Catabolic Defect of Branched-Chain Amino Acids Promotes Heart Failure

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Background—Although metabolic reprogramming is critical in the pathogenesis of heart failure, studies to date have focused principally on fatty acid and glucose metabolism. Contribution of amino acid metabolic regulation in the disease remains understudied.

Methods and Results—Transcriptomic and metabolomic analyses were performed in mouse failing heart induced by pressure overload. Suppression of branched-chain amino acid (BCAA) catabolic gene expression along with concomitant tissue accumulation of branched-chain α-keto acids was identified as a significant signature of metabolic reprogramming in mouse failing hearts and validated to be shared in human cardiomyopathy hearts. Molecular and genetic evidence identified the transcription factor Krüppel-like factor 15 as a key upstream regulator of the BCAA catabolic regulation in the heart. Studies using a genetic mouse model revealed that BCAA catabolic defect promoted heart failure associated with induced oxidative stress and metabolic disturbance in response to mechanical overload. Mechanistically, elevated branched-chain α-keto acids directly suppressed respiration and induced superoxide production in isolated mitochondria. Finally, pharmacological enhancement of branched-chain α-keto acid dehydrogenase activity significantly blunted cardiac dysfunction after pressure overload.

Conclusions—BCAA catabolic defect is a metabolic hallmark of failing heart resulting from Krüppel-like factor 15–mediated transcriptional reprogramming. BCAA catabolic defect imposes a previously unappreciated significant contribution to heart failure. (Circulation. 2016;133:2038-2049. DOI: 10.1161/CIRCULATIONAHA.115.020226.)

Key Words: amino acids ■ heart failure ■ metabolism ■ oxidant stress ■ pathogenesis ■ remodeling

Alterations in cardiac metabolism are hallmarks of the pathological changes in the failing heart, with studies over the past several decades centered on fatty acid and glucose utilization. Suppression of oxidative phosphorylation with reduced utilization of fatty acid in conjunction with increased glucose consumption is a common feature of heart failure. However, little is known about the metabolic changes of amino acid and their functional relevance in the pathogenesis of heart failure.

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Amino acids serve as building blocks for protein synthesis and energy-providing substrates, although the relative importance of a bioenergetic contribution by amino acids in the heart remains unclear under either physiological or pathological conditions. In addition, derivatives of amino acids such as taurine, creatine, carnitine, and glutathione are critical to bioenerogenesis and cellular function in the heart. An
early study by Peterson et al suggested that total free amino acid concentrations were increased in the failing right ventricle. In patients with mitral valve disease, higher glutamine and glutamate concentrations were detected in the dilated left ventricle. More recently, 2 reports using multisystems analysis in hypertrophied and early-stage failing mouse hearts after pressure overload or myocardial infarction also revealed profound metabolic derangement, including amino acid metabolism, associated with pathological remodeling. These observations indicate that amino acid homeostasis is perturbed in diseased heart tissue.

In this study, we found that the branched-chain amino acid (BCAA) catabolic pathway was the most significantly altered metabolic change in the mouse failing heart and that this coordinated suppression of BCAA catabolic pathway was regulated by Krüppel-like factor 15 (KLF15). Furthermore, we found the loss of BCAA catabolic gene expression and the resulting accumulation of intramyocardial levels of BCAA catabolic mediators such as branched-chain α-keto acids (BCKAs) were conserved metabolic signatures in human failing hearts. Impairment of BCAA catabolic pathway impaired heart function and promoted pressure overload–induced heart failure, associated with elevated superoxide production, oxidative injury, and profound metabolic changes in the heart. Finally, pharmacological enhancement of BCAA catabolic activity significantly preserved cardiac function after pressure overload. These findings established that defect of BCAA catabolism is an underappreciated integral part of the metabolic reprogramming in stressed hearts, that amino acid metabolism makes a significant contribution to the progression of heart failure, and that the BCAA catabolic pathway can serve as a potential therapeutic target for the disease.

Methods

Animals and Human Cohorts

PP2Cm germ-line knockout (PP2Cm-KO) mice were generated as previously described. Human cohorts of dilated cardiomyopathy and controls were obtained from Columbia and Duke with Institutional Review Board approval. All animal procedures were carried out in accordance with the guidelines and protocols approved by the University of California at Los Angeles Institutional Animal Care and Use Committee (IACUC). Transcardiac constriction (TAC) and cardiac echocardiography were performed as reported earlier on mice from different genotypes between 14 and 16 weeks of age. Compound BT2 (3,6-dichlorobenzo[b]thiophene-2-carboxylic acid) was purchased from Sigma-Aldrich and administered by oral gavage at 40 mg·kg⁻¹·d⁻¹ as previously described.

Molecular Methods and Reagents

The details of expression vectors, transfection methods, cell culture, immunoblotting, reverse transcription–polymerase chain reaction (RT-PCR), and chromatin immunoprecipitation methods were provided in the online-only Data Supplement. Superoxide measurement was performed by the electron spin resonance method, and BCKA and BCAA measurements from tissue or plasma were performed following the method published by Olson et al with modifications; details are given in the online-only Data Supplement. The global metabolomic analysis was carried out by Metabolon, Inc (Durham, NC) using heart tissues from PP2Cm-KO and wild-type male mice at 14 to 16 weeks of age. A detailed description of the analysis is given in the online-only Data Supplement.

Statistics

Unless otherwise specified, statistical analyses to compare 2 groups were performed with either the Student t test or the Wilcoxon rank-sum test (when n≤5 or when the variance distributions differed on the basis of the Bartlett test). When >2 groups were analyzed, standard ANOVA followed by the Newman-Keuls test was performed when n≤5 for all groups and passed by the Bartlett test of homogeneity of variances. Otherwise, the Kruskal-Wallis test was performed followed by the Dunn multiple-comparison test. Presented values are mean with standard deviation or standard error of the mean. A linear mixed-effect model test was performed for repeated measurements over time. A value of P<0.05 was considered statistically significant.

Results

BCAA Catabolic Gene Regulation in Developing and Pathologically Stressed Hearts

It has been well established that postnatal maturation of developing heart results in dynamic shifts from glucose to fatty acid utilization, a phenotype that is reversed in the diseased heart. These changes are orchestrated, at least in part, at the transcriptional level as part of the so-called fetal-like gene expression reprogramming. From cardiac transcriptome in pressure overload–induced failing mouse hearts, we performed functional annotation analysis using the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov) to identify Kyoto Encyclopedia of Genes and Genomes pathways significantly overrepresented in differentially expressed genes. The analysis of downregulated genes in the failing heart revealed >20 specific metabolic pathways that were significantly enriched (Table I in the online-only Data Supplement). Unexpectedly, among them, the valine, leucine, and isoleucine (or BCAAs) catabolic pathway demonstrated the most significant changes associated with heart failure (Figure 1A).

A total of 25 of 46 genes in the Kyoto Encyclopedia of Genes and Genomes BCAA catabolic pathway showed reduced expression in the failing heart compared with the sham controls (Figure 1A and Table II in the online-only Data Supplement). The reduced expression of these key BCAA catabolic enzymes, including BCAT2, BCKD subunits E1α, E1β and E2, and the BCKD phosphatase PP2Cm, was verified at both the mRNA and protein levels (Figure 1B and 1C). In contrast, no reduction was seen in the expression of BCKD kinase (BCKDK; Figure 1B and 1C and Figure 1A in the online-only Data Supplement). However, we observed a coordinated induction of the same set of genes during postnatal maturation from neonatal to adult. This dynamic expression pattern is comparable to what is observed for glucose and fatty acid metabolic genes, including Glut4 (glucose transporter 4) and Mcad (medium-chain acyl-CoA dehydrogenase), along with other well established fetal-like marker genes, including Nppa and Mvyh (Figure 1B). Therefore, the rate-limiting and downstream steps of the BCAA catabolic pathway are coordinately downregulated as part of the fetal-like transcriptome remodeling in failing heart.
phosphorylation of BCKD regulatory subunit E1α in the failing hearts comparing with controls (Figure IB in the online-only Data Supplement). Phosphorylation level of BCKD E1α subunit inversely correlates with the BCKD enzymatic activity; consequently, the levels of the intramyocardial BCKA were significantly increased in the mouse failing hearts (Figure 1D), whereas the total BCAA levels remained unchanged (Figure II in the online-only Data Supplement).

