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Deletion of BCATm increases insulin-stimulated glucose oxidation in the heart

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ABSTRACT

Backgrounds: Branched chain amino acid (BCAA) oxidation is impaired in cardiac insulin resistance, leading to the accumulation of BCAAs and the first products of BCAA oxidation, the branched chain ketoacids. However, it is not clear whether it is the BCAAs, BCKAs or both that are mediating cardiac insulin resistance. To determine this, we produced mice with a cardiac-specific deletion of BCAA aminotransferase ($BCATm^{-/-}$), the first enzyme in the BCAA oxidation pathway that is responsible for converting BCAAs to BCKAs.
Methods: Eight-week-old BCATm cardiac specific knockout ($BCATm^{-/-}$) male mice and their α-MHC (myosin heavy chain) - Cre expressing wild type littermates ($WT\text{-}Cre^{+/+}$) received tamoxifen (50 mg/kg *i.p.* 6 times over 8 days). At 16-weeks of age, cardiac energy metabolism was assessed in isolated working hearts.
Results: $BCATm^{-/-}$ mice have decreased cardiac BCAA oxidation rates, increased cardiac BCAAs and a reduction in cardiac BCKAs. Hearts from $BCATm^{-/-}$ mice showed an increase in insulin stimulation of glucose oxidation and an increase in p-AKT. To determine the impact of reversing these events, we perfused isolated working mice hearts with high levels of BCKAs, which completely abolished insulin-stimulated glucose oxidation rates, an effect associated with decreased p-AKT and inactivation of pyruvate dehydrogenase (PDH), the rate-limiting enzyme in glucose oxidation.
Conclusion: This implicates the BCKAs, and not BCAAs, as the actual mediators of cardiac insulin resistance and suggests that lowering cardiac BCKAs can be used as a therapeutic strategy to improve insulin sensitivity in the heart.

1. Introduction

The essential branched chain amino acids (BCAAs) are important in protein synthesis and turnover, are important in neurotransmitter synthesis, and act as bioactive molecules in metabolism through various different signaling pathways [1,2]. However, a number of studies have suggested that increased levels of BCAAs are linked to the development of insulin resistance [3–6]. For instance, BCAA supplementation increases the severity of insulin resistance in liver and muscle [1,7]. BCAA supplementation in addition to a western diet in mice also results in an impaired glucose tolerance and insulin resistance [8]. In contrast, removing individual BCAAs from the diet of *db*/*db* mice improves systemic insulin sensitivity [9]. This suggests a potential feedback regulation between glucose and BCAA metabolism.

The heart is very sensitive to insulin, with insulin both increasing cardiac glucose oxidation and decreasing cardiac fatty acid oxidation [6,10,11]. Insulin resistance can also occur in the heart, such as in the setting of obesity or heart failure [6,12,13]. In both obesity and heart failure, insulin stimulation of glucose oxidation in the heart is markedly

Abbreviations: Akt, protein kinase A; BCAA, branched chain amino acids; BCKA, branched chain keto acids; BCATm, mitochondrial branched chain aminotransferase; BCKDH, branched-chain α-keto acid dehydrogenase; BCKDK, branched-chain α-keto acid dehydrogenase kinase; IRS-1, insulin receptor substrate 1; KLF15, Krüppel-like factor 15; p38MAPK, p38 mitogen-activated protein kinases; TCA, tricarboxylic acid; PP2Cm, 2C-type serine-threonine protein phosphatase; GTT, glucose tolerance test.; PDH, pyruvate dehydrogenase; mTOR, mammalian target of rapamycin.

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impaired [6,10,12]. Interestingly, this is associated with an impaired oxidation of BCAAs by the heart [5,10] and an accumulation of BCAAs in the heart [4–6], suggesting a role of altered BCAA metabolism in mediating insulin resistance in both obesity and heart failure.

BCAA oxidation in the heart mainly occurs in the mitochondria, although some initial steps in BCAA oxidation can occur in both the cytosol and mitochondria [4,14]. For BCAA oxidation to occur, BCAAs (leucine, isoleucine and valine) are first reversibly converted to branched chain keto acids (BCKAs) (α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate respectively) through BCAA aminotransferase (BCAT). Unlike most amino acid catabolism, the first step of BCAA catabolism occurs mostly in the extrahepatic tissues due to the low BCAT activity in the liver [15]. Cytoplasmic BCAT, BCATc, is restricted to the brain, but mitochondrial BCAT, BCATm, is ubiquitously expressed, and is primarily responsible for BCAT activity in the heart [16]. The second step of BCAA catabolism involves the mitochondrial BCKA dehydrogenase (BCKDH) that commits the BCKAs to oxidation, thereby producing either acetyl-CoA or succinyl-CoA for the TCA cycle [4,14,17]. BCKDH catalyzes the oxidative decarboxylation of BCKAs to isovaleryl-CoA, α-methylbutyryl-CoA and isobutyryl-CoA. The second catabolic enzyme BCKD activity is higher in the liver than in the heart. However in skeletal muscles, adipose tissue, and brain BCKD activity is lower than the heart [14]. The BCKDH kinase (BCKDK) phosphorylates and inhibits BCKDH, while protein phosphatase 2Cm (PP2Cm) dephosphorylates and activates BCKDH to enhance BCAA oxidation [18]. We and others have shown that in obesity and heart failure, a decrease in BCAA oxidation occurs, partly due to an increase in phosphorylation and inhibition of BCKDH [4,5]. This results in the accumulation of both BCAAs and BCKAs in the heart [5,6].

The mechanism(s) by which altered BCAA oxidation mediate insulin resistance in the heart has not been fully elucidated. Decreases in BCKDH activity can result in both BCAA and BCKA accumulation in the heart [4,5]. Both BCAAs and BCKAs are bioactive molecules that can modulate the mammalian target of rapamycin (mTOR) activity, which has multiple functions to regulate cell growth/proliferation and insulin signaling [3,7,19]. Activation of BCKDH can improve insulin sensitivity and improve heart function in obesity and heart failure [5]. However, activation of this downstream enzyme involved in BCAA oxidation would be expected to decrease both BCAAs and BCKAs in the heart. Interestingly, an accumulation of BCKAs with unaltered BCAAs has been reported in heart failure patients [4]. Alternatively, studies in mice with a whole body BCATm knockdown suggests that BCKAs must be converted back to BCAAs for insulin resistance to occur [20]. The aim of this study is to determine whether the accumulation of BCKAs or BCAAs is a critical factor responsible for cardiac insulin resistance. One potential approach to determine whether the accumulation of BCKAs or BCAAs is a critical factor responsible for cardiac insulin resistance, would be to inhibit the first enzyme involved in BCAA oxidation, BCATm. We therefore generated mice in which a cardiac selective BACTm deletion was produced ($BCATm^{-/-}$), which resulted in an accumulation of BCAAs and a reduction in BCKAs in the heart. We also employed another approach to increase the levels of cardiac BCKAs by perfusing isolating working heart with high levels of BCKAs that should result in accumulation of BCKAs.

2. Methods

2.1. Animal

Mice were housed at the Health Sciences Lab Animal Services Facility at the University of Alberta in a temperature and humidity-controlled room with a 12 h light dark cycle. All the animals were euthanized in a non-fasted state at the end of experiments. The experimental timeline is presented in Fig. 1A. All procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85–23, revised 1985).

