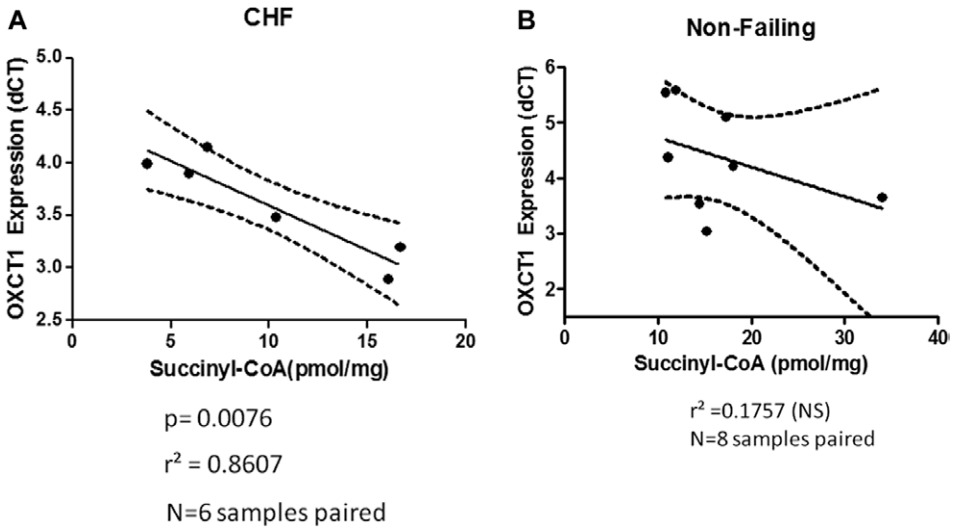

Figure 8. A, A positive correlation of increased OCXT1 gene expression when succinyl-CoA is abundant. Expression is reciprocal because it is reported as ΔCt. *P*=0.0076; r^2=0.8607; n=6. A Pearson correlation was calculated and the *P* value reported is calculated from an *F* test with the null hypothesis that the overall slope is zero. **B,** No correlation between the expression of SCOT and the concentration of succinyl-CoA is identified in the nonfailing myocardium. CHF indicates congestive heart failure; CoA, coenzyme A; Ct, cycle threshold; NS, not significant; OXCT1, 3-oxoacid-CoA transferase 1; and SCOT, succinyl-CoA:3-oxoacid CoA transferase.

data provide a summary of the metabolic signature or transition that is characteristic of a more advanced state of chronic human HF independent of diabetes mellitus.

Previous studies identifying excess lipotoxic species in human myocardium suggested a link, which had already been established in animal models of lipotoxicity,[15] between excess myocardial lipids and myocardial insulin resistance. Even recently, these studies included both diabetic and nondiabetic patients and targeted a restricted set of lipid species: diacylglycerols and ceramides.[2] Because diabetic patients are known to have distinct metabolic adaptations to support myocardial energetic demands irrespective of their HF status, the contributions of HF versus diabetes mellitus to toxic lipid species are difficult to define in these previous studies. By including an unbiased lipidomic survey of end-stage failing myocardium and nonfailing hearts in nondiabetic subjects, the present study addresses the deficiencies of previous studies.

Decreased Myocardial Acylcarnitines in Advanced Human HF

Despite increased levels of circulating NEFAs in serum and a significant increase in the gene expression of the main transporter of albumin-bound NEFAs, the cardiomyocyte-specific FA transport protein (SLC27A6, online-only Data Supplement Figure VIII), the myocardial concentration of acylcarnitines is significantly decreased in the end-stage failing heart. Unlike the recent report by Lai et al[16] in a mouse model of hypertrophy and early HF which identified increased levels of many acylcarnitine species as the distinguishing metabolite signature of the failing group, our data in nondiabetic advanced human HF are consistent with significantly reduced abundance of myocardial acylcarnitines, especially the ones that are esterified from the major free FAs in the human diet: palmitic and oleic acid (palmitoylcarnitine and palmitoleoylcarnitine). Beyond species differences, a possible explanation of the discrepancy between our data and the metabolite signature by Lai et al, is the stage and severity of HF that has been implicated as an important factor in determining lipid substrate utilization.[17,18]