**Defect of BCAA Catabolism in Human Failing Hearts**

Human cardiomyopathy hearts demonstrated a striking parallel to the observations in rodents, with coordinated

![Diagram](https://example.com/diagram.png)

**Figure 1.** Remodeling of branched-chain amino acid (BCAA) catabolism in murine failing heart. A. The downregulated genes in failing heart were mapped into BCAA catabolism pathway by Kyoto Encyclopedia of Genes and Genomes. B. Real-time reverse transcription–polymerase chain reaction result of specific genes using mRNA from myocardium of neonatal (n=3), normal (adult sham; n=3), and failing (adult failing; n=3) mouse hearts. The y axis represents the relative mRNA level. ANOVA followed by the Newman-Keuls (Continued)
reduction of all key BCAA catabolic gene products, including BCAT2, BCKD subunits, and PP2Cm, whereas BCKD activity expression was slightly increased (Figure 2A). Importantly, intramyocardial levels of BCKA were also significantly increased in human cardiomyopathy hearts (Figure 2B). A significantly higher level of KMV but not KIC or KIV was also observed in plasma from humans with heart failure (Figure III in the online-only Data Supplement). In contrast, intramyocardial BCAA levels were not significantly altered (Figure IV in the online-only Data Supplement). Therefore, impairment of BCAA catabolic activity and intramyocardial accumulation of BCKA metabolites are conserved metabolic alterations in mouse and human failing hearts.
KLF15 Regulates Cardiac BCAA Catabolic Gene Expression

Coordinated regulation of BCAA gene products suggests a shared regulatory mechanism at the transcriptional level. We performed an upstream regulator analysis using Ingenuity Pathway Analysis software (http://www.ingenuity.com) for the genes showing altered expression in the mouse failing heart.23 The analysis of downregulated genes in the failing heart predicted numerous factors involved in their regulation (Table III in the online-only Data Supplement). The top 3 candidates were MAP4K4, KLF15, and PPARα. KLF15 was reported to be a direct transcriptional activator of BCAT2.25–27 In cultured cardiomyocytes, overexpression of KLF15 significantly induced the mRNA expression of BCAT2, BCKD subunits, and PP2Cm, with a notable exception of BCKDK (Figure 3A). Ectopic expression of KLF15 also induced the expression of these targets in non-myocytes (Figure 3B and Figure V A in the online-only Data Supplement). Using a PP2Cm promoter luciferase reporter, we showed that KLF15 directly induced the transcriptional activity of the PP2Cm promoter containing putative KLF15 binding motifs (Figure 3C and Figure VB in the online-only Data Supplement). Finally, chromatin immunoprecipitation analysis revealed a significant accumulation of KLF15 binding to the endogenous PP2Cm promoter in neonatal rat ventricular myocytes after KLF15 overexpression. The experiment was repeated twice with similar results.

Figure 3. Krüppel-like factor 15 (KLF15) regulates branched-chain amino acid (BCAA) catabolic gene expression. A, Real-time reverse transcription–polymerase chain reaction result of specific genes using mRNA from neonatal rat ventricular myocytes with (KLF15) or without (vector) KLF15 overexpression (n=6). *P<0.05 vs vector control. B, Western blotting result of proteins involved in BCAA catabolism (GAPDH as loading control) using cellular lysates from KLF15-overexpressed Hela cells. C, Illustration of partial mouse PP2Cm promoter fragments with 2 GC-rich sites and luciferase assay result of PP2Cm promoter–luciferase in HeLa cells cotransfected with either KLF15 or corresponding empty vector. The data represented the average values, with the SD of triplicate samples from 1 experiment representative of 3 independent experiments. *P<0.05 vs same promoter without KLF15 overexpression; #P<0.05 vs 468-bp promoter with KLF15 overexpression (n=3). D, Representative result of chromatin immunoprecipitation–polymerase chain reaction validation for KLF15 binding to the PP2Cm gene promoter in neonatal rat ventricular myocytes after KLF15 overexpression. The experiment was repeated twice with similar results.
pathway and show that loss of KLF15 is a potential molecular mechanism underlying stress-induced BCAA catabolic defects in the diseased heart.

**BCAA Catabolic Defect Impaired Cardiac Contractile Function**

To directly assess the effect of BCAA catabolic defect on heart function, we used a mouse model carrying the genetically inactivated PP2Cm coding gene ppm1k (PP2Cm-KO) in which BCKD activity is significantly inhibited because of the constantly elevated E1α phosphorylation. Indeed, compared with wild-type controls, intramyocardial BCKA and BCAA levels were significantly increased in the PP2Cm-deficient hearts from mice fasted for 6 hours (Figure 5A) at levels (<5 nmol·L⁻¹·g⁻¹) comparable to what was observed in mouse and human failing hearts (Figures 1D and 2B). However, cardiac BCKA concentrations became much higher (15–45 nmol·L⁻¹·g⁻¹) in the PP2Cm-KO heart under feeding conditions (Figure 5B and Figure VIIA in the online-only Data Supplement), highlighting the potential dietary influence on BCKA accumulation in the diseased heart when BCAA catabolic activity is compromised. Echocardiogram measurements showed a modest but statistically significant reduction in cardiac systolic function in the PP2Cm-deficient mice at 3 months of age (Figure 5C). By 18 months of age, their cardiac function was further reduced compared with the age-matched wild-type controls (Figure 5D). However, young PP2Cm-deficient mice exhibited no major changes in cardiac morphology, histology, and ultrastructure, as well as molecular markers of myocardial remodeling (Figure 5E–5G and Figure VIIB in the online-only Data Supplement). Therefore, abnormal BCAA catabolism is sufficient to promote contractile dysfunction over time in the absence of any external pathological stressor.

**BCAA Catabolic Defect Enhances Susceptibility to Heart Failure in Response to Pathological Stress**

We then subjected wild-type and PP2Cm-KO mice (3–4 months of age) to pressure overload. From the second week of TAC, PP2Cm-deficient mice exhibited a marked reduction in contractile function (Figure 6A and Figure VIII in the online-only Data Supplement). A repeated-measures linear model analysis demonstrated that changes in cardiac echocardiogram parameters such as left ventricular internal dimension in systole for group×day ($P=3.54e^{-6}$) and left ventricular fractional shortening ($P=1.27e^{-3}$) were significant between the PP2Cm-deficient and wild-type mice. At 8 weeks after TAC, PP2Cm-deficient mice exhibited signs of heart failure, as evidenced by significantly reduced left ventricular ejection fraction, chamber dilation, and elevated wet lung weights, an indicator of severe pulmonary congestion resulting from heart failure (Figure 6). Collectively, these data indicate that deficient BCAA catabolism can directly impair cardiac function and accelerates pressure overload–induced cardiomyopathy.
Impaired Metabolic and Redox Homeostasis by BCAA Catabolic Deficiency in Heart

The impact of BCAA catabolic defects on cardiac function is consistently correlated with elevated BCKA metabolites in the diseased heart tissue. We found that in isolated cardiac mitochondria, BCKAs directly inhibited complex I– but not complex II–mediated respiration (Figure 7A and Figure IXA and IXB in the online-only Data Supplement). The inhibition was dose dependent with a marked decrease observed at concentrations as low as 20 μmol/L BCKAs (Figure 7B). In the meantime, BCKAs also promoted superoxide production in isolated cardiac mitochondria in a dose-dependent manner (Figure 7C). A significant increase in superoxide production was detected from the PP2Cm-deficient mitochondria (Figure 7D) and myocardium (Figure 7E), associated with the enhanced oxidative injury to cardiac proteins (Figure 7F). These data suggest that accumulated BCKAs resulting from BCKD inactivation may directly affect cardiac mitochondrial activity and redox homeostasis.