C57BL/6J wild-type (WT), alpha-myosin heavy chain (αMHC)-MerCreMer mice were purchased from the Jackson Laboratory, USA and BCATmflox mice were a kind gift from Dr. Susan M. Hutson (Virginia Polytechnic Institute and State University). The procedure to generate $BCATm^{-/-}$ (a cardiac specific BCATm knockout) were followed as described previously [21]. To generate cardiac specific BCATm knockout mice, αMHC-MerCreMer transgenic mice expressing tamoxifen-inducible Cre in cardiac myocytes were bred with BCATmflox mice. Cre-induced inactivation of the BCATm gene was carried out via 6 intraperitoneal (i.p.) injections of tamoxifen (50 mg/kg) spread over 8 days in male mice starting at 8 weeks of age. All mice were allowed 6 weeks washout post-tamoxifen administration prior to experimentation. For the second cohort of perfusions with the high BCKAs levels, we used male C57BL/6J mice at 10–12 weeks of age.

2.2. Glucose tolerance tests

At 14 week of age animals were weighed and fasted for 6 h to perform a glucose tolerance test (GTT). GTT procedures were followed as described previously [22]. After the establishment of a baseline glucose level, mice were challenged with an i.p. glucose bolus (2 g/kg body weight). Blood glucose concentrations were measured at 15, 30, 60, 90, 120, and 180 min after glucose administration. Two days following GTT, an echocardiograph analysis was conducted.

2.3. Mice echocardiography and tissue doppler imaging

Echocardiographic analysis on mice was performed at 14–15 weeks of age, by using a (30–40 MHz; Vevo 3100, Visual Sonics, Toronto, Canada) high-resolution echocardiography imaging system. M-mode images were obtained for measurements of ejection fraction (%EF), LVPW;d, LVPWD;s, and LV mass. A non-invasive measurement of area under the curve (mmHg) in pulse wave Doppler-mode was used to measure the pressure gradient across the transverse aortic constriction site.

2.4. Tissue collection

One-two weeks' post-ultrasound echocardiography analysis, all mice were anaesthetized with i.p. sodium pentobarbital (60 mg/kg), following which an isolated working heart perfusion was conducted, as described previously [5,12]. At 16/17 weeks of age, a blood sample was collected immediately after removing the heart for the isolated working heart perfusions. Liver, quadriceps muscle and kidney were dissected and weighed. Left ventricle tissue was isolated from the isolated working hearts frozen at the end of the perfusions. All tissue was snap frozen using liquid N_2 and then stored at −80 °C. Tissues were ground using a Tissue Pulverizer (Bessman) on dry ice and liquid N2.

2.5. Tissue (BCAA and BCKA extraction)

~40 mg of powdered cardiac tissue from the frozen isolated working hearts, 120 uL of internal standard (ISTD; 4 μg/ml in H_2O containing leucine-d3 (CDN Isotopes, D-1973) and 0.8 ng/uL in H_2O containing sodium-2-Keto-3-methyl-d3-butyrate-3,4,4,4d4 (KIVd7; CDN Isotopes, D-6855), 120 μl of 6 mol/L perchloric acid (VWR, CA71007–908) were combined and homogenized with a tissue homogenizer. Proteins were precipitated in two sequential steps followed by centrifugation at 16,500*g* for 15 min at 4 °C. Supernatant collected from both steps were combined and split into two portions for measuring BCAAs and BCKAs. For BCAAs, the extract was neutralized with 2 mol/l KOH (VWR, CABH9262-500G) to a pH of 6–10, vortexed and centrifuged at 13,000*g* for 5 min at 4 °C. The supernatant collected were freeze dried and reconstituted in 50:50 Methanol (MeOH): water (VWR, CAMX0486-6) to yield 4 μg/ml of internal standard. Samples were derivatized according to previously established protocols [23–26].

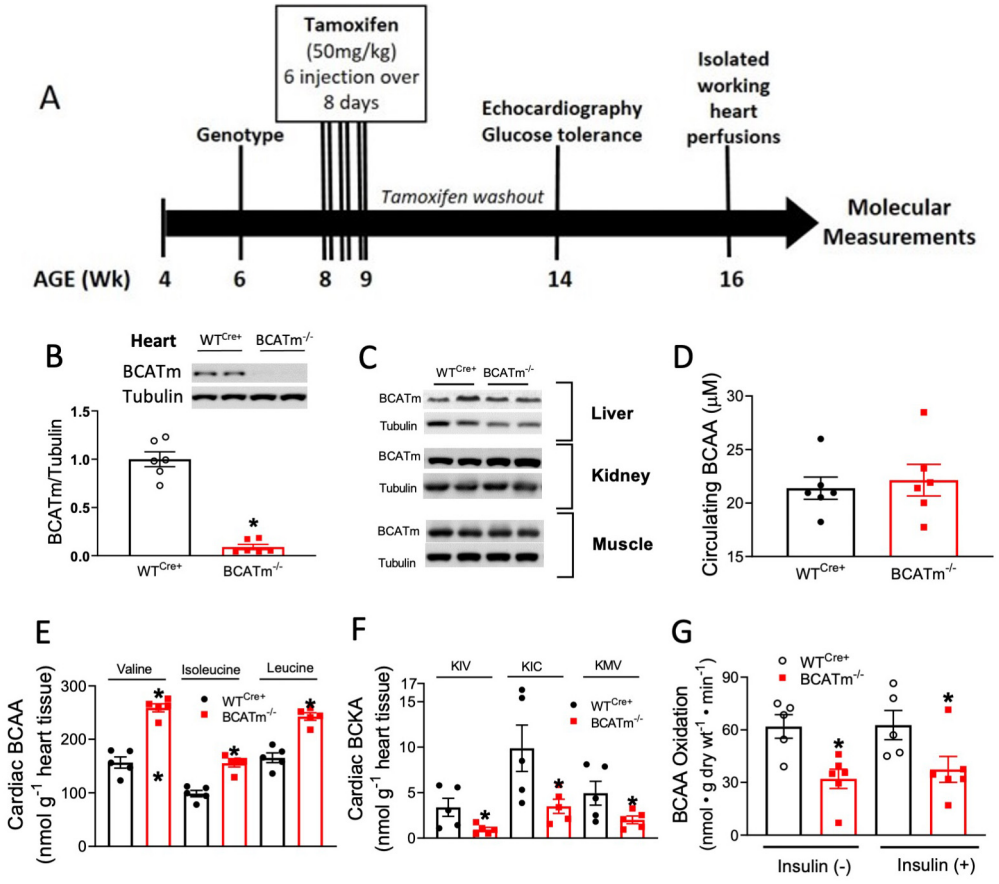

Fig. 1. A successful cardiac-specific deletion of BCATm and significant reduction of cardiac BCAA oxidation in mice. (A) Schematic drawing of the experimental protocol to generate cardiac specific BCATm knockout mice. Biopsies were collected after birth (and weaning), depending on the genotype for genotype assessment. At 8 week of age mice were subjected to 6 tamoxifen injections of 50 mg/kg body weight. Mice were allowed a 6-week washout period following the last tamoxifen injection prior to experimentation. At 14 weeks of age, a GTT was conducted followed by 6 h fasting (using a 2 g/kg body weight glucose bolus dose), after 2 days, an echocardiograph analysis was conducted. Two weeks' post-ultrasound echocardiography analysis, all mice were anaesthetized with *i.p.* sodium pentobarbital (60 mg/kg), following which an isolated working heart perfusion was conducted. Heart, liver, kidney, quadriceps muscle and plasma was also collected. (B) Densitometry analysis (WT^{Cre+}, $n = 6$; $BCATm^{-/-}$, n = 6) and representative blots of BCATm enzyme in the heart (blunted expression in the heart) to confirm cardiac specific deletion. (C) Representative blots of BCATm in liver, muscle and kidney tissues are presented along with Tubulin as the loading control for each tissue type. (E-F) Levels of cardiac BCAAs and BCKAs (WT^{Cre+}, $n = 5$; $BCATm^{-/-}$, n = 5) in myocardial biopsies collected at the end of the perfusion protocol, where cardiac energy metabolism was measured. (G) Rates of BCAA oxidation in normal hearts (WT^{Cre+}, n = 5; $BCATm^{-/-}$, $n = 6$). Data are presented as mean ± SEM. Data were analysed by *t*-test using GraphPad Prism® software. $^{*}p < 0.05$ was considered statistically significant.