The decrease in myocardial acylcarnitines cannot be explained by changes in the outer or inner mitochondrial membrane carnitine palmitoyltransferases, CPT-1 or CPT-2, for several reasons. First expression of CPT-1a, CPT-1b, or CPT-2 were not decreased in end-stage failing myocardium, nor was the expression of the regulator PPARα. In fact, expression of the peroxisome proliferator-activated receptor γ coactivator orchestrating mitochondrial biogenesis and FA oxidation, was found to be increased by 1.5-fold in end-stage failing myocardium (Figure 5) as previously reported.[19,20] Moreover, the highly regulated metabolite malonyl-CoA which inhibits CPT-1 was not increased in the myocardium of nondiabetic advanced HF patients (Table 2). Finally, the abundance of long-chain acyl-CoA species was not increased in the failing heart, which would be expected with myocardial suppression of the carnitine palmitoyltransferase system.

Our data do support another potential mechanism to explain decreased myocardial acylcarnitines in advanced HF: the decreased abundance of the plasmalemmal carrier organic cation/carnitine transporter novel type 2 (OCTN2) (SLC22A5: fold change 0.54, P=0.00046, see Figure 5) responsible for cellular carnitine uptake. OCTN2 is regulated by PPARα. There is strong evidence from murine PPARα agonist and PPARα knockout models that OCTN2 expression contributes to tissue, including myocardial, carnitine levels.[21] This mechanism deserves further investigation, given the known association of primary carnitine deficiency attributable to mutations in the OCTN2 gene with cardiomyopathic failure[22] and the reduced expression of OCTN2 in endomyocardial biopsy specimens of patients with chronic DCM.[23]

Our results were unequivocal in identifying marked and consistent reductions in myocardial acylcarnitines in advanced HF across a broad spectrum of carbon chain lengths (Figure 2B, online-only Data Supplement Figure II). Transgenic models with impaired fatty acid oxidation such as the long-chain acyl-CoA–deficient and PPARα knockout mice have each demonstrated increased accumulation of long-chain acylcarnitines in the myocardium.[24,25] Together, these results raise the question about whether, in the more advanced stages of HF, where increased myocardial insulin resistance has been observed,[2] there is increased reliance on lipid and ketone body substrates to generate acetyl-CoA.[17]

Evidence for Disruption in the Krebs Cycle in Advanced HF

Our study identifies an impairment in the Krebs cycle linking substrate oxidation to oxidative phosphorylation, providing initial evidence for a mechanism of altered mitochondrial metabolism in human HF already reported in animal models of cardiac injury and failure.[26,27] Specifically, the increased myocardial acetyl-CoA coupled with decreased levels of metabolites incorporated in the second span of the Krebs cycle (succinyl-CoA, succinate, and fumarate) suggest that the end-stage failing human myocardium has deficiencies in the Krebs cycle. This also includes a depleted pool of intermediate metabolites that feed the Krebs cycle through anaplerosis (propionyl-CoA), supporting recent observations that a disruption in Krebs cycle activity is an important signature of progression to HF.[3,26] Although our data are supportive of a possible bottleneck at the level of acetyl-CoA which disrupts the flux of substrate oxidation, we do not have evidence to determine whether the increased acetyl-CoA is derived from glycolytic flux or increased oxidative flux of free FAs or ketones.

The ratio of [succinyl-CoA] to [acetyl-CoA], which is significantly decreased in the end-stage failing myocardium, may also depict an imbalance of the intracellular levels of important regulatory intermediates that regulate mitochondrial and other cellular metabolism through succinylation and acetylation.[28–30] The importance of maintaining adequate levels of succinyl-CoA that are commensurate with cellular energetic demands is noteworthy, because succinylation in many key mitochondrial pathways (including FA oxidation, branched-chain amino acid catabolism, and the Krebs cycle) is primarily a nonenzymatic process that could be dependent on compartmentalized concentrations of succinyl-CoA.[29] In a similar manner, increasing the intracellular pools of acetyl-CoA could increase protein acetylation, which is also known to have broad regulatory effects on cellular metabolism.