We performed additional targeted metabolomic analysis on >300 metabolic intermediates in hearts from wild-type and PP2Cm-KO mice. Principal component analysis revealed a divergent separation between wild-type and PP2Cm-deficient mitochondria in a dose-dependent manner (Figure 7C). A significant increase in superoxide production was detected from the PP2Cm-deficient mitochondria (Figure 7D) and myocardium (Figure 7E), associated with the enhanced oxidative injury to cardiac proteins (Figure 7F). These data suggest that accumulated BCKAs resulting from BCKD inactivation may directly affect cardiac mitochondrial activity and redox homeostasis.

Inhibition of BCKDK Promoted BCKA Degradation and Preserved Heart Function

Given the significant contribution of BCAA catabolic defect to cardiac dysfunction, we investigated the impact of enhancing BCAA catabolic activity on pressure overload–induced heart failure in mice. 3,6-Dichlorobenz[b]thiophene-2-carboxylic acid (BT2) is a highly specific and potent inhibitor of BCKDK. Administration of BT2 in mice significantly reduced the phosphorylation of BCKD subunit E1α in heart (Figure 8A and 8B) and dramatically enhanced cardiac BCKD activity in both wild-type (≈7-fold) and the PP2Cm-KO mice (≈9-fold; Figure 8C). Consequently, the plasma BCKA level in both wild-type and PP2Cm-KO mice was markedly reduced (Figure 8D) but with modest impact on plasma BCAA level (Figure XI in the online-only Data Supplement). More important, at 4 weeks after TAC, BT2-treated mice displayed significantly preserved left ventricular ejection fraction and reduced chamber dilation (Figure 8D–8F and Figure XII in the online-only Data Supplement). These results suggested that enhancing BCKA degradation...
by targeted inhibition of BCKDK significantly preserved cardiac function in response to pathological stress.

Discussion

In the present study, we reveal that BCAA catabolic gene expression is coordinately suppressed in both murine and human failing hearts as part of fetal-like gene expression and metabolic reprogramming. KLF15-mediated transcriptional regulation is central for this coordinated reduction of BCAA catabolism. Genetic and cellular analyses suggest that BCAA catabolic defects and the resulting accumulation of BCKA metabolites cause cardiac reactive oxygen species injury and global metabolic alteration and significantly contribute to the progression of heart failure.

Figure 6. Branched-chain amino acid (BCAA) catabolic defect promotes heart failure progression. A and B, Time course for left ventricular fractional shortening (LV%FS; A) and left ventricular internal dimension (in millimeters) at systole (LVIDs; B) from wild-type (WT; n=11–15) and PP2Cm germ-line knockout (PP2Cm-KO) mice (n=13–19) with transaortic constriction (TAC) surgery. The x axis shows the time in weeks after surgery. C and D, Representative M-mode echocardiographs (C) or ratio of lung weight to body weight (LV/BW; D) WT sham, n=9; KO sham, n=10; WT TAC, n=8; KO sham, n=8) from WT and PP2Cm-KO mice at 8 weeks after surgery. Error bars represent SEM. Statistical analyses were performed with the Student t test (A and B) to compare the values of WT and PP2Cm-KO at the same time point (#P<0.05, *P<0.05) or the Kruskal-Wallis test followed by the Dunn multiple-comparison test (D; *P<0.05 vs KO sham). A repeated-measures linear model was also fitted for LVIDs (A) and LV%FS (B).

Figure 7. Disturbed metabolic and redox homeostasis by branched-chain α-keto acids (BCKAs). A, Oxygen consumption in mitochondria isolated from wild-type (WT) hearts in the absence or presence of 500 μmol/L BCKAs. B, Relative oxygen consumption rate in the absence or presence of BCKAs at different concentrations (n=3–8 in each group; *P<0.05 vs control). C, Superoxide production in isolated cardiac mitochondria (n=4–7 in each group; *P<0.05 vs control). D and E, Superoxide production in isolated mitochondria (D, n=5–6 in each group) and myocardium (E, n=3 in each group) from WT and PP2Cm germ-line-deficient (KO) mice. F, Immunoblotting of total protein oxidation detected by carbonyl groups (left) from tissue lysates of WT and PP2Cm-KO mouse hearts. G, Principal component analysis of metabolomic profiles revealed a distinct genotype-based separation for the heart samples (WT, n=8; KO, n=7). H, List of the top 30 biochemicals that separated different genotypes based on their importance. Error bars represent SEM (B–E).
Amino acids serve as both important nutrients and potent signaling molecules. However, compared with the extensive knowledge of fatty acid and glucose metabolism, current understanding of amino acid metabolic regulation under normal development or pathological conditions is very limited. BCAAs, including leucine, isoleucine, and valine, are essential amino acids with a shared catabolic pathway. In addition to participating in de novo protein synthesis, BCAAs function as potent nutrient signal molecules for cellular metabolism and growth. Through the mechanistic target of rapamycin pathway, BCAAs (particularly leucine) can regulate vital cellular processes, including protein translation, autophagy, and insulin signaling, affecting glucose and fatty acid metabolism, muscle anabolism, and life span. Genetic defect of BCAA catabolism leads to maple syrup urine disease. Recently, abnormal plasma BCAA levels have been associated with neurological, cardiovascular, metabolic diseases, and cancer in numerous studies. These findings highlight the importance of BCAA metabolism in normal physiology and a broad spectrum of human diseases. Hence, BCAA catabolic defect is another metabolic hallmark of heart diseases that may be exploited as additional metabolic biomarkers for cardiac pathology.

The coordinated loss of BCAA catabolic gene expression suggests a common regulatory machinery for the pathway. This notion is consistent with 2 recent studies reported in hypertrophied and early-stage failing heart. From both bioinformatic and genetic approaches, we identify KLF15 as a master transcription factor responsible for BCAA catabolic gene expression in heart. The functional role of KLF15 is well documented in cardiac hypertrophy, heart failure, and cardiac fibrosis. In addition to hypertrophic genes, KLF15 serves as a key regulator of glucose, fatty acid, and amino acid metabolism. Genetic defect of BCAA catabolism leads to maple syrup urine disease. Recently, abnormal plasma BCAA levels have been associated with neurological, cardiovascular, metabolic diseases, and cancer in numerous studies. These findings highlight the importance of BCAA metabolism in normal physiology and a broad spectrum of human diseases. Suppressed BCAA catabolic activity appears to be a common feature in the stressed heart. Earlier reports by Kato et al using Dahl salt-sensitive rats demonstrate that cardiac valine, isoleucine, and leucine levels are elevated after a high-salt diet. Several other studies, including our present study in both rodents and humans, have now linked high levels of BCAAs with cardiac diseases. Therefore, BCAA catabolic defect is another metabolic hallmark of heart diseases that may be exploited as additional metabolic biomarkers for cardiac pathology.

Figure 8. Inhibition of BCKD kinase (BCKDK) by BT2 (3,6-dichlorobenz[b]thiophene-2-carboxylic acid) promotes branched-chain α-keto acid (BCKA) degradation and preserves cardiac function in the pressure-overloaded heart. A, Immunoblot for total and phosphorylated BCKD subunit E1α in heart from wild-type (WT) mice treated with vehicle (veh; n=4) or BT2 (n=5). B, The average phosphorylation level of E1α vs total E1α is presented with the SEM. Error bars represent SEM. *P<0.05 between vehicle- and BT2-treated samples. C, BCKD activity in cardiac tissues from WT or PP2Cm germ-line knockout mice (KO) mice treated with vehicle or BT2 (n=4–5 in each group). *P<0.05, vehicle- vs BT2-treated groups. D, Individual BCKA concentration in plasma from WT and PP2Cm-KO (n=4–6) mice treated with vehicle or BT2: Vehicle WT group, n=5; BT2 group, n=6. *P<0.05, vehicle- vs BT2-treated groups of the same genotype. E, Representative M-mode echocardiographs of mouse hearts after sham surgery or transaortic constriction (TAC) treated with vehicle or BT2. F, Left ventricular ejection fraction (%LVEF; n=6–8) and (G) left ventricular internal dimension at systole (LVIDs; n=6–8) from mice with sham or TAC surgery for 4 weeks, treated with or without BT2 as indicated. Error bars represent SEM. *P<0.05 between designated groups.
in contrast to what we observed in both end-stage human cardiomyopathy hearts and mouse failing hearts 8 weeks after TAC. It is plausible to speculate that BCAA catabolic reprogramming is a compensatory mechanism at least at the initial stage of the response of the myocardium to stress, given that BCAA preservation would redirect amino acids from catabolic consumption to protein synthesis and cell growth during cardiac hypertrophy. Perturbation of BCAA catabolic activity may have a significant impact on mechanistic target of rapamycin signaling, leading to potential changes in cardiac growth, metabolism, and survival. However, defective BCKD activity also causes accumulation of BCKA in hearts, which may lead to a detrimental effect resulting from cytotoxic effects on mitochondrial function and reactive oxygen species homeostasis. Indeed, a direct and dose-dependent impact of BCKA treatment on mitochondrial function and reactive oxygen species production as demonstrated in this study highlights the potential contribution of BCKA as the true pathogenic culprit underlying BCAA catabolic defects in the progression of heart failure. BCKA and BCKA-mediated mitochondrial and cellular defects should be further explored as both metabolic biomarkers and therapeutic targets for heart failure.