2.6. BCAA derivatization and quantification

AccQ-Tag derivatization kit (Waters, 186003836) was used for derivatizing cardiac BCAAs. 10 μl of reconstituted extract was transferred to an autosampler vial and was combined with 70 μL of borate buffer followed by 20 μl of AccQ-Tag derivatization agent. Samples were derivatized at 55° C for 10 min and vortexed thoroughly. Derivatized samples were quantified with a Waters Acquity UPLC, Xevo-μ Tandem Mass Spectrometer and an AccQ-Tag Ultra RP column (130 Å, 1.7 μm, 2.1 mm X100 mm) using multiple reaction monitoring (MRM) and internal standard calibration [23,26].

2.7. BCKA derivatization and quantification

150 μl of extract and 500 μl of 25 mM OPD in 2 M HCl (made from o-Phenylenediamine, 98%; VWR, CAAAA11946-30) were combined. The mixture was vortexed and then incubated at 80 °C for 20 min followed by incubation on ice for 10 min. The derivatized extract was centrifuged at 500*g* for 15 min and the supernatant transferred to a tube containing 0.08 g sodium sulfate (VWR, CA71008-804) and 500 μl of ethyl acetate (ethyl acetate; VWR, CABDH83621.100) following which they were centrifuged at 500*g* at RT for 15 min. This step was repeated twice and the supernatant collected were vacuum centrifuged at 30 °C for 45 min Samples were then reconstituted in 64 μl of 200 mM ammonium acetate (made from; ammonium Acetate, 98%; VWR, CA97061–014) and transferred to amber glass UPLC vials (Waters, 186001130C). BCKAs were quantified with a Waters Acquity UPLC, Xevo-μTandem Mass Spectrometer and an Acquity UPLC BEH C18 (1.7 μm, 2.1 mm × 50 mm; Waters, 186004660) and ACQBEHC18 VanGuard (130 Å, 1.7 μm, 2.1 mm × 5 mm; Waters, 186003975) using multiple reaction monitoring (MRM) and internal standard calibration as per a prior study [24,25].

2.8. Assessment of cardiac energy substrate oxidation

The isolated working heart perfusions were performed as described previously [27]. The working heart perfusions allowed for quantitative measurement of $^{14}CO_2$ generated from ^{14}C-labelled carbon substrates. The system also allowed the measurement of $3H_2O$ generated from 3H-labelled energy substrates. Isolated working hearts were perfused with Krebs Henseleit solution (118.5 mmol/L NaCl, 25 mmol/l $NaHCO_3$, 1.2 mmol/l $MgSO_4$, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 2.5 mmol/l $CaCl_2$) supplemented with 0.8 mmol/l palmitate bound to 3% fatty acid free bovine serum albumin, 5 mmol/l glucose, 0.15 mmol/l leucine, 0.15 mmol/l isoleucine, and 0.2 mmol/l valine. One set of hearts from each experimental group was perfused with [5-^{3}H] glucose and [U-^{14}C] glucose to measure glycolysis and glucose oxidation rates, respectively, while another set of hearts was perfused with [9,10-^{3}H]

palmitate and [U-^{14}C] valine/leucine/isoleucine to measure palmitate and BCAA oxidation rates, respectively.

In another series of experiments, isolated working hearts were perfused with BCKAs to assess what effect BCKAs have on cardiac insulin signaling pathway. To achieve this, we randomised two sets of working mice hearts to be perfused with either vehicle (distilled water) or high levels of BCKAs (a mixture of α-keto-β-methylvalorate (KMV, 100 μmol/L), α-keto-isocaproate (KIC, 80 μmol/L) and α-keto-isoovalorate (KIV, 70 μmol/L) for 60 min (levels of BCKAs that can be seen in diabetes and obesity) [28,29].

The isolated working heart perfusion to measure BCAA oxidation was described previously [5]. The hearts were perfused for 30 min, after which 100 μU/ml insulin was added to the heart perfusate and the heart was perfused for an additional 30 min. Cardiac function was monitored throughout the ex vivo heart perfusion experiments and cardiac work was calculated from left ventricular developed pressure and cardiac output. At the end of perfusion protocol, the heart was snap frozen with tongs cooled to the temperature of liquid nitrogen and stored at −80 °C for further molecular characterization.

2.9. Western blot analysis

Frozen heart tissues were homogenized in a buffer containing 50 mmol/l Tris HCl, 1 mmol/l EDTA, 10% glycerol, 0.02% Brij-35, 1 mmol/l DTT, protease and phosphatase inhibitors (Sigma). Thirty μg of protein from the resulting supernatant were subjected to SDS-PAGE followed by western blotting procedures. The following antibodies were used to detect the proteins of interest: acetyl lysine (Millipore Sigma, 06-933), P-AKT Ser473 (Cell Signaling, 9271S), AKT (Cell Signaling, 9272S), BCATm (Thermo Scientific, PA5-21549), BCKDH (Abcam, ab138460), P-BCKDH Ser293 (Abcam, ab200577), BCKDK (Abcam, ab128935), βHAD (Abcam, ab37673), CD36 (Santa Cruz, 14309), CS (Cell Signaling, 14309), GLUT4 (Cell Signaling, 2213S), P-GSK-3α/β Ser21/9 (Cell Signaling, 9331S), GSK-3α/β (Cell Signaling, 9315S), P-IRS Ser636/639 (Cell Signaling, 2388S), P-IRS Tyr628 (Millipore Sigma, 09-433), IRS (Cell Signaling, 2382S), KLF15 (Abcam, ab185958), LCAD (Abcam, ab129711), MPC2 (Millipore Sigma, HPA056091), P-mTOR Ser2448 (Cell Signaling, 2971S), mTOR (Cell Signaling, 4517S), OXPHOS (Abcam, Ab110413), P-p38MAPK Thr180/Tyr182 (Cell Signaling, 9211S), p38MAPK (Cell Signaling, 9212S), P-p70S6K Thr389 (Cell Signaling, 9205S), p70S6K (Cell Signaling, 2708S), P-PDH (Millipore Sigma, ABS204), PDH (Cell Signaling, 3205S), PP2Cm (Abcam, ab135286), succinyl Lysine (PTM BIO, PTM-401), α-tubulin (T6074, Sigma). Membranes were then incubated with the appropriate secondary antibodies from (Sigma, goat anti-rabbit 7074P2; goat anti-mouse 31430; goat anti-chicken A16054) for 1–2 h. Enhanced chemiluminescence (Perkin Elmer) was used to visualize protein bands on autoradiography films, and quantification of the protein bands was performed with Image J.

2.10. Immunoprecipitations

Immunoprecipitation procedures were as described previously [12]. Briefly, preclearing and washing tissue lysate (containing 300 μg of protein) was performed using A/G PLUS-Agarose beads (Santa Cruz Biotechnology). The protein sample was then incubated with anti-acetyl-lysine antibody (2 μL, Millipore Sigma) during the process. The final pellet was washed three times with the homogenization buffer. Laemmli buffer was then added to the pellet and the mixture, the proteins were blotted following the same protocol for immunoblotting in the previous section. Negative control sample contained A/G PLUS-Agarose beads and anti-acetyl-lysine antibody with no protein sample, while positive control sample contained protein sample and Laemmli buffer which has not been incubated with either A/G PLUS-Agarose beads or anti-acetyl-lysine antibody.

2.11. Quantification and statistical analysis

Data are presented as the mean ± SEM. Data were analysed by student *t*-test. *P* value of <0.05 were considered statistically different. All data passed the D'Agostino-Pearson omnibus normality test in GraphPad Prism® software.

