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Defective Branched-Chain Amino Acid (BCAA) Catabolism Disrupts Glucose Metabolism and Sensitizes the Heart to Ischemia-reperfusion Injury

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Summary

Elevated levels of branched-chain amino acids (BCAAs) have recently been implicated in the development of cardiovascular and metabolic diseases but the molecular mechanisms are unknown. In a mouse model of impaired BCAA catabolism (KO), we found that chronic accumulation of BCAAs suppressed glucose metabolism and sensitized the heart to ischemic injury. High levels of BCAAs selectively disrupted mitochondrial pyruvate utilization through inhibition of pyruvate dehydrogenase complex (PDH) activity. Furthermore, downregulation of hexosamine biosynthetic pathway in KO hearts decreased protein O-linked-N-acetylglucosamine (O-GlcNAc) modification and inactivated PDH resulting in significant decreases in glucose oxidation. Although the metabolic remodeling in KO did not affect baseline cardiac energetics or function, it rendered the heart vulnerable to ischemia-reperfusion injury. Promoting BCAA catabolism or normalizing glucose utilization by overexpressing GLUT1 in the KO heart rescued the metabolic and functional outcome. These observations revealed a novel role of BCAA catabolism in regulating cardiac metabolism and stress response.

eTOC blurb

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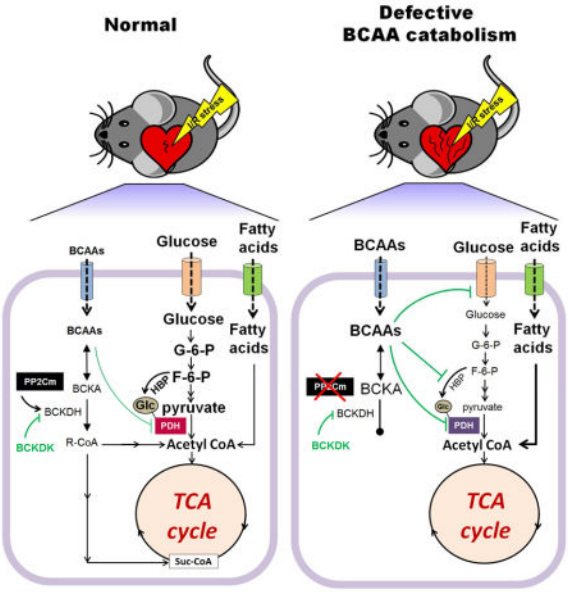
R.T., T.L. and Z.Z. designed all experiments. T.L., Z.Z., S.C.K., L.A., N.D.R., B.Z., Y.C., and J.R. performed the experiments. H.S. contributed the mouse model. H.G., D.R, and R.T. supervised the study. T.L., Z.Z., and S.C.K. analyzed the data. R.T. and T.L. wrote the manuscript.

Branched-chain amino acids (BCAAs) have been implicated in cardiovascular disease. Li, et al now reveal molecular mechanisms behind BCAA catabolism in regulating cardiac metabolism and stress response. Chronic accumulation of BCAAs downregulates the hexosamine biosynthetic pathway and inactivates pyruvate dehydrogenase, which renders the heart vulnerable to ischemic injury.



Introduction

Branched-chain amino acids (BCAAs), including leucine, isoleucine, and valine, are essential amino acids for mammals. The rate of protein turnover, BCAA intake and catabolism are key mechanisms in governing BCAA homeostasis in the body (Harper et al., 1984). In the BCAA catabolic pathway, BCAAs are first converted into branched-chain alpha-ketoacids (BCKAs) by branched-chain amino-transferase (BCAT) in a reversible reaction, followed by irreversible decarboxylation by branched-chain alpha-ketoacid dehydrogenase (BCKDH) complex and eventually metabolized to acetyl-CoA or succinyl-CoA for oxidation in the tricarboxylic acid (TCA) cycle. BCKDH, the rate-limiting enzyme of the pathway, is activated by dephosphorylation via a mitochondrial localized 2C-type ser/thr protein phosphatase (PP2Cm) (Lu et al., 2009). Loss of PP2Cm in genetic models impaired BCAA catabolism, increased oxidative stress, and in zebrafish caused abnormal cardiac and neural development (Lu et al., 2007; Lu et al., 2009).

The heart is a high energy-consuming organ, therefore, cardiac energy metabolism is essential for the normal biology and physiology of the heart. Alterations in cardiac substrate metabolism, in particular, glucose and fatty acid metabolism, have been recognized as important disease mechanisms (Kolwicz et al., 2013). Studies in the 1980s have reported that excessive BCAAs or their metabolites BCKAs could inhibit pyruvate dehydrogenase complex *in vitro* (Jackson and Singer, 1983; Randle et al., 1963; Randle et al., 1964; Williamson et al., 1979). It is unknown whether this is an effect of BCKAs directly or

indirectly owing to their conversion and accumulation of BCAAs. Importantly, the role of amino acids metabolism in the diseased heart has rarely been studied.

Emerging evidence shows that levels of circulating BCAAs and related metabolites are strongly associated with insulin resistance and coronary heart disease (Newgard, 2012; Newgard et al., 2009; Shah et al., 2010; Turer et al., 2009). On the other hand, BCAA supplementation is widely used to promote exercise capacity and has also been shown to improve mitochondrial function in middle ages animals (Crowe et al., 2006; D'Antona et al., 2010). The molecular mechanisms by which the BCAAs or their metabolites mediate these seemingly contradictory effects, however, remain elusive. In the present study, we sought to elucidate the impact of BCAA catabolism on cardiac energy metabolism and function using PP2Cm deficient mice. Our results identified novel mechanisms by which defective BCAA catabolism suppresses glucose metabolism and sensitizes the heart to ischemia-reperfusion (I/R) injury.

Results

BCAAs regulate glucose metabolism in the heart

We utilized multinuclear magnetic resonance (NMR) spectroscopy to investigate the impact of BCAA catabolism on cardiac energy metabolism in a mouse model of PP2Cm deficiency (KO). By performing phosphorous-31 (^{31}P) NMR spectroscopy of isolated perfused mouse heart, we found that the contractile function was similar in WT and KO hearts at 2–3 months of age, and the phosphocreatine to ATP ratio (PCr/ATP), an estimate of myocardial energetics, was also comparable between the two groups (Figures 1A and 1B). However, carbon-13 (^{13}C) NMR isotopomer analysis showed a ~50% decrease in glucose oxidation in KO as compared with WT hearts, which was concomitant with a reciprocal increase in fatty acid oxidation (Figures 1C and S1A).

As expected, impaired BCAA catabolism in KO resulted in elevated levels of BCAAs in the blood and cardiac tissue (Figures 1D and S1B). Addition of BCAAs to the perfusate to match the physiological blood level induced a reduction of glucose oxidation in WT hearts (P<0.05, WT vs. WT+BCAA) but no further decrease in the KO (P=NS, KO vs. KO +BCAA; Figure 1C). The ^{13}C isotopic enrichment of lactate or alanine in the perfused heart in the KO or the tissue level of lactate was also similar (Figures S1C–S1E). Cardiac glycogen content was significantly reduced in the non-perfused KO hearts (Figure 1E). Furthermore, supplementation of BCAAs in the perfusate significantly inhibited glycogen synthesis from exogenous glucose (Figure 1F). Despite the significant shift in substrate metabolism, expression of genes involved in glucose uptake, glycolysis, glycogenesis and fatty acid oxidation remained unaffected in the KO heart (Figure S1F). Together, these results suggest that BCAAs compete against glucose metabolism in the heart through non-transcriptional mechanisms.

Defective BCAA catabolism suppresses glucose uptake

We next assessed whether decreased glucose utilization in the KO hearts was due to inhibition of glucose uptake. Glucose uptake was measured in isolated perfused hearts using

non-tracer 2-deoxyglucose (2-DG) and ^{31}P NMR spectroscopy (Figure 1G) (Luptak et al., 2007b). A significant reduction in glucose entry was observed in KO hearts perfused with or without insulin stimulation, and acute addition of BCAAs to the perfusate did not change glucose uptake rate (Figures 1H and 1I). Of note, insulin stimulated a ~ 3-fold increase in glucose uptake in both WT and KO hearts, suggesting that defective BCAA catabolism in the KO did not cause insulin resistance. This was confirmed by normal blood glucose and insulin levels *in vivo* and unaltered glucose tolerance test in KO mice compared to the age-matched WT (Figures S1G–S1I). Further study demonstrated that the protein expressions involved in insulin signaling, including mTOR, p-mTOR, Akt, p-Akt, PI3K and p-PI3K in KO hearts were also comparable to those of WT (Figure S1J). Taken together, these results suggest that defective BCAA catabolism suppresses glucose uptake independent of insulin signaling.