Our genetic data from this report clearly implicate BCAA catabolic defect as a significant contributor to the pathogenesis of heart failure. The results from our study using BCKD inhibitor (Figure 8) clearly demonstrate the translational value of targeting BCAA catabolism as a therapy for heart failure. They also raise the question of the potential impact of dietary influence on disease progression. Tanada et al showed that BCAA supplement ameliorated the progression of heart failure in rats associated with preserved skeletal muscle weight and mitochondrial function. It is not clear if the benefit of BCAA supplement at the early stage of heart failure is derived from enhanced BCAA flux and preserved BCAA catabolic activities. Clearly, more preclinical and clinical studies are needed to fully establish the therapeutic window and approaches to manipulate BCAA catabolism in heart failure.

**Conclusions**

We have elucidated a previously unappreciated role for BCAA catabolism in the cardiac metabolic adaptation to stress. This insight can be further exploited for future diagnostic and therapeutic development.

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**Disclosures**

None.

**References**


Heart failure is a leading cause of mortality and hospitalization, and effective therapies remain elusive. The present study uncovers that branched-chain amino acid (BCAA; including leucine, isoleucine, and valine) catabolic defect is a hallmark of metabolic changes in murine failing heart and human dilated cardiomyopathy hearts. Accumulation of branched-chain α-keto acids resulting from BCAA catabolic defect directly impairs mitochondrial activity, induces oxidative stress, and promotes cardiac dysfunction. More importantly, restoration of BCAA catabolism by pharmacological agents blunt disease progression in pressure overload–induced heart failure. Therefore, defects in BCAA/branched-chain α-keto acids catabolism are not only a new metabolic biomarker for heart failure, but also a significant contributor to heart failure. Promoting BCAA catabolic activity with a pharmacological agent can be a potentially effective therapeutic strategy to ameliorate the pathogenic progression of heart failure. Finally, considering the dietary source of BCAA, the present study indicates a role of protein intake in the disease progression of heart failure and thus serves as a preclinical basis to develop a more appropriate nutritional intervention approach for this disease.
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Branched-Chain Amino Acid Catabolic Reprogramming in Heart Failure

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Supplementary Material
ON-LINE MATERIAL AND METHODS

Animals

PP2Cm KO mice were backcrossed for more than 8 generations into a C57BL/6 background. Wildtype C57BL/6 mice and PP2Cm KO mice were housed at 22°C with a 12-hour light, 12-hour dark cycle with free access to water and standard chow. Studies were performed with male mice. All animal procedures were carried out in accordance with the guidelines and protocols approved by the University of California at Los Angeles Institutional Animal Care and Use Committee (IACUC).

Transverse Aortic Constriction

In mice, transverse aortic constriction (TAC) was performed as described (Gao, 2015 #808) in anesthetized (pentobarbital 60 mg/kg, IP) and ventilated mice (age 14–16 weeks) to induce hypertrophy and heart failure. After left anterolateral thoracotomy with blunt dissection through the intercostal muscles, aortic constriction was induced by ligating the transverse aorta around a 27 1/2-guage blunt needle using 6-0 silk suture. The needle was subsequently removed. Sham-operated mice underwent a similar surgical procedure without constriction of the aorta. All mice were maintained in the same environment with regular lab chow and water ad libitum. At the end of the experiments, animals were euthanized and the hearts and lungs were removed and weighed. Hearts were dissected and tissues were either immediately immersed into 4% buffered formaldehyde or quickly frozen in liquid nitrogen for further experiments.

BT-2 Treatment
Compound BT2 (3,6-dichlorobenzo[b]thiophene-2-carboxylic acid) was purchased from Sigma-Aldrich. Administration of BT2 was performed as previously described \(^1\) except that animals (age ~8 weeks) were dosed daily by oral gavage at 40 mg/kg/day. Administration of BT2 started one week before TAC surgery and continued for 4 weeks post-TAC. Measurements of BCKD activity in mouse cardiac tissue and plasma BCKA/BCAA concentrations were performed as previously described \(^1\).

**Echocardiography**

The mice were anesthetized and maintained with 1-2% isofluorane in 95% oxygen. Echocardiography was performed with a VisualSonics Vevo 770 (VisualSonics Inc, Toronto, Canada) equipped with a 30-MHz linear transducer. A parasternal short axis view was used to obtain M-mode images for analysis of fractional shortening, ejection fraction, and other cardiac parameters.

**Mitochondrial Assay**

The isolation of mitochondria to measure oxygen consumption was performed as described elsewhere \(^2\). Briefly, mitochondria were isolated from heart tissue and oxygen consumption was measured using an Ocean Optics fiber optic spectrofluorometer. Mitochondria (0.25 mg/ml) were added to the assay buffer (125 mM KCl, 10 mm HEPES-KOH, pH 7.4). The oxygen concentration in the buffer was continuously recorded via an Ocean Optics FOXY fiber optic oxygen sensor. Pyruvate, malate, and glutamate were added as free acids buffered with Tris (pH 7.4) for Complex I activity assay. Addition of 0.2mM ADP initiated oxygen consumption. NaCl or BCKA-Na mixture was added to the reaction system after the first pulse of ADP was consumed. Then the
second pulse of ADP was added. The oxygen consumption rate (OCR) was calculated with each ADP addition. The relative rate of oxygen consumption was calculated by dividing the OCR of second pulse of ADP by the OCR of the first pulse of ADP. Succinate was used for Complex II activity assay in presence of rotenone (1μM). The oxygen consumption rate (OCR) was calculated with each ADP addition. The presented data represented the average values of three independent experiments.

**Western Blot Analysis**

Proteins from heart tissue or cells were harvested in buffer (50mM HEPES [pH7.4], 150mM NaCl, 1% NP-40, 1mM EDTA, 1mM EGTA, 1mM glycerophosphate, 2.5mM sodium pyrophosphate 1mM Na3VO4, 20mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL of aprotinin, leupeptin, and pepstatin). Samples were separated on 4-12% Bis-Tris gels (Invitrogen), and transferred onto a nitrocellulose blot (Amersham). The blot was probed with the indicated primary antibodies. Protein signals were detected using HRP conjugated secondary antibodies and enhanced chemiluminescence (ECL) western blotting detection regents (Pierce). Rabbit polyclonal antisera against the E1 and E2 subunits of BCKD complex is a kind gift from Dr. Yoshiharu Shimomura (Nagoya Institute of Technology). PP2Cm and phosphor-E1a antibodies were generated in the lab. The KLF15 primary antibody was purchased from Abcam.

**Real-time RT-PCR and Microarray analysis**

Total RNA was extracted from cells or tissues using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. For neonatal mouse, 3-5 hearts were combined to extract RNA as one individual sample. Total RNA was reverse-transcribed into the first-strand cDNA using
the Superscript First-Strand Synthesis Kit (Invitrogen). Then, cDNA transcripts were quantified by the Step-One Plus Real-Time PCR System (ABI) using SYBR Green (ABI). 18sRNA were used for normalization except where indicated. PCR primer information is available upon request. The cDNA from wildtype and PP2Cm deficient mice was applied to Illumina MouseRef-8 v2.0 Expression BeadChips at the UCLA DNA Microarray Core Facility for whole-genome expression profiling. The data were analyzed with BeadStudio Gene Expression Module v3.4 program.