3. Results

3.1. Cardiac specific deletion of BCATm decreases cardiac BCAA oxidation

To investigate the importance of BCAAs versus BCKAs in mediating insulin resistance, we produced a cardiac specific deletion of BCATm in mice (Fig. 1A -C; Fig. S1A, B). To confirm the cardiac specific BCATm knockout (KO) in the mouse, BCATm protein expression in the heart was shown to be blunted in BCATm$^{-/-}$ mice hearts (Fig. 1B). In contrast, BCATm protein expression in the liver, kidney, and muscle in BCATm$^{-/-}$ mice was similar to levels expressed in WT^{Cre+} control mice (Fig. 1C). We also found that BCATm expression was unchanged in the various control littermates, such as wild-type mice (WT^{Cre-}), and BCATm-Flox mice (FLOX^{Cre-}) produced during the Cre-lox technology mice breeding (Fig. S1B). Previous studies have shown that whole body BCATm deletion mice increases plasma BCAAs followed by metabolic consequences including decreased body weight, improvements in glucose and insulin tolerance, and protection from diet-induced obesity [30]. In this current study, we show that the cardiac specific deletion of BCATm does not affect these parameters in mice (Fig. S1C-F).

To determine if the cardiac specific BCATm deletion affected BCAA catabolic enzyme in other tissues, we analysed the major catabolic enzyme involved in the BCAA catabolic pathway (BCKDH, phosphorylation of BCKDH, BCKDK and PP2Cm) in liver, muscle and kidney (Fig. S2A—I, Fig. S3A-E). Again, this analysis did not show any significant difference in the expression or phosphorylation of these enzymes. This confirms that BCATm was only knocked out in the myocardium of BCATm$^{-/-}$ mice. Circulating BCAAs levels were also similar in both BCATm$^{-/-}$ and WT^{Cre+} mice (Fig. 1D).

While a cardiac specific BCATm deletion did not alter circulating BCAA levels (Fig. 1D), it did have profound effects on the levels of cardiac BCAAs and BCKAs. The individual BCAAs (valine, isoleucine, and leucine) in the heart were increased in BCATm KO mice, and their respective BCKAs were decreased in the BCATm$^{-/-}$ mice (Fig. 1E, F). Circulating BCAA levels are dynamic and may influence cardiac BCAA and BCKA levels. However, we did not see any changes in circulating BCAAs in plasma collected just before the mouse hearts were perfused for measurement of mitochondrial oxidation rates. Also, heart tissue was collected right after the perfusion for measurements of the BCAAs and BCKAs, and to determine the relationship between these levels and mitochondrial oxidation rates. As would be expected, BCAA oxidation rates were also significantly decreased in the BCATm$^{-/-}$ mice (Fig. 1G). Consistent with our previous findings [5,10], we also did not see any effect of insulin on BCAA oxidation rates in the heart. There were also no significant differences observed with cardiac BCKDK (Fig. S4A and B), BCKDH or p38MAPK expression between BCATm$^{-/-}$ and WT^{Cre+} mice (Fig. S4A, C and E). In addition, there were no significant differences seen in cardiac P-BCKDH and P-p38MAPK (Fig. S4A, D and F). However, expression of PP2CM (Fig. S4A and G) along with the BCAA metabolic pathway upstream regulator, KLF15 (Fig. S4A and H) were significantly increased in the BCATm$^{-/-}$ mice.

3.2. Cardiac specific BCATm deletion increases insulin stimulation of glucose oxidation in mice hearts

Similar to previous studies [10,12], glucose oxidation rates significantly increased in isolated working hearts following addition of insulin to the perfusate (Fig. 2A). However, BCATm$^{-/-}$ mice showed a significant increase in glucose oxidation rates compared to control with the

addition of insulin (Fig. 2A and F). We saw a similar outcome when we calculated the fold change due to insulin supplementation between the groups compared to control WT^{Cre+} (pre-insulin) mouse hearts (Fig. S5A). This shows the $BCATm^{-/-}$ mouse hearts had a two fold higher glucose oxidation upon insulin exposure compared to WT^{Cre+} mouse hearts. After insulin stimulation glucose oxidation rates were 2.5 fold higher in $BCATm^{-/-}$ mouse hearts compared WT^{Cre+} mouse hearts pre insulin. Additionally, to further show that the BCATm−/− mouse hearts are more insulin sensitive, we plotted glucose oxidation rates from individual hearts to visualize the pre- and post- insulin effect on glucose oxidation rates in the WT^{Cre+} and $BCATm^{-/-}$ hearts (Fig. 2B). This, together with the fold change, shows the $BCATm^{-/-}$ mouse hearts were more insulin sensitive compared to the WT^{Cre+} mouse hearts. Insulin inhibition of fatty acid oxidation was not dramatically different between $BCATm^{-/-}$ WT^{Cre+} mouse hearts (Fig. 2C and F). However, when examining overall TCA cycle activity we saw a switch from the contribution of glucose oxidation, which is increased in these hearts (Fig. 2F). Glycolysis rates showed no significant changes between groups (Fig. 2D). Cardiac work in the BCATm−/− mouse hearts was similar to that seen in the WT^{Cre+} mouse hearts, regardless of the presence or absence of insulin (Fig. 2E).

The contribution of acetyl-CoA for the TCA cycle from the oxidation of BCAAs, palmitate and glucose confirm that the contribution of BCAA oxidation to acetyl CoA supply for the TCA cycle is minimal (Fig. 2F). However, insulin did increase the contribution of glucose oxidation to TCA cycle acetyl-CoA supply (Fig. 2F) and ATP production rates (Fig. S5B), which was accompanied by a decrease in the contribution of fatty acid oxidation to TCA cycle acetyl-CoA supply and ATP production. Altogether, the actual glucose oxidation rates (Fig. 2A), the slope analysis (Fig. 2B), the contribution of acetyl-CoA for the TCA cycle (Fig. 2F) and the ATP production (Fig. S5B) clearly demonstrated that the $BCATm^{-/-}$ mice hearts had the higher insulin-stimulated glucose oxidation.

3.3. Minimal changes in enzymes of oxidative metabolism and contribution of BCAA oxidation to ATP production

We previously showed that in heart failure and HFD-induced obese animal models that impaired glucose and palmitate oxidation rates in the heart are associated with changes in the activity of a number of the oxidative enzymes including the mitochondrial pyruvate career (MPC2) (responsible for carrying pyruvate in to the mitochondria), long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (βHAD) (both responsible for fatty acid oxidation), and the phosphorylation of PDH (inactivation of PDH activity) and pyruvate dehydrogenase kinase (PDK4) (responsible for inactivating PDH, which is responsible for inhibiting PDH activity) [6,31]. However, in the $BCATm^{-/-}$ mice there were no significant changes observed between groups in PDH, PDK4, MPC2, LCAD, and βHAD or phosphorylation of PDH (Fig. S6A-G).