Mitochondrial pyruvate utilization is inhibited in the KO heart

To understand the mitochondrial mechanism(s) underlying the suppression of glucose utilization by BCAAs, we examined mitochondrial morphology and function in the KO heart. Electron microscopic images showed normal mitochondrial size and density in the KO hearts (Figures 2A and 2B). Succinate-initiated complex II respiration and maximal respiratory capacity upon addition of the chemical uncoupler trifluorocarbonylcyanide phenylhydrazone (FCCP) were also comparable in isolated WT and KO mitochondria (Figure 2C). However, pyruvate/malate-supported complex I respiration and the respiratory control ratio (RCR) were significantly reduced in the KO in comparison with WT (Figures 2D, 2E and S2A). Interestingly, decreased respiration was not observed when using other complex I substrates, e.g. glutamate/malate or palmitoyl-carnitine/malate, suggesting a selective inhibition of pyruvate utilization in the KO (Figures 2E and S2A). Additionally, the total and the rotenone specific complex I activity were also normal in the KO group (Figure 2F). Moreover, acute exposure to BCAAs induced a >30% reduction of pyruvate-stimulated state-3 respiration in WT, but it did not further suppress respiration in KO mitochondria (Figures 2G–2I and S2B). Consistent with the above findings, addition of BCAAs did not alter respiration supported by succinate, glutamate/malate or palmitoyl-carnitine/malate in either groups (Figures S2C–S2E). Taken together, these data indicate that BCAAs selectively decrease mitochondrial pyruvate utilization without altering the structure or function of the respiratory complexes.

BCAAs directly inhibit pyruvate dehydrogenase (PDH) activity

PDH controls the flux of pyruvate into the mitochondria, serving as a key regulator for glucose oxidation. We found that PDH activity was significantly lower in the KO heart (Figure 3A). Consistently, ^{13}C-isotopomer analysis also showed a reduced PDH flux relative to tricarboxylic acid (TCA) cycle flux (V_{PDH}/V_{TCA}) in the KO heart (Figure 3B). Supplementation of BCAAs in the perfusate suppressed the V_{PDH}/V_{TCA} in the WT heart, indicating that BCAA exhibited an inhibitory effect of PDH flux *in vivo* (Figure 3B). To determine whether BCAAs directly inhibit PDH activity, purified porcine PDH enzyme was incubated with increasing concentrations of BCAAs (range from 4–5 folds above and below the physiological blood level in mice) at 37°C for 30 min (Herman et al., 2010; Newgard et al., 2009). We found that the PDH activity was inhibited dose-dependently by BCAAs but

not by other hydrophobic amino acids such as alanine *in vitro* (Figures 3C, 3D and S3A). The inhibition was preserved at physiological level of intracellular Ca^{2+} (Figure 3E). We also tested the effects of BCKAs, the first metabolites in BCAA catabolism pathway, at high physiological concentrations (Sun et al., 2016). Increasing doses of BCKAs did not exert any inhibitory effect on PDH activity (Figures S3B and S3C). These data collectively indicate that BCAAs rather than their metabolites directly inhibit PDH activity, which contributes to the reduced PDH flux and decreased glucose oxidation in the heart.

Defective BCAA catabolism modulates PDH activity through protein O-linked-N-acetylglucosamine (O-GlcNAc) modification

Since the PDH activity was lower in cardiac tissue of the KO *in vitro*, we hypothesized that in addition to the direct inhibition by BCAAs, the PDH activity was also altered by chronic modifications due to defective BCAA catabolism. It has been well documented that the PDH activity is rapidly regulated by reversible phosphorylation of its E1α component (Holness and Sugden, 2003; Kaplon et al., 2013; Kolobova et al., 2001). However, there were no differences in total PDH protein expression and E1α phosphorylation between WT and KO, under either fed or fasting condition although fasting induced a significant increase of phosphorylation as expected (Figure S3D). Isolated mitochondria from the KO heart also exhibited similar PDH E1α phosphorylation level as compared to that of the WT (Figure S3E). Furthermore, the mRNA levels of *PDH phosphatase 1* (*PDP1*), *PDP2* and *PDH kinase 4* (*PDK4*), as well as mitochondrial pyruvate carrier (*MPC1* and *MPC2*) were not different between WT and KO (Figure S3F). Thus, it is unlikely that decreased PDH activity in KO is attributable to altered phosphorylation.

To determine whether PDH can be modified by glycosylation, we immunoprecipitated PDH from WT or KO LV tissue lysates and probed with anti-O-GlcNAc antibody. The PDH from KO heart exhibited a lower signal of O-GlcNAc modification (Figures 3F and 3G). Conversely, we immunoprecipitated all the O-GlcNAcylated protein and probed with anti-PDH antibody. Less PDH was O-GlcNAc modified in the KO heart (Figure S3G). PDH complex is comprised of subunits from multiple catalytic enzymes: pyruvate dehydrogenase (E1α/β), dihydrolipoamide transacetylase (E2), and dihydrolipoamide dehydrogenase (E3), as well as the tethering protein, E3-binding protein (E3bp). We detected O-GlcNAc modification signal at the E2 and E3/E3bp subunits but not the E1α subunits. To test whether O-GlcNAcylation regulates PDH activity, we incubated the LV tissue lysates with uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the O-GlcNAc precursor and measured the PDH activity *in vitro*. UDP-GlcNAc elevated protein O-GlcNAcylation (Figure S3H) and the PDH activity in both WT and KO groups in a dose-dependent fashion (Figure 3H). Notably, PDH activity in KO heart was normalized to the WT level after incubation with UDP-GlcNAc, suggesting that PDH activity is positively related to the level of O-GlcNAc modification. Increased glycosylation also blunted the inhibitory effects of BCAAs on PDH (Figure 3I). Together, these data indicate that chronic accumulation of BCAAs due to defective catabolism reduces protein O-GlcNAc modification and impairs PDH activity.

Chronic accumulation of BCAAs suppresses protein glycosylation

We observed a ~40% decrease in protein O-GlcNAc modification in the KO heart (Figures 4A and 4B). This decrease was companied by a downregulation of protein abundance of multiple enzymes in the hexosamine biosynthetic pathway (HBP). Glutamine: fructose 6-phosphate aminotransferase (GFAT) 1 and GFAT2, the rate-limiting enzymes of the HBP, were significantly reduced in the KO heart (Figures 4C, 4D and S4A). The protein levels of hexokinase (HK) II and O-GlcNAc transferase (OGT), which generates glucosamine-6-phosphate (GlcN-6-P) from glucosamine or conjugates O-GlcNAc groups to target protein, respectively, were also decreased. The mRNA levels of these enzymes were not changed though in the KO heart (Figure S4B). To determine whether BCAAs or their metabolites were responsible for the reduced protein O-GlcNAc modification in KO, we incubated isolated adult rat cardiomyocytes with increasing concentrations of BCAAs for 24 hours. BCAAs elicited a downregulation of protein O-GlcNAc modification at high concentrations, while incubation with increasing doses of BCKAs did not lead to such a change (Figures 4E–4G).

The KO hearts have normal baseline function but show increased sensitivity to I/R injury

Despite the reduction of glucose metabolism in the KO hearts, we did not observe any changes of cardiac function *in vivo* under unstressed condition. Echocardiographic measurements showed normal fractional shortening (FS), left ventricular (LV) posterior wall thickness and LV chamber dimension in KO mice at 2 and 6 months of age (Figures 5A–5C). There is no evidence of pathological hypertrophy or fibrosis in the KO hearts at gross morphological, histological or molecular level up to 12 months of age (Figures 5D, 5E, S5A and S5B).