**Bioinformatics Analysis**

The transcriptomes of sham and failing mouse hearts were analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov). The lists of genes showing either up-regulation (782 genes) or down-regulation (653 genes) in failing hearts were separately entered into the DAVID and subjected to Functional Annotation Chart analysis with a EASE score of 0.05 using the KEGG pathway database. KEGG pathway tool was utilized through DAVID online tools to visually map down-regulated genes involved in BCAA degradation pathway in failing heart.

To predict upstream regulators of target genes, the down-regulated gene list (653 genes) was analysed using Ingenuity Pathway Analysis (IPA) Software (http://www.ingenuity.com). A Fisher’s Exact Test p-value is calculated to assess the significance of enrichment.

**Expression constructs, cell culture, transient transfection and luciferase Assay**

Mouse KLF15 cDNA was generated from heart mRNA, inserted into the pFLAG-CMV-4 expression vector, and used to generate adenovirus. Utilizing the NCBI GenBank, we identified the genomic sequence of mouse PP2Cm (Ppm1k). We amplified five proximal 5’ regions (-468, -
412, -296, -254 to +20 bp relative to transcript start site, respectively) by PCR. Promoter PCR product was cloned into a firefly luciferase reporter pGL3-Basic vector (Promega, Madison, WI) to drive luciferase expression (PP2Cm-luc). The site-specific deletion was accomplished with the Agilent QuikChange XL site directed mutagenesis kit. Neonatal rat ventricular myocytes (NRVM) were isolated and cultured as previously described. HeLa cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100U/mL penicillin, and 100μg/ml streptomycin. Transient transfections were performed with the use of Lipofectamine 2000 reagent (Invitrogen). HeLa cells were seeded into 12 well plates at a density of 2×10^5 cells per well the day before transfection. For each well of cells 0.2 μg of the promoter constructs were co-transfected with 0.02 μg of the pSV40-Renilla vectors. The transfected cells were collected after transfection 48 hours. Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promenade). To normalize for transfection efficiency, the promoter activity was expressed as the ratio of firefly activity to renilla activity. For each construct, more than three independent experiments were performed in triplicate.

**Chromatin immunoprecipitation (ChIP) assay**

Neonatal Rat Ventricular Myocytes (NRVM) (4.0*10^7) were mock or FLAG-KLF15 infected with adenovirus. 48 hr post infection, cells were cross-linked with 1% formaldehyde for 10min at room temperature. ChIP analysis was performed using SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling) according to the manufacture’s protocol. Briefly, cross linking was terminated by adding glycine into cells for 5min at room temperature. Cells were harvested by PBS/PMSF and chromatin DNA was extracted. Following Micrococcal nuclease treatment, chromatin DNA was further sheared by sonication. Immuno-precipitation was performed using
Normal Rabbit IgG (Santa Cruz) or DYKDDDDK antibody (Cell Signaling) with ChIP Grade Protein G Magnetic Beads overnight. Following immune-precipitation, magnetic beads were washed with low salt buffer and high salt buffer according to the protocol, and the cross-linking was reversed by proteinase K digestion at 65 degree for 2hr. The eluted DNA was further purified using column provided in SimpleChIP Enzymatic Chromatin IP Kit. PCR was performed to detect enrichment of rat PP2Cm promoter region. For PP2Cm, forward primer sequence is 5’ACAAATTAAGACTAAAAAGT3’ and reverse primer sequence is 5’CCCACAGGAACCTAGTGAGG3’. PCR products were separated using agarose gel electrophoresis and visualized. IgG was used as negative control for ChIP specificity.

**Measurement of BCAA and BCKA concentrations in hearts**

Mice on normal chow diet were either fasted for 6 hours or overnight followed with a high protein diet (40%, Teklad) feeding for two hours before tissue collection. BCKA level in KLF15 knockout heart was measured in mice fasted for 48 hours. Human left ventricular RNA samples were obtained as previously described. In brief, myocardial specimens were collected before and after left ventricular assisted device (LVAD) implantation and explantation as a bridge to transplantation for end-stage HF patients. Control heart samples were obtained from non-failing hearts as previously described. The use of all mouse and human samples was approved by the Institutional Review Board of Columbia University and Case Western Reserve University (IRB-AAAEE7393). Freeze clamped mouse hearts were crushed with a metal mortar and pestle that was maintained at the temperature of liquid nitrogen. The powdered tissue was transferred to a tared tube, the weight recorded, and then processed with perchloric acid as previously described. A ratio of 300 μl perchloric acid per 100 mg of tissue was used. The aliquotted perchloric acid
supernatant was stored at -80°C until further assay. For BCAA determinations, a 20 µl perchloric acid supernatant aliquot of the mouse heart was thawed and neutralized with 0.25M MOPS-3M KOH buffer for analysis of free amino acids in the heart. The amino acids were derivatized and extracted as previously described using internal and external standards. The samples were analyzed using a Waters Synapt HDMS hybrid QTOF with Ion Mobility. BCKA measurements were performed as described elsewhere. Briefly, the perchloric acid heart supernatants were derivatized by O-phenylenediamine (OPD), extracted with ethyl acetate, and dried down in glass tubes in an unheated vacuum centrifuge. Following drying, the ketoacids were reconstituted in 200mM ammonium acetate, pH 6.8, and analyzed using a Shimadzu ultra-fast liquid chromatography (UFLC) 20ADXR LC system in-line with an AB-Sciex 5600 TripleTOF Q-TOF mass spectrometer (MS). Both instruments used in this analysis were housed in the Penn State College of Medicine Macromolecular Core Facility. BCKA concentration were measured and normalized to the weight of tissue. For human heart result, statistical analyses were performed with Student’s t-test after log transforming the data.

**BCKA Measurements in mouse Plasma:** The method published by Olson, et al was followed with modification. Briefly, plasma was cleared of proteins by adding an equal volume of methanol, followed by two rounds of centrifugation. The supernatant was lyophilized and re-suspended in distilled water (dH20). Stock solutions of each keto acid were prepared in dH20 and stored at -80C until they were used once and not refrozen. 10 ng of [13C] KIV was added to each vial of sample and standard which were then derivatized with freshly prepared O-phenylenediamine (OPD) and extracted twice with ethyl acetate as described. The pure organic phase was transferred to an eppendorf tube and dried under mild heat (40°C). The samples were re-suspended
in 50:50 MeOH:5 mM NH4 acetate and analyzed by LC-MS/MS using a Sciex 3200 Q-Trap coupled to a Shimadzu Prominence LC. An Agilent C18 XDB 5 micron packing column (50 X 4.6 mm) was used for chromatography with the following conditions: Buffer A: 5 mM NH4 acetate, Buffer B: methanol, 0 - 2.0 min 50% B, 2.0 - 2.5 min gradient to 100% B, 2.5 - 3.5 min 100% B, 3.5 - 3.6 min gradient to 50% B, 3.6 - 4.6 min 50% B. The derivatized keto acids were detected with the mass spectrometer in positive MRM (multiple reaction monitoring) mode using the following transitions: KIC 203.1 to 161.1 (retention time: 2.91 min); KIV 189.145 to 119.2 (retention time 2.84 min); KMV 203.065 to 174.2 (retention time 2.99 min); [13C] KIV 194.109 to 120.1 (retention time: 2.84 min).