3.4. BCATm deletion leads to mTOR and AKT activation in the mouse heart

We and others have shown that BCAA catabolism is impaired in heart failure in both humans and animals [4–6]. Impaired cardiac function in heart failure patients and increased left ventricular mass in heart failure mice are both associated with the accumulation of cardiac BCAAs and mTOR activation [5]. We therefore determined if the accumulation of cardiac BCAAs in $BCATm^{-/-}$ mice had any effect on cardiac function, hypertrophy and mTOR activation. Echocardiographic analysis of $BCATm^{-/-}$ hearts showed that ejection fraction (Fig. 3A), diastolic function (E'/A') and fractional shortening (Fig. S5C and D) were not significantly different between $BCATm^{-/-}$ and WT^{Cre+} mice hearts. This supports the findings in ex vivo isolated working heart perfusions, indicating that cardiac work was not different between $BCATm^{-/-}$ and WT^{Cre+} mice (Fig. 2E). However, the measured left ventricular (LV)

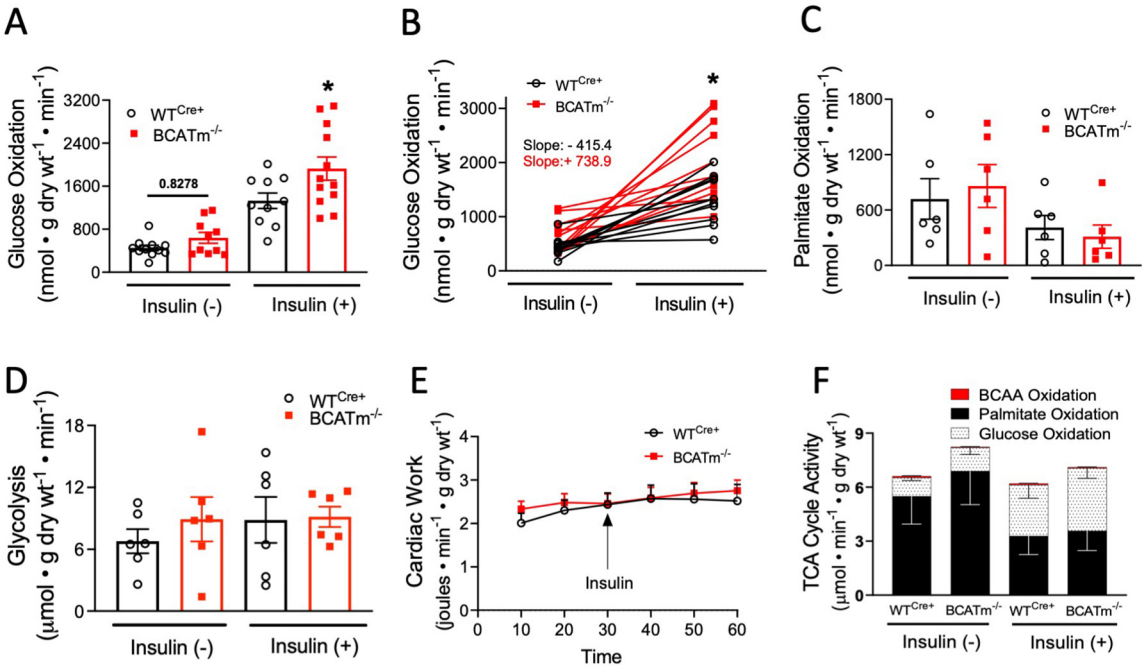

Fig. 2. Cardiac-specific BCATm knockout increases insulin sensitivity in mice hearts. (A) Rates of glucose oxidation in hearts (WT^{Cre+}, $n = 10$; $BCATm^{-/-}$, n = 10). (B) B) The magnitude of insulin stimulation on glucose oxidation rates in the individual WT^{Cre+} and $BCATm^{-/-}$ hearts (WT^{Cre+}, n = 10; $BCATm^{-/-}$, n = 10). *$p < 0.05$ indicates the significance of the combined BCATm−/− hearts versus the WT^{Cre+} hearts post-insulin, NS = not significant. (C) Rates of palmitate oxidation in hearts (WT^{Cre+}, $n = 6$; $BCATm^{-/-}$, n = 6). (D) Rates of glycolysis in hearts (WT^{Cre+}, n = 6; $BCATm^{-/-}$, n = 6). (E) Cardiac work was measured as a functional parameter during ex vivo working heart perfusions. (F) TCA cycle activity from glucose oxidation ($n = 5$–6/group), palmitate oxidation (n = 6/group) and BCAA oxidation (n = 6/group). Data are presented as mean ± SEM. Data were analysed by *t*-test using GraphPad Prism® software. *$p < 0.05$ was considered statistically significant.

mass from dissected hearts (Fig. 3D) and LV mass measured by in vivo by echocardiography (Fig. 3B-C) were significantly increased in BCATm$^{-/-}$ mice hearts compared to WT^{Cre+} mice. The increase in cardiac mass was accompanied by an increase in LV systolic and diastole posterior wall thickness in BCATm$^{-/-}$ mice compared to WT^{Cre+} control mice (Fig. 3E-F). In contrast, no differences in LV anterior wall thickness were observed between the BCATm$^{-/-}$ and WT^{Cre+} mice (Fig. S5E—F). Due to the association of mTOR activation and hypertrophy, we also measured the phosphorylation of mTOR. Despite no change in mTOR protein expression (Fig. S4 I), a significant increase in mTOR phosphorylation in BCATm$^{-/-}$ mice was seen compared to the WT^{Cre+} mice (Fig. 3G). This finding is in line with the increased LV mass or hypertrophy seen in the BCATm$^{-/-}$ mice. No phosphorylation changes in p70S6K, or in both IRS serine and tyrosine sites were observed (Fig. 4A-D), although a significant increase in AKT phosphorylation was observed (Fig. 4E). There were no significant differences observed in total protein expression of p70S6K, IRS, AKT, GSK3β or GLUT4 in the BCATm$^{-/-}$ mice hearts (Fig. S7A-E and Fig. 4G) in the BCATm$^{-/-}$ mice. Taken together, these data indicate that a reduction of BCKAs and elevated BCAAs are causing AKT phosphorylation and mTOR activation. Therefore, we next sought to delineate whether it is BCAAs or BCKAs that activate AKT and/or mTOR.

3.5. Cardiac specific deletion of BCATm is not associated with a significant change in total acetylation or succinylation in the heart

We also determined if cardiac specific BCATm deletion causes any other post-translational modifications. There were no significant differences seen in total acetylated or succinylated lysine between BCATm$^{-/-}$ and WT^{Cre+} mice hearts (Fig. 5A-B). Additionally, we immunoprecipitated acetylated PDH and LCAD from BCATm$^{-/-}$ and WT^{Cre+} mice heart tissue to determine whether the acetylation status of these enzymes were altered in the BCATm$^{-/-}$ hearts. However, we did not observed any significant changes in the acetylation level of PDH and LCAD between the BCATm$^{-/-}$ and WT^{Cre+} mice (Fig. 5C-E). We also determined whether this deletion of cardiac specific mitochondrial BCATm, triggers any modifications in the mitochondrial electron transport chain or markers for mitochondrial function such as citrate synthase (CS) or the key regulator for NAD^+, CD38. We did not observe any changes in the expression of the different complexes, CS or CD38 (Fig. S8A-G).