We next investigated whether the metabolic reprogramming in the KO heart would affect the response to stress by subjecting the isolated perfused hearts to 25-minute low flow ischemia (1% of baseline) and 40-minute reperfusion. The baseline LV-developed pressure (LVDevP), LV-end diastolic pressure (LVEDP), and rate pressure product (RPP) were not different between WT and KO. The KO hearts showed a greater increase of diastolic pressure during ischemia, which persisted throughout the reperfusion (Figure 5F). Furthermore, the contractile function in KO barely recovered while it recovered to ~40% of baseline in WT at the end of reperfusion (Figures 5G and 5H). Simultaneous assessment of cardiac energetics revealed that the KO heart had an accelerated rate of ATP depletion coupled with a greater increase of inorganic phosphates during ischemia (Figures 5I–5K). The recovery of high energy phosphate content during reperfusion was also impaired in the KO hearts (Figures 5I–5K).

Excessive BCAAs worsen I/R injury which is rescued by promoting BCAA catabolism

To determine whether BCAA or its catabolism was responsible for the vulnerability of the heart to I/R insult, we sought to increase BCAA level or BCAA catabolism in mice. Supplementing BCAAs for 7 days (1.5mg/g body weight per day) in non-transgenic mice did not alter body weight, blood glucose and the BCAA catabolism-related mRNA expression (Figures S5C–S5E) but elevated circulating BCAA concentrations for 6 hours (Figures 6A). When these mice were subjected to *in vivo* I/R injury within the 6-hour time

frame, a greater infarct size was observed, accompanied by increased oxidative stress (Figures 6B–6D, S5F and S5G). Simultaneous stimulation of BCAA catabolism via BT2 treatment, an activator of BCKDH (Figures 6E–6G), led to a decrease in infarct size, suggesting that promoting BCAA catabolism was able to rescue the adverse effect of excessive BCAAs (Figures 6B–6D). Similarly, KO mice exhibited a larger infarct size, which was significantly reduced by BT2 treatment (Figures 6H–6J and S5H). Interestingly, BT2 alone did not change the infarct size in the control mice (Figures 6B–6D and 6H–6J). Also, supplementation of BCAAs in the KO mice did not further increase the infarct size, suggesting that the cardiac toxicity of excessive BCAAs could be saturated (Figures 6H–6J). In isolated perfused hearts, inclusion of physiological level of BCAAs in the perfusate did not change the responses to I/R in either WT or KO hearts (Figures S5I–S5K). These results collectively show that high BCAA level but not catabolism of BCAAs adversely affects the outcome of I/R insult.

Exacerbated I/R injury in the KO heart can be rescued by enhancing glucose metabolism

We hypothesized that impaired glucose metabolism due to defective BCAA catabolism accounted for exacerbated I/R injury, and therefore tested whether enhancing glucose metabolism would rescue the phenotype in the KO. We crossed the KO with a mouse line with cardiac-specific overexpression of GLUT1, an insulin independent glucose transporter (KO/TG) (Liao et al., 2002). Overexpression of GLUT1 rescued the KO heart from the I/R injury, as evidenced by improved recovery of myocardial function and energetics in isolated perfused hearts from TG and KO/TG mice (Figures 7A–7D, S6A and S6B). In mice subjected to 30-minute ischemia and 24-hour reperfusion *in vivo*, the infarct size of KO/TG was also significantly reduced in comparison with the KO (Figures 7E–7G). This rescue was associated with increases in glucose uptake, glycolysis, glycogen synthesis and glucose oxidation in KO/TG either in the presence or absence of BCAAs (Figures 7H, 7I, and S6C–S6F).

It has been shown that protein O-GlcNAc modification is a critical protective mechanism against the I/R insult (Champattanachai et al., 2007; Wang et al., 2014). We found that decreased protein O-GlcNAcylation in the KO heart persisted during I/R (Figures 7J–7M). Overexpression of GLUT1 in KO normalized the expression of key enzymes in HBP (Figures 7N and 7O) and restored the level of protein O-GlcNAcylation (Figures 7J–7M). Measurement of HBP metabolites confirmed the downregulation of glucosamine (GlcN), glucosamine-6-phosphate (GlcN-6-P) and acetylglucosamine-1-phosphate (GlcNAc-1-P) in KO, while overexpression of GLUT1 significantly increased and/or normalized these metabolites, including GlcN, GlcN-6-P, and GlcNAc (Figure 7P).

We have previously reported that overexpression of GLUT1 promotes myocardial glucose utilization and protects against I/R injury (Luptak et al., 2007b). Here, we also found that the TG hearts showed better recovery compared to the WT after I/R. Importantly, the KO/TG recovered to the same level as the TG during the reperfusion, suggesting that reversing the suppression of glucose metabolism is sufficient to rescue the pathological mechanism(s) caused by defective BCAA catabolism.

Discussion

In the present study, we demonstrate that BCAA catabolism plays an important role in regulating glucose metabolism in the heart (Figure S7). In normal hearts, BCAAs directly inhibit PDH activity hence decrease glucose oxidation and promote fatty acid oxidation. In hearts defective of BCAA catabolism there are marked decreases in glucose uptake, oxidation, glycogen content and protein glycosylation. Chronic accumulation of BCAAs downregulates hexosamine biosynthetic pathway and inactivates PDH by reducing the O-GlcNAc modification of E2 and E3/3bp subunits. Even though the metabolic remodeling caused by defective BCAA catabolism is well tolerated under unstressed conditions, it renders the heart vulnerable to ischemic injury, which is completely rescued by either improving BCAA catabolism or enhancing glucose utilization. Our results, therefore, reveal a critical role of BCAA catabolism in regulating cardiac metabolism and stress response.

Unlike most of the amino acids, which are degraded in the liver, BCAAs are oxidized extensively in extra-hepatic tissue although the significance of such a metabolic pattern has not been completely understood (Harper et al., 1984). The heart expresses the highest levels of the enzymes in the BCAA catabolic pathway but BCAA oxidation contributes to a very small fraction of energy production in the heart (Brand, 1981; Harper et al., 1984), suggesting that this pathway has other functions in addition to energy generation. The rate-limiting step in BCAA catabolism is BCKA decarboxylation in the mitochondria catalyzed by BCKDH complex. PP2Cm is responsible for the dephosphorylation of the E1α subunit (Ser293) of the BCKDH and hence activation of the complex (Lu et al., 2009). As expected, deletion of PP2Cm in mice impairs BCAA catabolism resulting in a higher level of circulating and tissue BCAAs in the heart. Interestingly, inability to oxidize BCAAs does not affect myocardial energetics or insulin sensitivity in these mice but significantly remodeled substrate metabolism by suppressing glucose metabolism and shift the oxidative metabolism towards fatty acid oxidation.

Our study identified PDH as a key regulatory point through which BCAAs modulate cardiac metabolism. Results obtained from experiments using purified PDH, isolated mitochondria and intact beating hearts consistently show that physiological level of BCAAs directly inhibits PDH activity. As a rate-limiting enzyme for pyruvate oxidation, the activity of PDH serves as a set point for the relative contributions of carbohydrates versus fatty acids for the oxidative metabolism in the mitochondria (Stanley et al., 2005). Thus, by competing against glucose for oxidative degradation through modulating PDH activity BCAAs and their catabolism play a pivotal role in the regulation of cardiac substrate metabolism.

While the inhibition of PDH by acute exposure to BCAAs appears to be competitive, chronic accumulation of BCAAs in the PP2Cm deficient hearts inactivates PDH via mechanism(s) that persist when BCAAs are removed from the assay. It is well known that PDH activity is regulated by covalent modification of the E1 subunits, primarily by phosphorylation (Holness and Sugden, 2003; Kolobova et al., 2001). Here we ruled out changes in E1α phosphorylation in the PP2Cm deficient hearts and, instead, showed that manipulations of O-GlcNAc modification of the E2/3/3bp subunits are sufficient to alter the PDH activity. It does not exclude the possible that other forms of post-translational

modification could affect PDH activity and flux during accumulation of BCAAs. Nevertheless, the present findings identify a novel mechanism for the regulation of PDH activity. It also reveals new candidate pathways through which BCAA catabolism regulates cardiac biology and metabolism.

It has long been established that PDH activity is a key determinant for myocardial I/R injury (Lewandowski and White, 1995; Stanley et al., 1997). Cardiac I/R insult is associated with decreased PDH activity and increased fatty acid oxidation both uncouples glycolysis from glucose oxidation (Kobayashi and Neely, 1983; Liu et al., 1996; Vary and Randle, 1985). Manipulation of PDH activity, such as increasing pyruvate supply, reducing PDH phosphorylation by dichloroacetate (DCA) or partial inhibition of fatty acid oxidation has been shown to improve I/R injury (Chandler et al., 2003; Kobayashi and Neely, 1983; Lewandowski and White, 1995).