In contrast to the decrease in succinyl-CoA, absolute levels of βhydroxybutyryl-CoA, a ketogenic substrate derived when FA oxidation rates exceed the tricarboxylic acid cycle, were higher in the failing group (Figure 3B).[31,32] FA oxidation to βhydroxybutyryl-CoA may constitute compensatory metabolic alteration that takes place in the setting of energetic failure by maintaining ATP production through the $FADH_2$-dependent complex II respiration.[32] Alternatively, the increased oxidation of ketones could also explain, when fatty acid oxidation is substrate limited, the increase in myocardial βhydroxybutyryl-CoA (also known as 3-hydroxybutanoyl-CoA; see Figure 7), which we identified in the end-stage failing group. Furthermore, βhydroxybutyryl-CoA formed by increased oxidation of FAs or ketones is metabolized to form acetoacetyl-CoA, which undergoes thiolase-mediated breakdown to acetyl-CoA. In this manner, the acetyl-CoA pool and cellular bioenergetics are maintained in the setting of advanced HF, where myocardial insulin resistance could inhibit the flux of glucose-derived acetyl-CoA.[2]

Evidence for Increased Myocardial Utilization of Ketones in HF

Our data suggest that the severely failing myocardium is utilizing the ketone body β-hydroxybutyrate (βOHB). In line with previous studies, our study found an increased concentration of βOHB in the serum of advanced HF patients with no history of diabetes mellitus.[33] Despite this increase in peripheral βOHB, we identified a decrease in myocardial βOHB in end-stage HF. This finding, along with a significant increase in the gene expression of BDH1, BDH2, and the rate-limiting enzyme succinyl-CoA:3-oxoacid-CoA transferase (SCOT, encoded by nuclear OXCT1) involved in the myocardial oxidation of βOHB, supports a hypothesis of increased utilization of this ketone body in the myocardium of end-stage HF. In turn, utilization of βOHB might therefore explain the increased acetyl-CoA levels from increased myocardial oxidation of ketone bodies derived from a peripheral ketotic milieu in advanced HF.

Chronic HF is characterized by the upregulation of neurohormonal factors, such as catecholamines and natriuretic peptides, which are known to activate adipocyte lipolysis[34,35] and release NEFAs. Our data demonstrated a 2-fold increase in the serum concentration of free FAs in nondiabetic HF patients at the time of cardiac transplantation (Figure 4), and a significant increase in the serum concentration of β-hydroxybutyrate, as well (Figure 6). We postulate that the increased circulating levels of NEFAs drive hepatic ketogenesis to synthesize ketones and, in the setting of an energetically compromised state, a transition occurs in the failing myocardium that allows the utilization of ketones to be oxidized as an important substrate, resulting in the decreased myocardial levels of the metabolized ketone β-hydroxybutyrate despite relatively high circulating ketone levels in end-stage HF.

Decreased myocardial succinyl-CoA levels may also be explained by the increased oxidation of ketones, driven in part by the increased abundance and possibly increased activation of SCOT that utilizes succinyl-CoA as a CoA donor in this rate-limiting step of ketolysis. The high degree of correlation between the expression of SCOT and the concentration of succinyl-CoA found only in the myocardium of end-stage failing patients (Figure 8) is highly suggestive that the myocardial levels of succinyl-CoA are associated with the abundance of SCOT. Previous studies in animals using the isolated working perfused heart model have demonstrated that the utilization of ketones in the form of acetoacetate is associated with a decreased pool of free or unesterified CoA with the sequestration of free CoA as acetoacetyl-CoA and acetyl-CoA and result in a depletion of succinyl-CoA both by inhibition of 2-oxoglutarate dehydrogenase with insufficient unesterified CoA or the increased activation of acetoacetate by the succinyl-CoA:3-oxoacid-CoA transferase (SCOT, see Figure 7).[36,37] Our data suggest that the end-stage failing heart is recapitulating this metabolic signature of utilizing ketones at the expense of a decreased myocardial concentration of unesterified CoA. The importance of ketone oxidation as a metabolic adaptation in the heart was recently demonstrated in a cardiomyocyte-specific knockout model of SCOT, where mitochondrial abnormalities and myocardial dysfunction were induced after increased load with transverse aortic constriction in the SCOT-knockout mice.[38] Further studies will be needed to define whether oxidation of ketones in advanced HF is energetically adaptive and up to what point that is the case.