**Human cohort for BCAA/BCKA measurements**

One hundred forty one subjects with no history of diabetes were selected from the CATHGEN bio-repository for ketoacid analysis based on cardiomyopathy phenotype. This study was approved by the Duke University Institutional Review Board. Heart failure (n=91) cases were defined as those having left ventricular ejection fraction (LVEF) less than or equal to 30%, no history of heart transplantation, no significant valvular disease, with or without history of myocardial infarction (MI), and New York Heart Association (NYHA) Functional Classification of 2 or greater. The control group (n=50) was composed of those with LVEF greater than or equal to 55%, no coronary vessels occluded greater than or equal to 50%, no history of MI, no history of percutaneous coronary intervention (PCI), no history of coronary artery bypass grafting (CABG), no history of congestive heart failure or heart transplantation, and no significant valvular heart disease.
Electron spin resonance measurement of superoxide production

Freshly isolated hearts were placed into chilled modified Krebs/HEPES buffer (composition in mmol/l: 99.01 NaCl, 4.69 KCl, 2.50 CaCl₂, 1.20 MgSO₄, 1.03 KH₂PO₄, 25.0 NaHCO₃, 20.0 Na-HEPES, and 5.6 glucose [pH 7.4]), cleaned of excessive adventitial tissue. The homogenates from heart tissues were prepared by homogenizing with a pestle (50 strokes) in fresh homogenization buffer (50 mmol/L of Tris-HCl, [pH 7.4] 0.1 mmol/L of EDTA, 0.1 mmol/L of EGTA) containing protease inhibitor cocktail and centrifuged at 800 g for 10 min. After centrifugation, supernatants were collected and then subjected for protein assay. The specific superoxide (O₂•⁻) spin trap methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH, 1mmol/L, Alexis) solution was prepared freshly in nitrogen gas bubbled Krebs/HEPEs buffer containing diethyldithiocarbamic acid (DETC, 5 μmol/L Sigma) and deferoxamine (25 μmol/L, Sigma). Homogenates (15 μg protein) was then mixed with the spin trap solution in the presence or absence of 100 units of SOD (manganese containing enzyme, Sigma) and loaded into a glass capillary (Fisher Scientific). For analysis of O₂•⁻ signal (CM⁺ formed after trapping O₂•⁻), the capillary was immediately loaded in an e-Scan electron spin resonance (ESR) spectrophotometer (Bruker Biospin, Germany). The ESR settings used were static-field, 3484 sweep width, 9.00 G (1 G = 0.1 mT); microwave frequency, 9.748660 GHz; microwave power 21.02 mW; modulation amplitude, 2470 mG; resolution in X, 512, number of X-scan, 10; and receiver gain, 1000. Data was presented as fold change versus WT. The superoxide production in isolated mitochondria was performed following a similar protocol except using an assay buffer containing 250mM sucrose, 10mM HEPES, 10mM Tris-HCl (pH7.4), and 4mM ADP.

Metabolomic analysis of heart tissue
The global metabolomic analysis was carried out by Metabolon, Inc. (Durham, NC) using heart tissues from PP2Cm KO and wildtype male mice at 14-16 weeks of age. Briefly, all samples were quickly frozen in liquid nitrogen and maintained at -80°C until processed. Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several types of controls were analyzed in concert with the experimental samples. The LC-MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo-Finnigan LTQ mass spectrometer operated at nominal mass resolution, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The samples destined for analysis by GC-MS were dried under vacuum prior to being derivatized under dried nitrogen using bistrimethyl-silyl trifluoroacetamide. Derivatized samples were separated on a 5% diphenyl / 95% dimethyl polysiloxane fused silica column and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI) and operated at unit mass resolving power. Raw data was extracted, peak-identified and QC processed using Metabolon’s hardware and software. Peaks were quantified using area-under-the-curve. A collection of information interpretation and visualization tools for use by data analysts. Welch’s two-sample t-test is used to test whether two unknown means are different from two independent populations. Principal component analysis (PCA) was used to visualize how individual samples in the cohort of wildtype and PP2Cm KO hearts differ from each other. The metabolites contributing the greatest to the differences among the two groups were determined in a random forest (RF) analysis. To determine which metabolites make the largest contribution to the classification, the “Mean Decrease Accuracy” is computed.

Statistics:
Unless otherwise specified, statistical analyses to compare two groups were performed with either the Student’s t-test or Wilcoxon Rank Sum test (when \( n < 5 \) or in which the variances distributions differed based on Bartlett test). When more than two groups were analyzed, standard ANOVA followed by Newman-Keuls test was performed when \( n > 5 \) for all groups and passed by the Bartlett test of homogeneity of variances. Otherwise, Kruskal-Wallis test followed by Dunn’s multiple comparison test was performed.

Presented values are mean with standard deviation or SEM (standard error of the mean). For repeated measurements over time as shown in longitudinal echocardiograph analysis, Linear Mixed Effect Model test using lmerTest package in R obtained from an on-line source was performed (Alexandra Kuznetsova, Per Bruun Brockhoff and Rune Haubo Bojesen Christensen (2015). lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-29. http://CRAN.R-project.org/package=lmerTest). A repeated measures linear model was fitted for echocardiograph parameters such as LVIDs and FS using animal ID as a random effect and day, group and group*day as fixed effects. A \( p \) value of less than 0.05 was considered statistically significant.
References cited in Methods:


SUPPLEMENTAL DATA:

FIGURE LEGEND:

Supplemental Table S1. The top 25 KEGG pathways identified by DAVID.

Supplemental Table S2. The 25 down-regulated genes were identified and mapped into BCAA catabolism pathway by KEGG (Kyoto Encyclopedia of Genes and Genomes).

Supplemental Table S3. The top 10 proteins identified by Upstream Regulator Analysis of IPA with the list of genes down-regulated in failing heart.

Supplemental Figure S1. A. The densitometric values of the bands in Figure 1C were analyzed. The densitometric value of each protein was normalized to GAPDH and presented as fold change versus Sham (n=3 in each group). The data represented the average values with standard deviation of three bands with p value labeled. B, Western blotting result of BCAA catabolic enzymes with GAPDH as loading control. The densitometric value of phosphorylated BCKDE1α was compared to that of total BCKDHE1α and the ratio was shown on top of the panels.

Supplemental Figure S2. Individual BCAA concentration in tissues from normal (Sham, n=10) and failing (Failure, n=7) murine hearts was measured and normalized to the weight of tissue. Error bars represent SEM.

Supplemental Figure S3. Individual BCKA concentration in plasma from human with normal (Ctrl, n=50) or failing (Failure, n=91) hearts was measured. Error bars represent SEM. *, p <0.05
compared to control.

**Supplemental Figure S4.** Individual BCAA concentration in tissues from normal (Ctrl, n=3) and failing (Failure, n=9) human hearts was measured and normalized to the weight of tissue. Error bars represent SEM.

**Supplemental Figure S5.** A, Real-time RT-PCR results of genes using mRNA from HeLa cells with (KLF15, n=3) or without (Vector, n=3) KLF15 overexpression. Y axis represents relative mRNA level. The data represented the average values with standard deviation of three samples. *, p <0.05 compared to vector control. B, Luciferase assay result of PP2Cm promoter-luciferase in HeLa cells co-transfected with either KLF15 or corresponding empty vector. 486bp, the 486bp promoter of PP2Cm; 486bpDD, the 486bp promoter with two GC-rich sites deleted. The data represented the average values with standard deviation of triplicate samples from one experiment representative of three independent experiments. *, p <0.05 compared to same promoter without KLF15 overexpression. #, p<0.05 compared to 468bp promoter with KLF15 overexpression.

**Supplemental Figure S6.** A, The densitometric values of the bands in Figure 34B were analyzed. The densitometric value of each protein was normalized to GAPDH. The data represented the average values of relative densitometry with standard deviation of four hearts. *, p<0.05 compared to WT control. B, Real-time RT-PCR result of KLF15 gene using mRNA from normal (Sham, n=3) and failing (Failure, n=3) heart induced by pressure overload. KLF15 mRNA level was normalized to 18sRNA. The data represented the average values with standard deviation of three hearts. *, p <0.05 compared to control.
Supplemental Figure S7. A, Individual BCAA concentration in tissues from PP2Cm knockout (KO, n=5) and wildtype (WT, n=5) mouse heart were measured with mass spectrometer and normalized to weight of tissue. Error bars represent SEM. *, p<0.05. B, Gene expression was examined by microarray using RNA from PP2Cm knockout (KO, n=3) and wildtype (WT, n=3) mouse heart. The data was presented as fold change versus WT. *, p<0.05.