O-GlcNAc modifications are catalyzed by OGT that uses UDP-GlcNAc, the final product of the hexosamine biosynthesis pathway (HBP), as its substrate. The HBP is an accessory pathway of glucose metabolism accounting for approximately 5% of glucose entering cells. The production of UDP-GlcNAc by HBP is, however, a primary determinant of O-GlcNAc modification that governs the function of numerous proteins under both physiological and pathological conditions (Guo et al., 2014; Wang et al., 2014; Zachara, 2012). Upregulation of HBP and increased O-GlcNAc modification have been shown protective against acute I/R injury (Champattanachai et al., 2007; Wang et al., 2014). Here we report that defective BCAA catabolism downregulates HBP and decreases O-GlcNAc modification resulting in higher susceptibility to stress. As the propensity to ischemic injury can be rescued by specifically overriding the suppression of glucose metabolism in the PP2Cm deficient heart, our results indicate a causal relationship linking defective BCAA catabolism to the regulation of glucose metabolism and stress response in the heart. These findings also suggest that promoting glucose metabolism under conditions of impaired BCAA catabolism, such as increasing glucose uptake and increasing the coupling of glycolysis and glucose oxidation via stimulation of PDH or partial inhibition of fatty acid oxidation, would be beneficial.

In conclusion, our study has identified PDH as a key target and HBP a novel candidate pathway that link BCAA catabolism with the regulation of substrate metabolism in the heart. Through these mechanisms, defective BCAA catabolism and chronic accumulation of BCAAs suppress glucose metabolism, reduce protein O-GlcNAc modification and exacerbate I/R injury. Thus, BCAA catabolism is essential for the homeostasis of cardiac metabolism although BCAA oxidation is dispensable for energy production.

Experimental Procedures

Animals

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Washington. PP2Cm germ-line knockout (KO) mice with C57BL/6 background were generated as previously described (Lu et al., 2009). To overexpress GLUT1, the PP2Cm-KO mice were crossed with cardiac-specific GLUT1-TG

mice for more than 6 generations into a C57BL/6 background. Mice were maintained on standard chow diet and water available ad libitum in a vivarium with a 12-hour light/dark cycle at 22°C. BCAAs (weight ratio, isoleucine: leucine: valine =0.8:1.5:1, Sigma-Aldrich) were administrated by oral gavage for 7 days at 1.5 mg/g body weight/day (D'Antona et al., 2010). Compound BT2 (3,6-dichlorobenzo[b]thiophene-2-carboxylic acid) were purchased from Santa Cruz biotechnology and administrated by oral gavage at 40 mg/kg body weight/day for 7 days as previously described (Sun et al., 2016). Blood samples for BCAA level measurement were collected via retro-orbital bleeding. Additional details are provided in the Supplemental Experimental Procedures.

Isolated mouse heart perfusion and ^{31}P NMR spectroscopy

To assess left ventricular function and cardiac energetics, we performed ^{31}P NMR spectroscopy of isolated isovolumic perfused hearts (Kolwicz et al., 2015; Luptak et al., 2007b; Yan et al., 2009; Yu et al., 2014). Glucose uptake in the perfused heart was measured using non-tracer glucose analogue 2-DG and ^{31}P NMR spectroscopy as previously described (Liao et al., 2002). The uptake rate was assessed by the time dependent accumulation of 2-DG-P and normalized to the heart weight. Additional details are provided in the Supplemental Experimental Procedures.

Cardiac I/R experiments in vivo and ex vivo

Cardiac I/R surgery (30-minute ischemia followed by 24-hour reperfusion) were performed as previously reported (Gao et al., 2010). For the mice with BCAAs and/or BT2 gavage, I/R surgery were performed between 1.5–3 hours after the final gavage. For the *ex vivo* cardiac I/R study, all isolated Langendorff-perfused hearts were stabilized for 25 minutes and then subjected to 25-minute low-flow (1% of baseline) global ischemia and 40-minute reperfusion. Additional details are provided in the Supplemental Experimental Procedures.

^{13}C NMR spectroscopy and isotopomer analysis

Isolated mouse hearts were perfused with unlabeled mixed-substrate buffer (in mM; NaCl 118, $NaHCO_3$ 25, KCl 4.1, $CaCl_2$ 2, $MgSO_4$ 1.2, KH_2PO_4 1.2, EDTA 0.5, glucose 5.5, mixed long-chain fatty acids bound to 1% albumin 0.4, lactate 1, and insulin 50 μU/mL) for 20 minutes and ^{13}C-labeled mixed-substrate buffer ([1,6-^{13}C] glucose and [U-^{13}C] mixed long-chain fatty acids) for another 40 minutes. The contributions of each labeled substrate and the total of unlabeled substrates to the oxidative metabolism were determined using the ^{13}C isotopomer peak areas of the C3- and C4-glutamate by modeling the TCA cycle fluxes as previously described (Luptak et al., 2007a). An in-depth description of the method can be found in the Supplemental Experimental Procedures.

In vitro mitochondrial Assays

Mitochondria were isolated as previously described (Boehm et al., 2001). Briefly, freshly isolated hearts were rinsed with sucrose-containing mitochondrial isolation buffer. Heart tissue was chopped into pieces, trypsinized, and homogenized by a Dounce homogenizer with Teflon pestle. Mitochondria were purified through centrifugations in sucrose gradient. Mitochondria respiration assay was performed using a Clark-type oxygen electrode

(Hansatech instrument, UK) (Kuznetsov et al., 2008). Procedures are described in detail in the Supplemental Experimental Procedures.

Measurement of BCAA concentration in cardiac tissue and serum

The sample for cardiac BCAA level measurement was prepared as reported previously (Marney et al., 2013). Briefly, frozen mouse heart tissue (20–25mg) was extracted via methanol/chloroform (1:2 v/v; 4°C). The aqueous layer was separated and dried. Blood serum samples were subjected to protein precipitation by methanol and the supernatant was separated and dried. After derivatized with MTBSTFA, samples were run on an Agilent 5977A Series GC/MSD system. Additional details are provided in the Supplemental Experimental Procedures.

RT-PCR, Western Blotting and Immunoprecipitation

The isolation of total RNA, real-time PCR, and Western blot were described in the Supplemental Experimental Procedures. The primers are described in the Table S1. Immunoprecipitation experiments were performed with a PDH Rodent Immunocapture kit (Abcam) or protein A/G agarose (Pierce) coupled with an anti-O-GlcNAc antibody (Abcam). Detailed descriptions can be found in the Supplemental Experimental Procedures.

Biochemical Assays

The glycogen content in cardiac tissue lysate was measured with colorimetric assay kit (BioVision). The PDH activity was determined using a coupled enzyme reaction, which results in a colorimetric product proportional to the enzymatic activity (Sigma). Mitochondria complex I enzyme activity was measured by spectrophotometer as previously reported (Spinazzi et al., 2012). The lactate level was measured with Lactate Reagent according to manufacturer's instructions (Trinity Biotech). The insulin level in serum sample was measured using a commercially available kit (Sigma). The blood glucose level was measured by ONE TOUCH Glucose Monitor (Lifescan Inc.) using blood samples obtained by tail bleeding.

Histology and Electron Microscopy

A detailed description of the procedures of histology and electron microscopy of heart tissues is available in the Supplemental Experimental Procedures.