Limitations

Our data provide a set of metabolic snapshots of highly relevant lipid pathways but do not yield any definitive measurements to derive the flux of carbohydrate, lipid, or ketone metabolism in the myocardium of end-stage HF. Nevertheless, the snapshot of a decreased myocardial concentration of β-hydroxybutyrate in the setting of an increased serum concentration, coupled with the increased gene expression of all the ketolytic oxidizing enzymes BDH1, BDH2, and OXCT1 (SCOT) suggest increased myocardial substrate utilization of ketones in advanced human HF. Under nonfailing conditions, myocardial expression of SCOT and ketone oxidation are actually suppressed when the heart is exposed to a peripheral ketotic milieu.[39] This distinction of the metabolic adaptation to a ketogenic environment in the nonfailing heart supports a hypothesis of metabolic reprogramming that allows for increased myocardial ketone utilization in advanced HF. Future studies are necessary to confirm through in vivo flux analyses if the failing human heart is actively and preferentially oxidizing ketones in its energy-deprived state.

Although we confirmed in a second cohort the depletion of lipid substrates characterizing the failing myocardium in the advanced stage of HF, we have not performed paired analyses in subjects who have demonstrated some degree of recovery of their myocardial function with therapies such as left ventricular assist device to observe if these disruptions in lipid metabolism are reversible. Furthermore, we have no data in patients with a less advanced form of this syndrome, such as ACC/AHA stage B or C patients.

Although we had only 2 female subjects in the failing group of patients, we observed the same pattern of lipid abundance, significant decrease in myocardial acylcarnitines, when the male subjects were analyzed separately. Future studies should determine whether there is a sex interaction that may differentiate the metabolic adaptation between male and female patients with advanced HF.

Conclusion

We have identified an abnormal lipid signature in the myocardium of nondiabetic, end-stage HF patients that is marked by a severe deficit in a broad range of myocardial acylcarnitines, an increase in acetyl-CoA, and a decrease in Krebs cycle intermediates including succinyl-CoA. Our metabolite and gene expression data support increased myocardial utilization of βOHB in end-stage HF and a potential link between increased ketone utilization and the abundance and function of regulatory intermediates such as acetyl-CoA and succinyl-CoA. Whether the disruptions in FA and ketone metabolism highlighted here are adaptive or maladaptive remains to be determined, but these findings nevertheless support the need to consider species-dependent, etiology-dependent, and severity-dependent distinctions as we elucidate the metabolic characteristics of the failing human heart and devise therapeutic strategies for improving the regulation of myocardial energetics.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Human heart failure is a progressive syndrome that remains associated with significant mortality. Our understanding of disease progression within the hemodynamic and neurohormonal paradigms has been elucidated to a great extent, allowing therapeutic advances that have improved symptoms and survival. However, the metabolic adaptations of the failing human heart remain elusive, precluding the development of therapeutic strategies that can enhance the efficiency and energetics of failing myocardium. Furthermore, our understanding of the transition from hypertrophy to failure is also rudimentary in humans, including the metabolic changes that take place. In this report, we describe the characteristic lipid signature of end-stage failing myocardium in advanced heart failure, marked by: (1) a significant decrease in long-chain acylcarnitines; (2) a decrease in the myocardial metabolite pool of downstream tricarboxylic acid cycle intermediates, including succinyl-coenzyme A, despite increased myocardial acetyl-coenzyme A; (3) increased myocardial utilization of β-hydroxybutyrate and upregulation of genes implicated in myocardial ketone oxidation including the succinyl-coenzyme A transferase (succinyl-CoA:3-oxoacid CoA transferase). Based on these novel observations, our enhanced understanding of the metabolic signature in nondiabetic heart failure may allow translation into therapeutic strategies that could decelerate disease progression or even reverse the cardiomyopathic burden in our patients with chronic heart failure.

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