Supplemental Figure S8. Time course for Ejection Fraction (%EF), left ventricular internal dimension at diastole (LVIDd), left ventricular posterior wall thickness at diastole (LVPWd), and left ventricular posterior wall thickness at systole (LVPWs) from TAC WT (n=11-15) and PP2Cm KO mice (n = 9-13). The X-axis show the time in weeks after surgery. *, p<0.05 compared to WT.

Supplemental Figure S9. A, Oxygen consumption in mitochondria isolated from wildtype hearts in absence or presence of BCKA-Na (500μM each of KIC, KIV, KMV mixed). NaCl (1.5mM) was used as control. Y axis: oxygen concentration (ppm) in assay buffer. The assay was completed in ~12 minutes. B, Relative oxygen consumption rate in the absence or presence of BCKA calculated based on results in A (n=3 in each group).

Supplemental Figure S10. Deletion of PP2Cm resulted in significant global perturbations in cardiac metabolism. A, Random Forest Confusion Matrix. Random Forest classification using named metabolites detected in heart tissue of wildtype and PP2Cm KO mice resulted in a predictive accuracy of 100%. B, Metabolomic analysis showed higher level of glucose, glycolytic intermediates, glucose-derived sugars such as fructose, and malate in PP2Cm deficient heart (red,
n=7) compared to that in wildtype heart (blue, n=8) at baseline. Statistical analyses were performed with Welch’s two-sample t-test. p<0.05 for all shown compounds.

**Supplemental Figure S11. Inhibition of BCKDK by BT2 reduced plasma BCAA level.** Individual BCAA concentration in plasma from wildtype and PP2Cm-KO (n=4-6) mice treated without (vehicle, veh group) or with BT2 (BT2 group). Error bars represent SEM. Statistical analyses were performed with Student’s t-test to compare the values of two groups. *, p<0.05 compare to KO Veh group.

**Supplemental Figure S12. Inhibition of BCKDK by BT2 preserves cardiac function.** A. Left ventricular internal dimension at diastole (LVIDd), B. Left ventricle pastier wall thickness at systole (LVPWs) and C. diastole (LVPWd) from mice following sham or post-TAC surgery at 4 weeks, treated with or without BT2 (n=6-8 in each group). Error bars represent SEM. Statistical analyses were performed with One-Way ANOVA followed by Newman-Keuls test for A and B, or Kruskal-Wallis test followed by Dunn’s multiple comparison test for C. *, p<0.05 between two groups.
**Supplemental Table S1.** The top 25 KEGG pathways identified by DAVID.

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**Supplemental Table S2.** The 25 down-regulated genes were identified and mapped into BCAA catabolism pathway by KEGG (Kyoto Encyclopedia of Genes and Genomes).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold decrease in failing heart</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abat</td>
<td>1.57</td>
<td>4-aminobutyrate aminotransferase</td>
</tr>
<tr>
<td>Acaa2</td>
<td>2.11</td>
<td>acetyl-Coenzyme A acyltransferase 2</td>
</tr>
<tr>
<td>Acadm</td>
<td>1.84</td>
<td>acyl-Coenzyme A dehydrogenase, medium chain</td>
</tr>
<tr>
<td>Acadsb</td>
<td>1.51</td>
<td>acyl-Coenzyme A dehydrogenase, short/branched chain</td>
</tr>
<tr>
<td>Acat1</td>
<td>1.54</td>
<td>acetyl-Coenzyme A acetyltransferase 1</td>
</tr>
<tr>
<td>Aldh2</td>
<td>1.68</td>
<td>aldehyde dehydrogenase 2, mitochondrial</td>
</tr>
<tr>
<td>Aldh6a1</td>
<td>1.84</td>
<td>aldehyde dehydrogenase family 6, subfamily A1</td>
</tr>
<tr>
<td>Aox1</td>
<td>2.63</td>
<td>aldehyde oxidase 1</td>
</tr>
<tr>
<td>Bckdha</td>
<td>1.88</td>
<td>branched chain ketoacid dehydrogenase E1, alpha polypeptide</td>
</tr>
<tr>
<td>Bckdhb</td>
<td>1.76</td>
<td>branched chain ketoacid dehydrogenase E1, beta polypeptide</td>
</tr>
<tr>
<td>Dbt</td>
<td>1.98</td>
<td>dihydrolipoamide branched chain transacylase E2</td>
</tr>
<tr>
<td>Dld</td>
<td>1.55</td>
<td>dihydrolipoamide dehydrogenase</td>
</tr>
<tr>
<td>Ehhadh</td>
<td>1.58</td>
<td>enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase</td>
</tr>
<tr>
<td>Hadh</td>
<td>1.72</td>
<td>hydroxyacyl-Coenzyme A dehydrogenase</td>
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<tr>
<td>Hadha</td>
<td>1.85</td>
<td>hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-</td>
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<tr>
<td></td>
<td></td>
<td>Coenzyme A hydratase</td>
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<tr>
<td>Hadhb</td>
<td>1.81</td>
<td>predicted gene 13910; similar to Hydroxyacyl-Coenzyme A dehydrogenase/3-</td>
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<tr>
<td></td>
<td></td>
<td>ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>beta subunit</td>
</tr>
<tr>
<td>Hibadh</td>
<td>1.55</td>
<td>predicted gene 11225; 3-hydroxyisobutyrate dehydrogenase</td>
</tr>
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<td>Hmgcs2</td>
<td>2.29</td>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2</td>
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<tr>
<td>Ivd</td>
<td>1.77</td>
<td>isovaleryl coenzyme A dehydrogenase</td>
</tr>
<tr>
<td>Mccc1</td>
<td>1.56</td>
<td>methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)</td>
</tr>
<tr>
<td>Mccc2</td>
<td>1.57</td>
<td>methylcrotonoyl-Coenzyme A carboxylase 2 (beta)</td>
</tr>
<tr>
<td>Mcee</td>
<td>1.54</td>
<td>methylmalonyl CoA epimerase</td>
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<tr>
<td>Mut</td>
<td>1.63</td>
<td>methylmalonyl-Coenzyme A mutase Mus musculus</td>
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<tr>
<td>Pcca</td>
<td>1.61</td>
<td>propionyl-Coenzyme A carboxylase, alpha polypeptide</td>
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<tr>
<td>Ppm1k</td>
<td>2.20</td>
<td>protein phosphatase 1K (PP2C domain containing)</td>
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</tbody>
</table>
### Supplemental Table S3.

The top 10 proteins identified by Upstream Regulator Analysis of IPA with the list of genes down-regulated in failing heart.