Statistical Analysis

All data are presented as mean ± SEM. An unpaired Student *t* test was used to detect significant differences when two groups were compared. One-way or two-way ANOVA was used to compare the differences among three or more groups. ANOVA with repeated measures was used for multiple group comparisons over multiple time points. Bonferroni post hoc analysis was used for all ANOVAs as applicable (SPSS 16.0 software). P values < 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Branched-chain amino acid (BCAA) catabolism regulates glucose metabolism in the heart
- High levels of BCAAs selectively disrupt mitochondrial pyruvate utilization
- Downregulation of HBP by chronic accumulation of BCAAs inactivates PDH
- Defective BCAA catabolism sensitizes the heart to ischemia-reperfusion insult

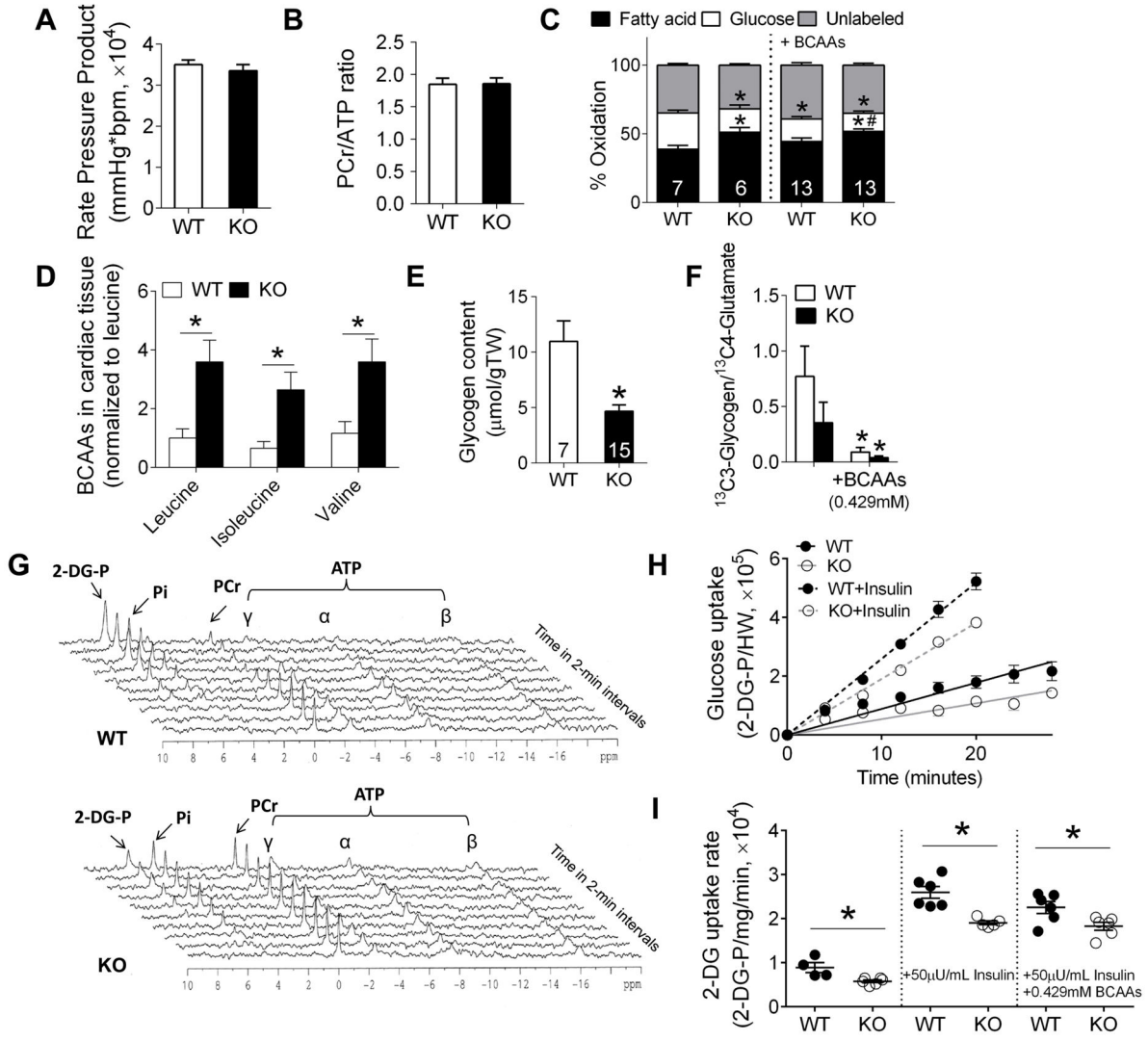

Figure 1. Defective BCAA catabolism alters cardiac glucose metabolism

(A) Contractile function of isolated perfused hearts from KO and WT mice measured as rate pressure product (the product of left ventricular developed pressure and heart rate) (n=15 per group).

(B) Phosphocreatine to ATP ratio (PCr/ATP) assessed by ^{31}P NMR spectroscopy in isolated perfused hearts (n=15 per group).

(C) Relative contribution of glucose, fatty acids and other substrates (lactate, endogenous) to the TCA cycle in hearts perfused with ^{13}C-labeled substrates without (n=6–7 per group) or with 0.429mM BCAAs (+BCAAs: n=13 per group).

(D) The BCAA levels in cardiac tissue extracts measured by GC-MS (n=4–5 per group).

(E) Glycogen content in WT and KO hearts (n=7–15 per group.

(F) Enrichment of ^{13}C3-glycogen normalized by ^{13}C4-Glutamate in perfused hearts (n=5–6 No BCAAs, and n=10–11 +BCAAs).

(G) Representative ^{31}P NMR spectra showing time-dependent accumulation of 2-DG-P in the WT and KO hearts. 2-DG-P, 2-deoxyglucose-6-phosphate. PCr, phosphocreatine. Pi, inorganic phosphate. γ, α and β represent the 3 phosphates on adenosine in ATP.
(H) Average time-dependent accumulation of 2-DG-P in KO and WT hearts (n=4–6 per group).
(I) The 2-DG uptake rate in hearts perfused by insulin-free and insulin-containing perfusion buffer with or without 0.429 mM BCAAs (n=4–6 per group). Data are shown as mean ± SEM. *P<0.05 vs. WT, $^{\#}P$<0.05 vs. WT+BCAAs. See also Figure S1.

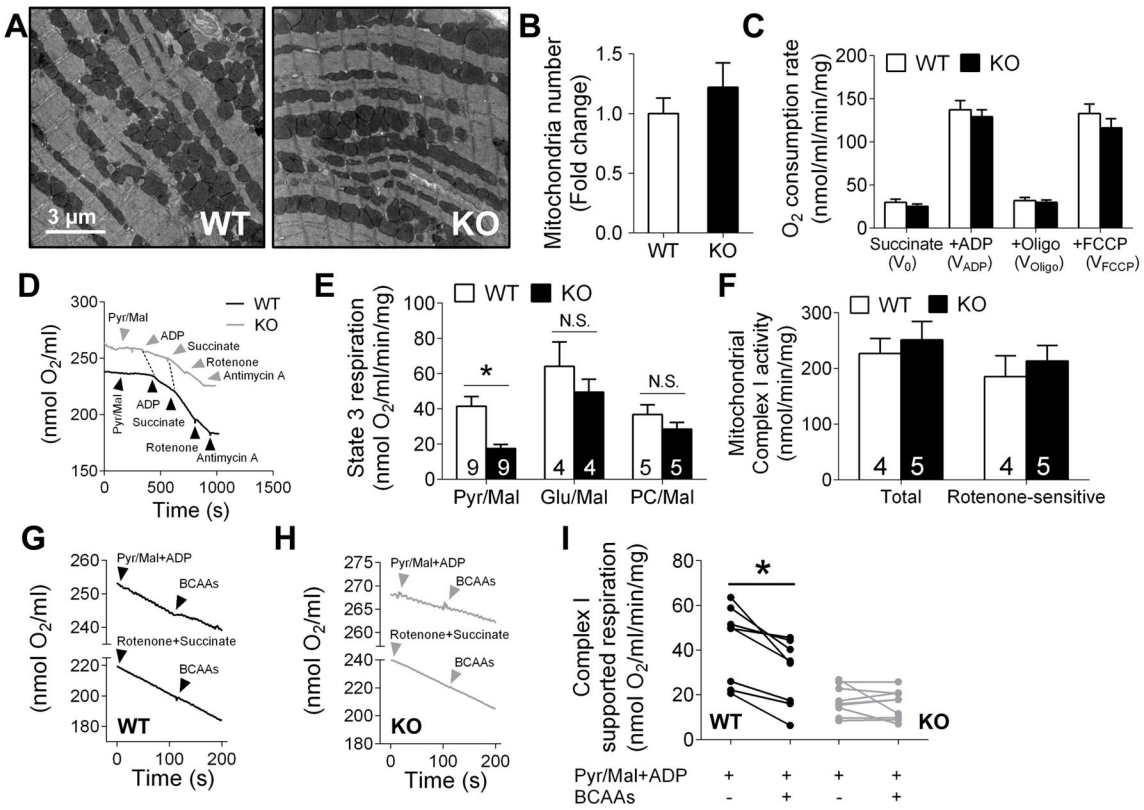

Figure 2. Mitochondrial pyruvate utilization is inhibited in the KO heart

(A) Representative electron microscopy images of WT and KO heart sections. Scale bar: 3 μm.