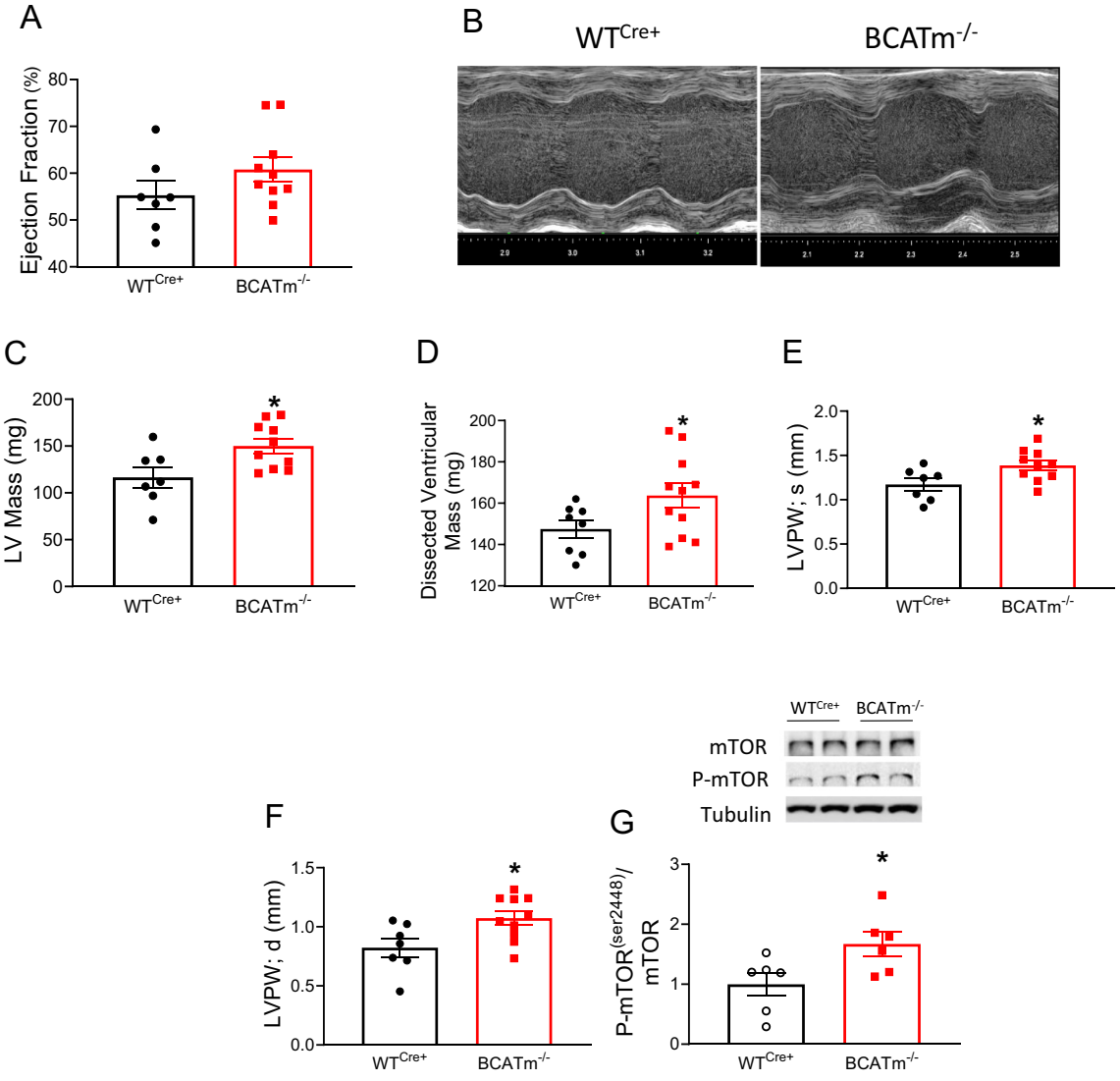

Fig. 3. Increasing left ventricular mass is associated with mTOR activation without affecting cardiac function in cardiac specific BCATm deleted mice. (A) Ejection fraction (%) (WT^{Cre+}, $n = 7$; BCATm$^{-/-}$, $n = 9$) based on echocardiographic analysis. (B) Representative image of an echocardiographic analysis showing increased wall thickness in BCATm$^{-/-}$ mice compared to WT^{Cre+} control mice. (C) Changes in the left ventricular mass, (D) Changes in the dissected ventricular mass and posterior wall thickness in diastole, (E) and systole (F) based on echocardiographic analysis. (G) Representative blots and densitometry analysis of P-mTOR$^{(Ser\ 2448)}$/mTOR along with Tubulin as the loading control. Data are presented as mean ± SEM. Data were analysed by t-test using GraphPad Prism® software. *$p < 0.05$ was considered statistically significant.

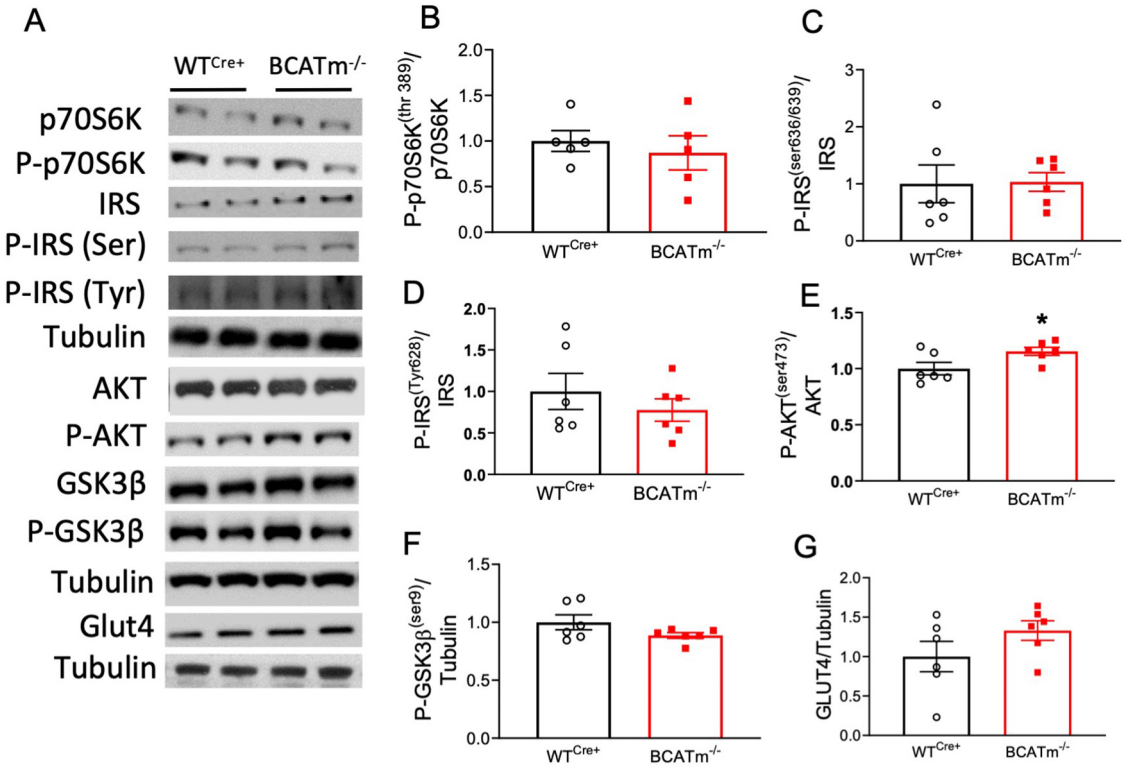

Fig. 4. Cardiac-specific BCATm deletion increases AKT phosphorylation in mice hearts. (A) Representative blots for the densitometry analysis in (B-G) along with Tubulin as the loading control. (B-G) Densitometry analysis of P-p70S6K$^{(Thr\ 389)}$, P-IRS1$^{(Ser636/639)}$, P-IRS1$^{(Tyr628)}$, P-AKT$^{(Ser473)}$, P-GSK3ß$^{(Ser9)}$ and GLUT4, respectively (n = 6/group). Data are presented as mean ± SEM. Data were analysed by t-test using GraphPad Prism® software. *p < 0.05 was considered statistically significant.