<table>
<thead>
<tr>
<th>Upstream Regulator</th>
<th>Molecule Type</th>
<th>p-value of overlap</th>
<th>Target molecules in dataset</th>
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</thead>
<tbody>
<tr>
<td>MAP4K4</td>
<td>kinase</td>
<td>1.31E-17</td>
<td>AA2A, ACACB, ACADVL, ALDH4A1, BCKDHA, CCBL2, COQ3, DHRS4, DLD, GCDH, GOT1, HADH, HADHA, HADHB, IVD, LD HB, MLXIPL, NDUFA5, NDUFS1, PEX11A, PHYH, PIGO, PLA2G12A, PXMP2, SLC2A4, SUCGL1</td>
</tr>
<tr>
<td>KLF15</td>
<td>transcription regulator</td>
<td>1.90E-15</td>
<td>ACADAM, ACADVL, Acot1, ACSS1, CPT2, DECR1, DGAT2, EHHADH, HADHA, HADHB, MLYCD, PXMP2, SLC25A20, SLC27A1, SLC2A4, UCP3</td>
</tr>
<tr>
<td>PPARA</td>
<td>ligand-dependent nuclear receptor</td>
<td>5.77E-15</td>
<td>ABCD3, ACAA2, ACADM, ACADVL, ACAT1, Acot1, ACOT2, ACSL1, ALDH2, ALDOB, C3, CPT2, DECR1, ECH1, EC11, EHHAD H, FDF1, FITM1, GPT, Gsta4, GSTK1, Gstt1, HADH, HADHA, HADHB, HMGCS2, HS17B11, KHK, LGALS4, MGST1, MLYCD, MT, CO2, PAQR9, PEX11A, PLIN5, PPARA, RETSAT, SELENBP1, SLC25A20, SLC27A1, SLC2A4, SORD, UCP3</td>
</tr>
<tr>
<td>HNF4A</td>
<td>transcription regulator</td>
<td>1.19E-10</td>
<td>ACA2, ACADM, ACADVL, ACOT2, ACSL1, ALDH2, ALDH5A1, ALDOB, AMACR, AS3MT, BCKDHA, BCL6, BOLA1, C21orf33, LOC102724023, C3, C4orf27, Cdkn1c, CLPX, CMSS1, COQ3, CPT2, CRAT, CREBL2, CUTC, CUZX, DBP, DBT, DEDD2, DHR S4, DNAJC28, DTWD1, ETFDH, FBXO31, FGFI3, FYCO1, GCHFR, GFM1, GOT1, GPT, GRB14, GSTK1, GTF2H4, HADHA, HADHB, HS11B1, HSPE1, HSPH1, IL15, KCN12, KIAA0141, KLF15, L2HDGDH, LARS2, MCCCI, MCEE, MGST1, MID1IP1, MIP EP, MLXIPL, MRPS21, MSRB2, MUT, NAMPT, NARS2, NDUFA5, NDUFS1, NUDT6, PANK1, PDSS2, PEX6, PFDN6, PHB, PIN K1, PNPO, PPARA, PPIB2, PPI1, PP1K2, PPL, PRDX5, RMND1, RPAP3, RPS6KA5, R1T4IP1, SCAND1, SLC22A3, SLC25A13, SLC25A20, SQRDL, STARD10, SUCLA2, SUCGL1, TBC1D16, TEF, TFTP, TME126B, UFP3B, UCQ1C, UROS</td>
</tr>
<tr>
<td>INSR</td>
<td>kinase</td>
<td>3.99E-10</td>
<td>ACA2, ACADM, ACADVL, ACOT2, ACSL1, ALDH2, ALDH6A1, ATP5G1, CPT2, CRAT, DECR1, EC11, EHHADH, FDF1, GADD45A, HADH, HADHB, HS1P1, ID1H, KLF9, MRPS21, NAMPT, PFDN6, PPARA, PRKCO, RTN2, SLC25A20, SLC2A4, SUCLA2, UCP3</td>
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<tr>
<td>PPARD</td>
<td>ligand-dependent nuclear receptor</td>
<td>6.48E-09</td>
<td>ACA2, ACADM, ACADVL, ACOT2, ACSL1, ALDH2, AMACR, ATTP2A2, ATP5G1, ATTPAF1, BCL6, CAMK2A, CAMK2B, CITED2, DBP, EC11, EHHADH, FDF1, FBXO32, FNDG5C, MAL, MAOB, MT-CO2, NDUFS1, PLIN5, PNLPA, PPARA, PRDX5, SDHA, SLC25A20, SLC2A4, SOD2, UCP3</td>
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<tr>
<td>PPARGC1A</td>
<td>transcription regulator</td>
<td>1.13E-08</td>
<td>ACACB, ACADM, ACADVL, ACAT1, C3, FBXO32, FNDG5C, MAL, MAOB, MT-CO2, NDUFS1, PLIN5, PNLPA, PPARA, PRDX5, SDHA, SLC25A20, SLC2A4, SOD2, UCP3</td>
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<tr>
<td>PRKAG3</td>
<td>kinase</td>
<td>1.26E-08</td>
<td>ACSL6, AMDC1, Cdkn1c, CES1, CLCN3, COQ3, GID4, GOT1, IL15, JPH1, MAGIX, MAP2K6, METTL7A, MID1IP1, NAMPT, SLC4 A1, SLC4A3, SORD, THRP, WNK2</td>
</tr>
<tr>
<td>HTT</td>
<td>transcription regulator</td>
<td>2.12E-08</td>
<td>ACADM, ACOT2, AMACR, AMDC1, ATP2A2, ATP5G1, ATTPAF1, BCL6, CAMK2A, CAMK2B, CITED2, DBP, EC11, EHHADH, FDF1, FBXO32, FNDG5C, MAL, MAOB, MT-CO2, NDUFS1, PLIN5, PNLPA, PPARA, PRDX5, SDHA, SLC25A20, SLC2A4, SOD2, UCP3</td>
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<tr>
<td>PPARG</td>
<td>ligand-dependent nuclear receptor</td>
<td>2.82E-08</td>
<td>ACA2, ACADM, ACAL1, ATTP2A2, BCL6, C3, Cdkn1c, CES1, CFD, CPT2, CRAT, CXCL14, DGAT2, EHHADH, FDF1, GADD45A, HADH, HADHB, HMGCS2, IVD, LOC102724788, PRODH, MGST1, MLYCD, NDUFA5, PEX11A, PEX6, PLIN5, PPARA, SLC25A20, SLC27A1, SLC2A4, UCP3</td>
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</tbody>
</table>
Supplemental Figure S1

A

Normalized density

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2Cm</td>
<td>1.50</td>
<td>0.20</td>
</tr>
<tr>
<td>E2</td>
<td>1.80</td>
<td>0.90</td>
</tr>
<tr>
<td>E1α</td>
<td>2.00</td>
<td>1.20</td>
</tr>
<tr>
<td>E1β</td>
<td>1.80</td>
<td>1.20</td>
</tr>
<tr>
<td>BCKDK</td>
<td>1.50</td>
<td>1.00</td>
</tr>
<tr>
<td>BCAT2</td>
<td>1.30</td>
<td>0.90</td>
</tr>
</tbody>
</table>

B

0.9  1.20  1.28  1.30  pE1α/E1α

Sham      Failure

GAPDH

Supplemental Figure S1
Supplemental Figure S2.

BCAA, μmol/g heart tissue

Sham Leu, Failure Leu, Sham Ile, Failure Ile, Sham Val, Failure Val
Supplemental Figure S3.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMV</td>
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<td></td>
</tr>
<tr>
<td>KIV</td>
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</tr>
<tr>
<td>KIC</td>
<td></td>
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</tr>
</tbody>
</table>

μmol/L Plasma
Supplemental Figure S4.
Figure S5

Panel A: Relative mRNA Level

- BCAT2
- E1α
- E1β
- E2
- PP2Cm
- Glut4

Vector vs. KLF15

Panel B: Relative Luciferase Expression

- Vector
- 486bp
- 486bpDD
Supplemental Figure S7.

A) BCAA, μM/g heart tissue

B) Relative mRNA Level

WT Leu
PP2Cm KO Leu
WT Ile
PP2Cm KO Ile
WT Val
PP2Cm KO Val

BCAA, μM/g heart tissue

WT
PP2Cm KO

* Significant difference

PP2Cm, E1α, E1β, BCKD, BCAT2, Myh7, Myh6, ANF, BNF

Supplemental Figure S7.
Supplemental Figure S9.

(A) Oxygen concentration in assay buffer (ppm) over time with mitochondria. Arrows indicate the addition of Succinate, BCKA/NaCl, and ADP.

(B) Rate of oxygen consumption comparison between ctr and BCKA.
### A

<table>
<thead>
<tr>
<th>Actual Group</th>
<th>KO</th>
<th>WT</th>
<th>Class Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO</td>
<td>7</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>WT</td>
<td>0</td>
<td>8</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Predictive accuracy = **100%**

### B

- **Glucose**
  - $\rightarrow$ Sorbitol
  - $\rightarrow$ glucose 6-P
  - $\rightarrow$ fructose 6-P
  - $\rightarrow$ fructose 1,6-bisP
  - $\leftarrow$ glyceraldehyde-3-P $\rightarrow$ DHAP
  - $\rightarrow$ 1,3-bisphosphoglycerate
  - $\rightarrow$ 3-phosphoglycerate
  - $\rightarrow$ 2-phosphoglycerate
  - $\rightarrow$ phosphoenolpyruvate $\leftarrow$ pyruvate $\rightarrow$ Malate
  - $\rightarrow$ Lactate
  - $\rightarrow$ acetyl CoA
Supplemental Figure S11.

Plasma Concentration (μM)

Leu/Ile

Val

WT Veh  WT B72  KO Veh  KO B72

WT Veh  WT B72  KO Veh  KO B72

*
Supplemental Figure S12.