(B) Fold differences in mitochondrial number. Mitochondrial number was counted in a total of 10 images per heart (45μm^2 per image at x12000 magnification, n=3 per group). Data were expressed as fold changes relative to WT.

(C) Mitochondrial respiration, after inhibition of complex I by rotenone (1μM), was measured with sequential addition of succinate (5 mM, V_0), ADP (150 μM, V_{ADP}), Oligomycin (1 mg/ml, V_{Oligo}) and FCCP (1 μM, V_{FCCP}) (n=9 per group).

(D) Representative oxygraph trace of WT and KO mitochondria after adding substrates and inhibitors as indicated.

(E) State 3 respiration of WT and KO mitochondria stimulated with ADP (2.5 mM) in the presence of pyruvate/malate (5 mM/2 mM), glutamate/malate (10 mM/2 mM), or palmitoylcarnitine/malate (50 μM/2 mM) (n=4–9 per group).

(F) The total complex I activity and rotenone-sensitive activity in isolated mitochondria from WT and KO hearts (n=4–5 per group).

(G and H) Representative oxygraph traces of State 3 respiration in WT (G) and KO (H) mitochondria supported by pyruvate/malate (upper) or succinate (lower) without or with 0.429mM BCAAs.

(I) The effect of BCAAs on pyruvate/malate-supported respiration in WT and KO mitochondria (n=8–9 per group). Data are shown as mean ± SEM. *P<0.05 for indicated comparisons, N.S.: no significant difference. See also Figure S2.

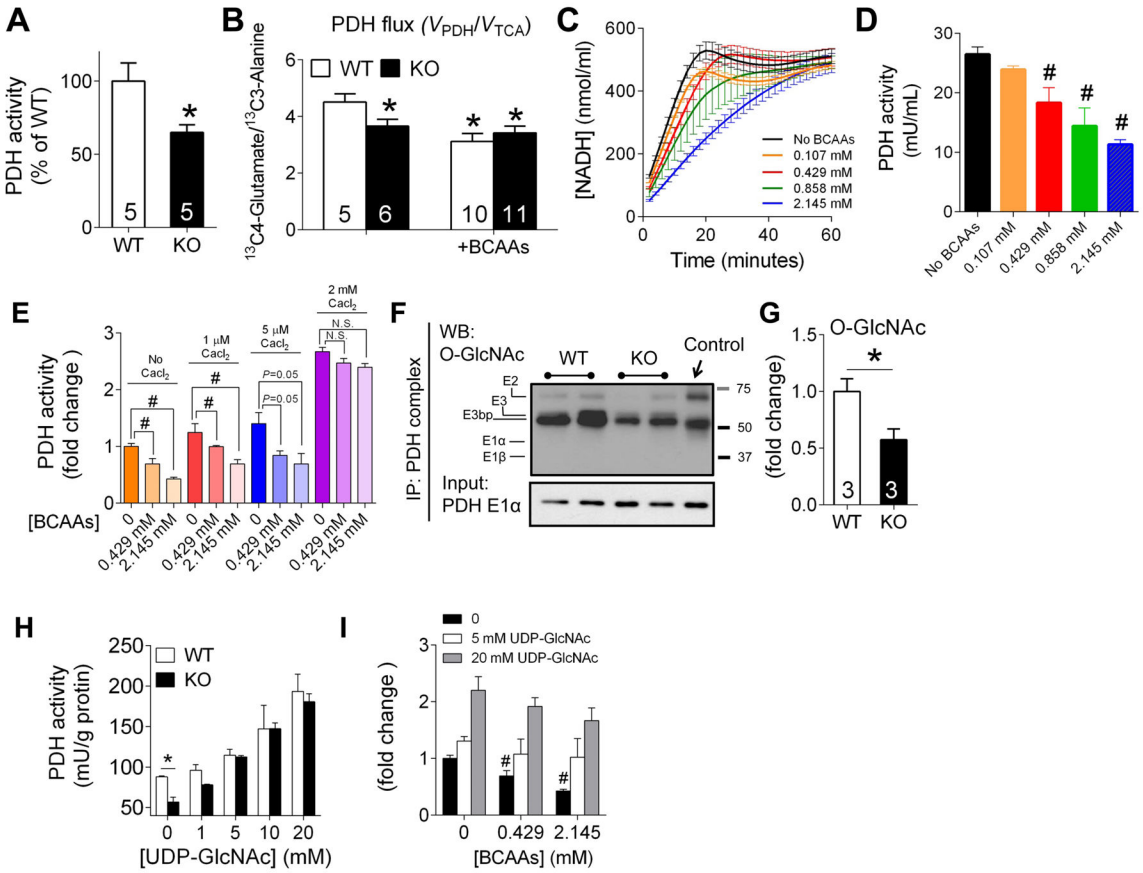

Figure 3. BCAA metabolism regulates PDH activity

(A) The PDH activity in the WT and KO hearts (n=5 per group).

(B) Relative PDH flux (V_{PDH}/V_{TCA}) of the WT and KO hearts perfused by ^{13}C-labelled substrate with or without 0.429 mM BCAAs (n=5–11 per group).

(C) The activity of purified porcine PDH complex measured by the rate of NADH generation in the presence of increasing concentrations of BCAAs (0, 0.107 mM, 0.429 mM, 0.858 mM, 2.145 mM) (n=3 per group).

(D) The average PDH activity at each [BCAAs] measured as nmol of NADH generated per minute at pH 7.5 at 37 °C (mU, n=3 per group).

(E) The effect of Ca^{2+} on PDH activity of tissue lysates from WT hearts in the presence of indicated concentrations of BCAAs (n=3–4 per group).

(F and G) Representative blots and quantitation of O-GlcNAc modified PDH subunits in WT and KO hearts (n=3 per group). Purified porcine PDH complex was used as positive control and PDH E1α subunit was blotted as input.

(H) PDH activity of tissue lysates from WT and KO hearts incubated with increasing concentration of UDP-GlcNAc (n=3–4 per group).

(I) The effect of UDP-GlcNAc on PDH activity of tissue lysates from WT hearts in the presence of indicated concentrations of BCAAs (n=3–4 per group). Data are shown as mean ± SEM. *P<0.05 vs. WT, #P<0.05 vs No BCAAs. See also Figure S3.

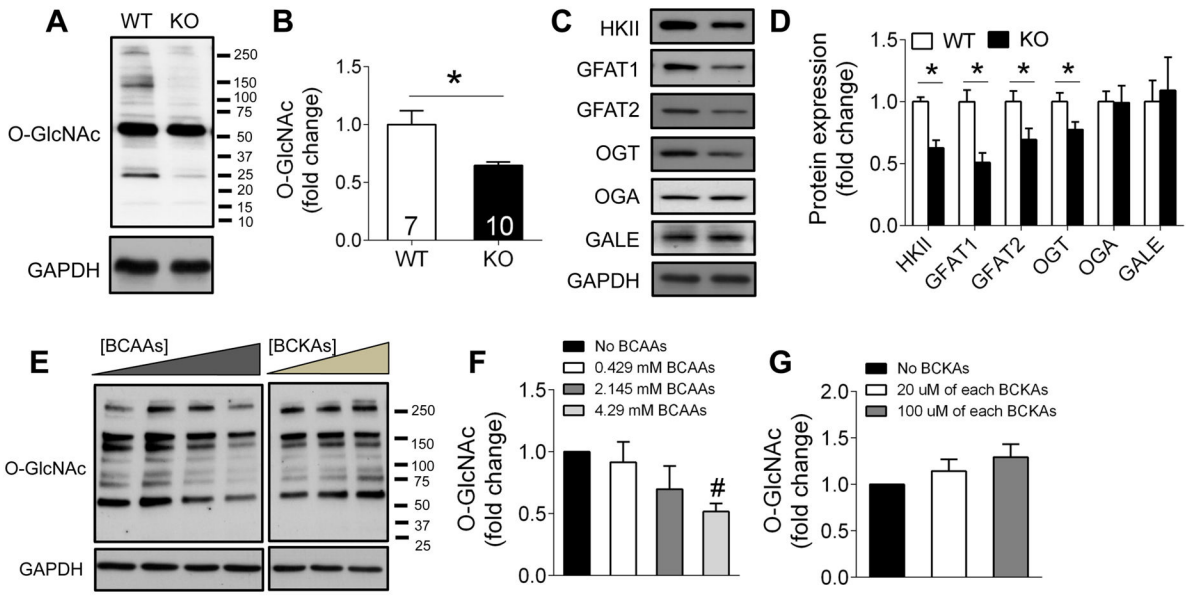

Figure 4. Protein O-GlcNAc modification in KO

(A and B) Western blot analysis of O-GlcNAc modified protein in hearts from WT and KO mice (n=7–10 per group).