3.6. Selective augmentation of cardiac BCKAs levels blunts insulin-stimulated cardiac glucose oxidation

Since cardiac specific deletion of BCATm$^{-/-}$ decreased cardiac BCKAs and increased insulin stimulated glucose oxidation, we determined directly what effect BCKAs have on cardiac insulin signaling and function. We exposed working mice hearts to high levels of BCKAs, levels that are seen in diabetes and obesity (Fig. 6A) [28,29,32]. High levels of BCKAs did not result in a significant change in cardiac work compared to vehicle-treated hearts (Fig. 6B). Consistent with the cardiac work, high BCKAs did not cause a significant change in the cardiac output compared to the vehicle-treated hearts (Supplemental Fig. S9L). However, augmenting cardiac BCKA levels completely blunted insulin-stimulation of glucose oxidation rates compared to vehicle-received hearts (Fig. 6C). Additionally, we plotted the pre- and post-insulin effect on glucose oxidations rates in the individual hearts, in the presence and absence of high BCKA levels to show the magnitude of the stimulation, following BCKA supplementation (Fig. 6D). BCAA levels in the perfusate at the end of the perfusion protocol for the vehicle and BCKA-treated hearts were compared with BCAA levels in the perfusate prior to perfusions (Fig. 6E). There was no significant difference in the BCAA levels in the perfusate between the vehicle-and the BCKA-treated hearts, confirming that the acute increase in cardiac BCKA levels does not cause a significant increase in cardiac BCAA levels. BCKA-induced inhibition of glucose oxidation was also associated with a significant inhibition of AKT and PDH activities (Fig. 6F-H). Of importance, is that these high levels of BCKAs were not associated with a significant change in the activity of mTOR (Fig. 6I). Consistent with the findings from our BCATm$^{-/-}$ hearts, there was no significant change in the phosphorylation of IRS-1 or P70S6K in the BCKA-treated hearts (Supplemental Fig. S9A, C and E). There was also no significant change in the total expression of either mTOR, IRS, p70S6K PDH or AKT in the high BCKAs-perused hearts compared to the vehicle-perfused hearts (Fig. S9A, B, D, F—H). We found that high BCKA levels did not have a significant effect on either PDK4 protein levels or FOXO1 status (Fig. S9I—K) Taken together, these findings emphasize that elevating levels of BCAAs, and not BCKAs, is triggering the hypertrophic signal in the BCATm$^{-/-}$ hearts. Along with our data from the BCATm$^{-/-}$ hearts, these findings also show that BCKAs, and not BCAAs, are a major mediator of cardiac insulin resistance via inhibiting AKT and PDH activities.

4. Discussion

We have recently shown that cardiac BCAA oxidation rates are impaired in murine models of high fat diet-induced obesity and heart failure, events where cardiac insulin resistance is present [5,10]. This is associated with accumulation of cardiac BCAAs, which suggested a possible link between BCAA accumulation and the development of cardiac insulin resistance. However, in this study we demonstrate it is not the accumulation of BCAAs or an inhibition of BCAA oxidation per se that is mediating cardiac insulin resistance, but rather the accumulation of BCKAs in the myocardium. In mice lacking cardiac BCATm, we show that BCAA oxidation rates decrease and BCAA levels increase in the heart, but this is associated with an actual increase in insulin stimulation of glucose oxidation. The increase in glucose oxidation is also associated with a decrease in BCKA levels due to the decrease in BCATm activity. Furthermore, addition of BCKAs to the heart results in a marked decrease in insulin stimulated glucose oxidation. An increase in BCKA levels also occurs in the failing heart and in diabetes/obesity [33], both of which are associated with cardiac insulin resistance [6,12,34–37]. As a result, we suggest that it is primarily an accumulation of BCKAs in the heart that mediates insulin resistance.

BCAAs can stimulate mTOR that, as part of a mTORC1 complex, has multiple functions in regulating cell growth/proliferation as well as insulin signaling. mTOR activates p70S6 kinase (S6K) that can phosphorylate insulin-receptor substrate-IRS and inhibit it to block insulin signaling [5,7,38]. For instance, repletion of dietary BCAAs can trigger

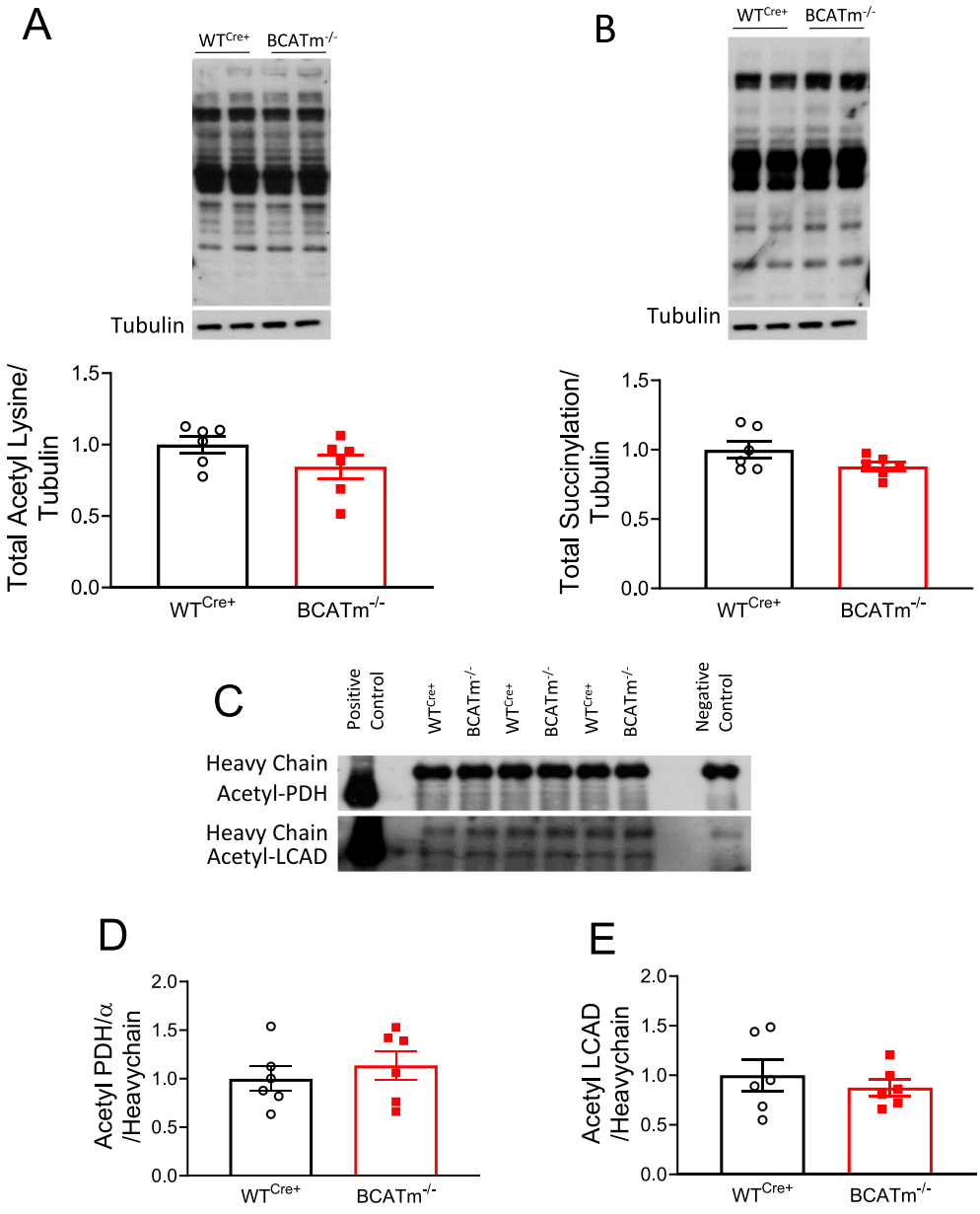

Fig. 5. No changes in acetylation or succinylation levels in the BCATm$^{-/-}$ hearts. (A-B) Densitometry analysis and representative blots for total protein acetylation and (acetyl-lysine) and succinylation (succinyl-lysine) for molecular weight range of 250–25 kDa (n = 6/group). (C-E) Representative blots and densitometry analysis of acetylated-PDH and acetylated-LCAD normalised to the heavy chain as control (n = 6/group). Data are presented as mean ± SEM. Data were analysed by t-test using GraphPad Prism® software. *$p < 0.05$ was considered statistically significant.