(C and D) Protein levels of HKII, GFAT1, GFAT2, OGT, OGA, GALE in hearts from WT and KO mice (n=4–9 per group).

(E–G) Representative blots and a quantitation graph of O-GlcNAc modified protein in isolated adult rat cardiomyocytes treated with increasing concentration of BCAAs or BCKAs (n=3 per group). Data are shown as mean ± SEM. **P*<0.05 vs. WT, #*P*<0.05 vs No BCAAs. See also Figure S4.

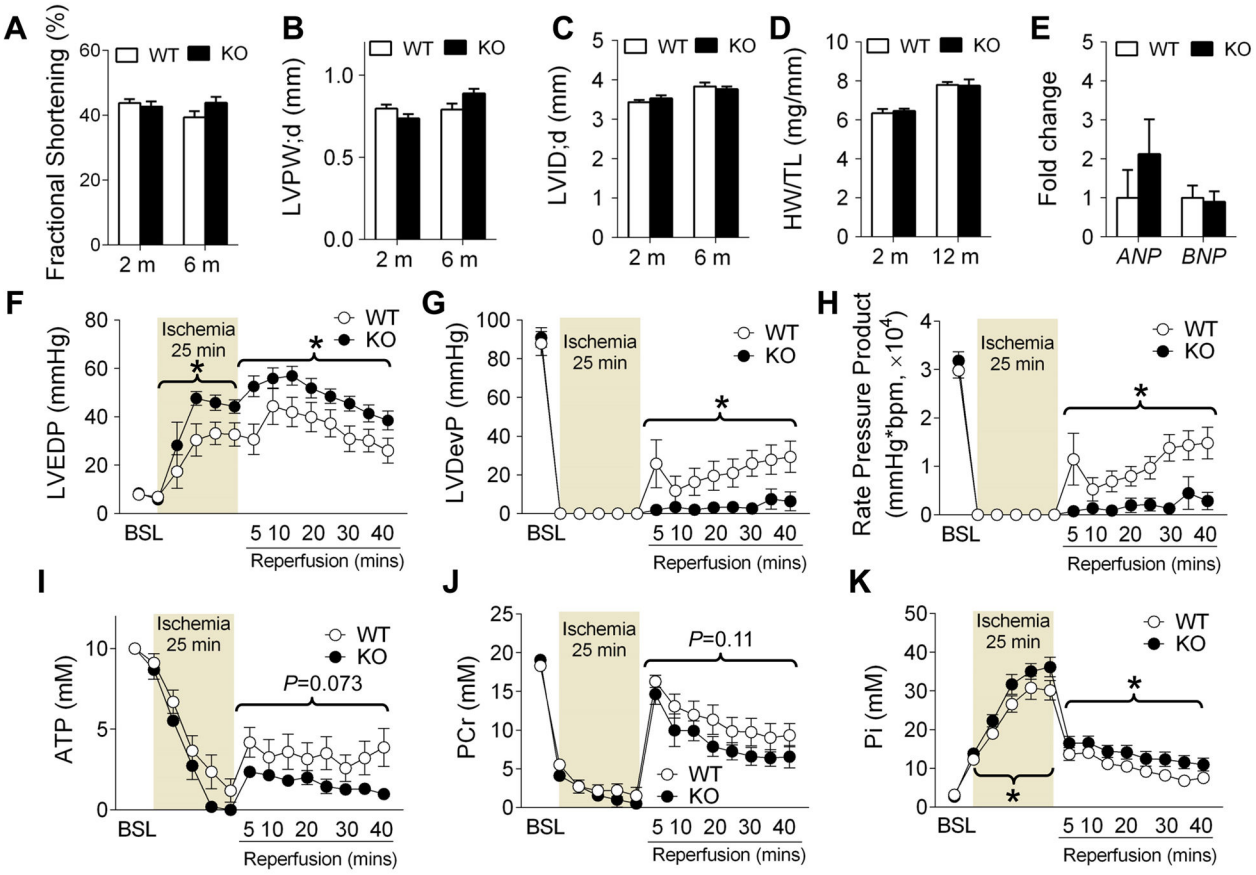

Figure 5. Cardiac function and responses to ischemia-reperfusion injury in KO hearts

(A–C) Echocardiographic data depicting fractional shortening (A), left ventricular-end diastolic posterior wall thickness (LVPW;d, B) and internal dimension (LVID;d, C) in WT and KO mice at 2 and 6 months (n=10–15 per group).

(D) Heart weight normalized to tibia length (HW/TL) of WT and KO mice at 2 and 12 months (n=8–11 per group).

(E) Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA values normalized to 18s and reported as fold changes over WT (n=4–5 per group).

(F–H) Left ventricular end-diastolic pressure (LVEDP, F), left ventricular developed pressure (LVDevP, G) and rate pressure product (H) of isolated perfused hearts subjected to low flow ischemia (1% of baseline coronary flow) and reperfusion (n=7–9 per group).

(I–K) Concentrations of ATP (I), phosphocreatine (PCr, J), and inorganic phosphate (Pi, K) measured by ^{31}P NMR spectroscopy in isolated perfused hearts (n=7–8 per group). Data are expressed as mean ± SEM. *P<0.05 vs. WT by repeated measures ANOVA. See also Figure S5.

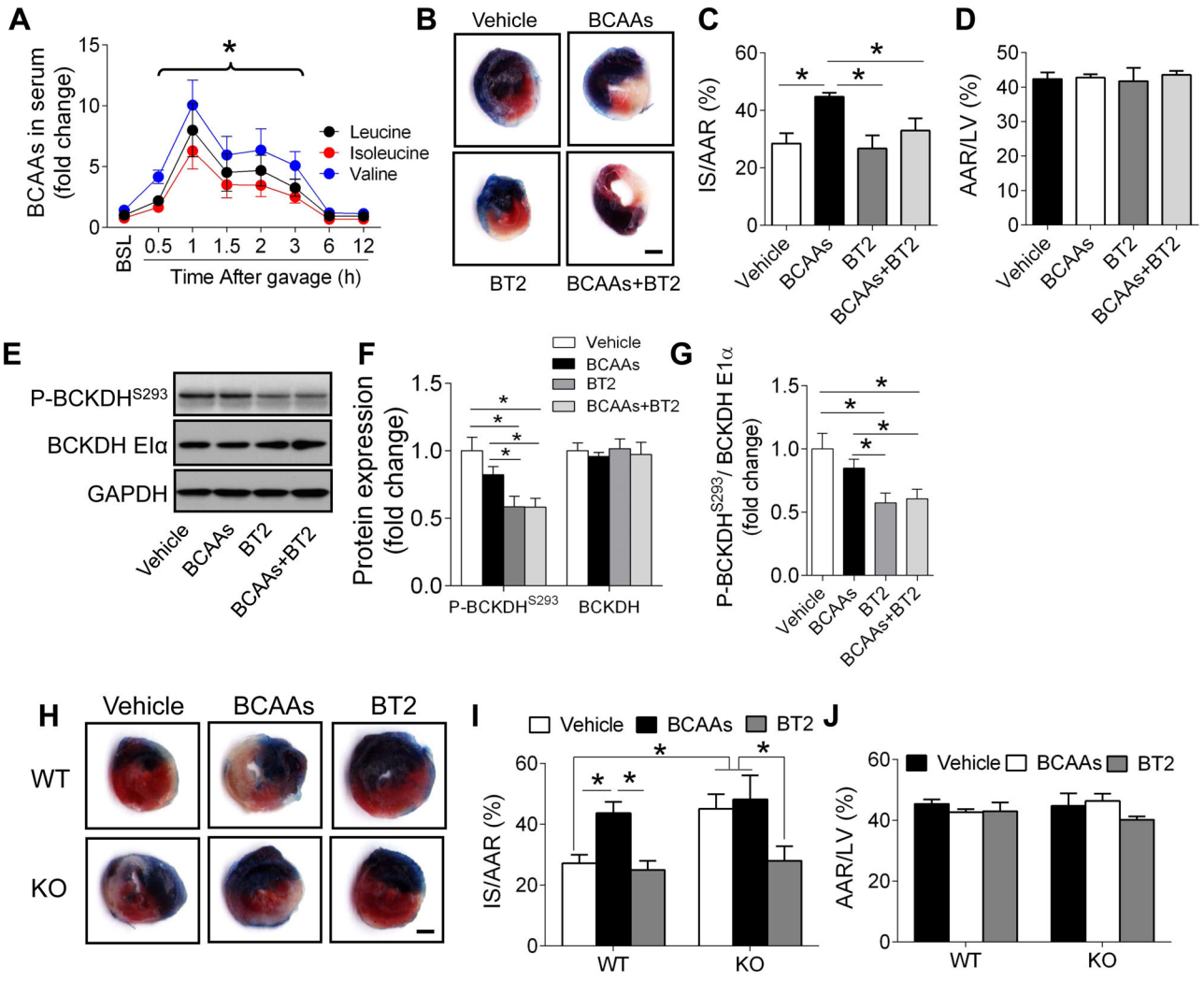

Figure 6. Excessive BCAAs worsen I/R injury which is rescued by promoting BCAA catabolism

(A) The time course of serum BCAA concentration after a single bolus of BCAA supplementation. Serum of WT mice was obtained before (BSL, n=11) and at different time intervals (30 min, 1 h, 1.5 h, 2 h, 3 h, 6 h and 12 h; n=4–7 for each time point) after a single bolus of BCAA gavage (1.5 mg/g body weight).

(B) Representative photographs of Evans blue and TTC double-stained heart sections from mice treated with vehicle, BCAAs, BT2 and BCAAs+BT2. Blue, unaffected, viable tissue; white, infarct area; red+white, area at risk. Scale bar, 1 mm.

(C and D) Infarct size (IS) relative to area at risk (AAR) and AAR relative to left ventricle (LV) were quantified.

(E–G) Western blot analysis of BCKDH phosphorylation after 7 days gavage (n=6 per group).

(H) Representative photographs of Evans blue and TTC double-stained heart sections from WT and KO mice after supplementation of BCAAs or BT2 for 7 days (n=4–5 per group).

(I and J) Infarct size (IS) relative to area at risk (AAR) and AAR relative to left ventricle (LV) were quantified. Data are shown as mean ± SEM. *P<0.05 vs. BSL or for indicated comparisons. See also Figure S5.

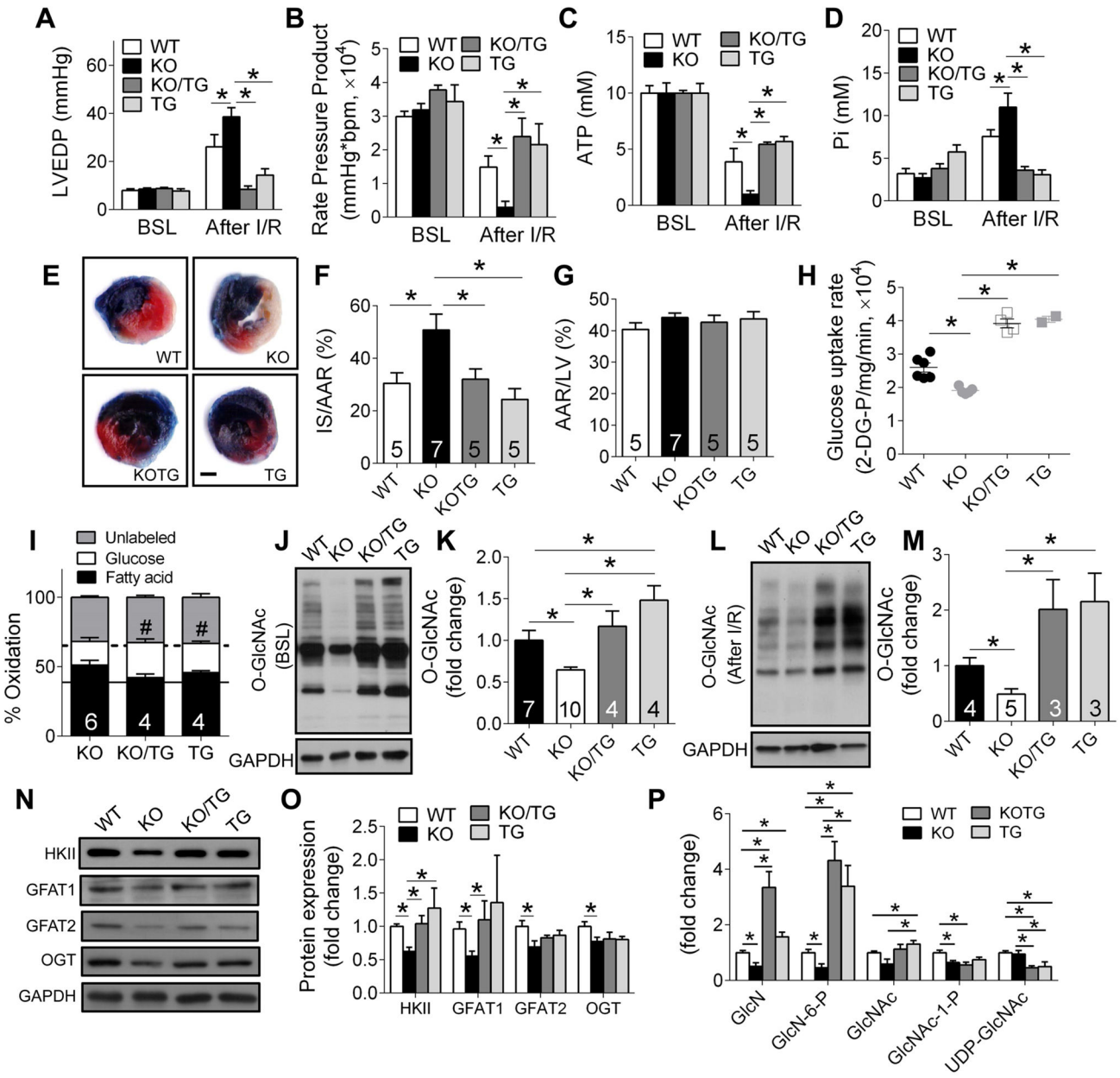

Figure 7. Exacerbated I/R injury in the KO heart can be rescued by enhancing glucose metabolism

(A and B) LVEDP (A) and rate pressure product (B) of isolated perfused hearts subjected to low flow ischemia and reperfusion (n=5–9 per group). See legends of Figure 5 for details.

(C and D) Concentrations of ATP (C) and PCr (D) measured by ^{31}P NMR spectroscopy in isolated perfused hearts at baseline and the end of reperfusion (n=5–8 per group).

(E) Representative photographs of Evans blue and TTC double-stained heart sections from WT, KO, KO/TG and TG hearts. Blue, unaffected, viable tissue; white, infarct area; red +white, area at risk. Scale bar, 1 mm.

(F and G) Infarct size (IS) relative to area at risk (AAR) and AAR relative to left ventricle (LV) were quantified.

(H) Glucose uptake in TG and KO/TG hearts perfused with buffer containing 50mU/L insulin (n=2–6 per group).
(I) Relative contribution of substrates to the TCA cycle in KO, KO/TG and TG hearts (n=4–6 per group). The solid line represents the relative contribution of fatty acids and the dash line represents the relative contribution of glucose in WT heart.
(J and K) Western blot analysis of protein O-GlcNAc modification after overexpression of GLUT1 (n=4–10 per group).
(L and M) Western blot analysis of O-GlcNAc modified protein in hearts after low flow ischemia and reperfusion (n=3–4 per group).
(N and O) Representative blots and quantitation of enzymes for the hexosamine biosynthesis pathway (HBP) in WT, KO, KO/TG and TG hearts (n=3–7 per group).
(P) Metabolites of the HBP in WT, KO, KO/TG and TG hearts (n=4–6 per group). Data are shown as mean ± SEM. *P<0.05 for indicated comparisons, #P<0.05 vs KO. See also Figure S6.