hepatic and peripheral mTORC1 signaling in obese New Zealand mice [39]. However, research has focused mainly on BCAAs to demystify the role of impaired BCAA catabolism in the development of insulin resistance, while very little attention has been paid to the role that BCKAs may play in the occurrence of and/or aggravating insulin resistance. Therefore, it is not clear whether BCAAs and/or BCKAs are prerequisite to trigger mTOR activity and/or induce insulin resistance. Our study is the first to identify a distinct role of BCKAs and BCAAs in mediating cardiac insulin signaling pathway and the mTOR signaling pathway, respectively, in vivo. Using our novel cardiac-specific BCATm$^{-/-}$ mouse model offers a unique opportunity to selectively increase BCAAs levels and decrease BCKAs levels in the heart, which was confirmed using mass spectrophotometry. Interestingly, we found that the accumulation of BCAAs due to BCATm deletion was associated with an activation of mTOR along with LV mass enlargement, suggesting that accumulation of BCAAs is triggering the hypertrophic signal in the heart. It is important to note that we have not observed any significant decrease in function in the BCATm$^{-/-}$ hearts. This may suggest that these BCATm$^{-/-}$ hearts are in a compensatory stage and it is not clear whether these animals will develop a pathological hypertrophy with time. While our data is consistent with the role of BCAAs in modifying mTOR activity, whether BCAAs modulate AKT activity to trigger mTOR activity and/or cardiac hypertrophy is not clear. In cultured cells, it has been shown that BCKAs impair insulin signaling while increasing insulin-stimulated mTORC1 activity [33,40]. However, it is hard to dissociate the effect of BCKAs from that of BCKAs-derived BCAAs in these cell models since the reversible BCATm enzyme is present in these cells, and can produce BCAAs from BCKAs. In order to dissociate the role of BCAAs and BCKAs in triggering the activity of mTOR and AKT, we performed additional experiments where normal mice hearts were acutely perfused with high levels of BCKAs in the absence of BCAAs. We found a significant reduction in AKT activity in the hearts treated with BCKAs, with no change in mTOR activity. This demonstrates a cause-and-effect relationship between BCAAs and mTOR activity in

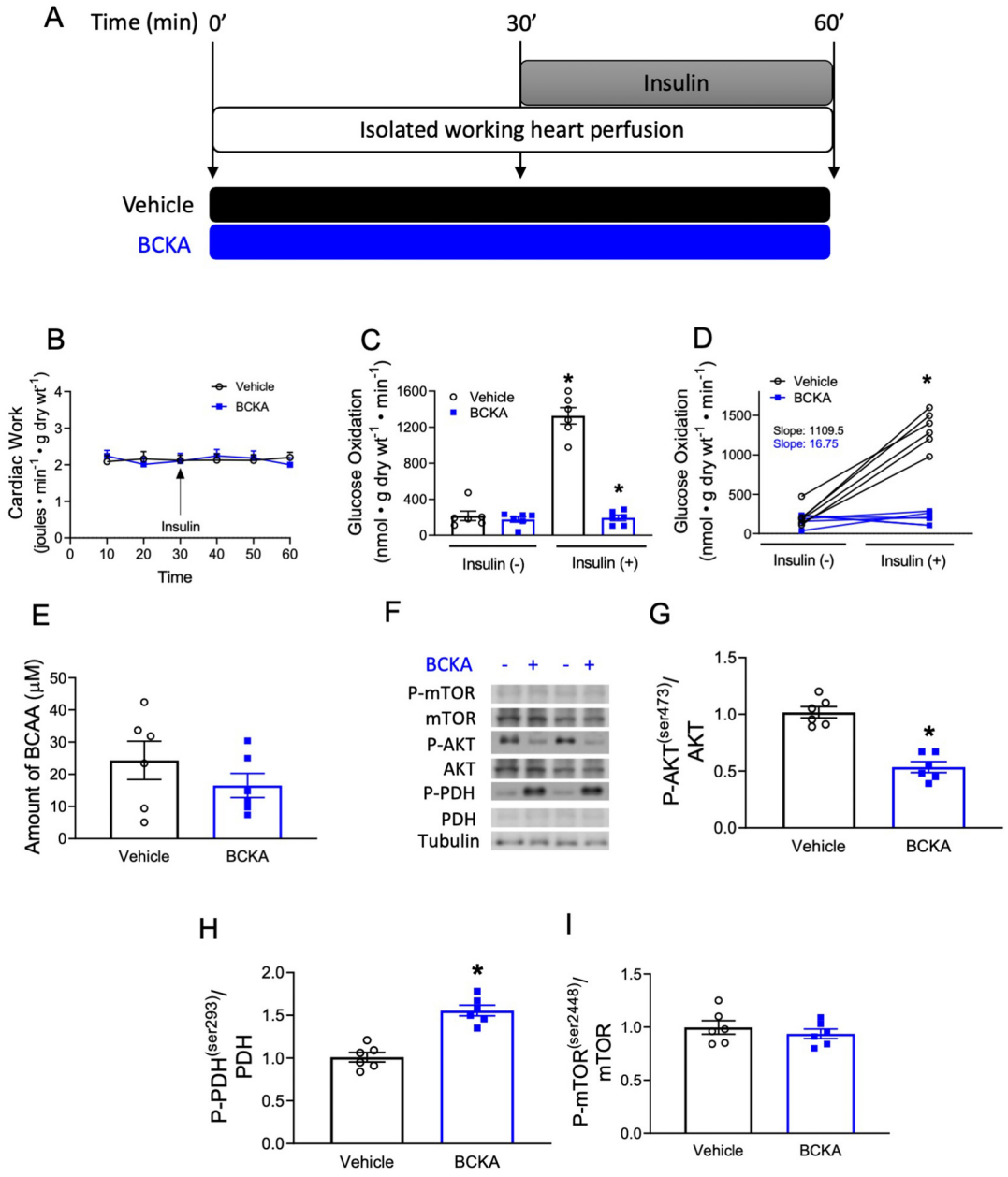

Fig. 6. High levels of BCKA significantly decrease insulin sensitivity in the mice hearts. (A) Schematic drawing of the experimental protocol to perfuse the mouse heart with either vehicle or high levels of BCKA. Male C57BL/6J mice at 10–12 weeks of age were used to perform these experiments. (B) Cardiac work was measured as a functional parameter during ex vivo working heart perfusions. (C) Rates of glucose oxidation in hearts (Vehicle, n = 6; BCKA, n = 6). (D) The magnitude of insulin stimulation on glucose oxidation rates in the presence and absence of high BCKA levels (Vehicle, n = 6; BCKA, n = 6). *$p < 0.05$ indicates the significance of the combined BCKA treated hearts versus the Vehicle treated hearts post-insulin, NS = not significant. (E) Levels of cardiac BCAAs and BCKAs (WT^{Cre+}, n = 5; $BCATm^{-/-}$, n = 5) in left ventricular tissue isolated at the end of the perfusion protocol, where cardiac energy metabolism was measured (F–I) Representative blots and densitometry analysis of P-$AKT^{(Ser473)}$/AKT, P-$PDH^{(Ser293)}$/PDH and P-$mTOR^{(Ser2448)}$/mTOR, respectively along with Tubulin as the loading control. Data are presented as mean ± SEM. Data were analysed by t-test using GraphPad Prism® software. *$p < 0.05$ was considered statistically significant.

the heart. This data also suggests that BCKAs could be more important than BCAAs in modulating AKT activity.

We observed elevated levels of BCAAs in the $BCATm^{-/-}$ hearts, however, this rise in cardiac BCAAs is not due to the change in the circulating BCAAs, as we did not see any changes in plasma concentration, which clearly suggests this is a consequence of lack of BCATm. Furthermore, we found that low levels of cardiac BCKAs in the $BCATm^{-/-}$ hearts was associated with enhanced insulin-stimulated glucose oxidation rates as well as its contribution to total ATP production rates. It is important to note that the impact of BCATm deletion on the basal levels of the insulin signaling pathway activity and/or glucose oxidation rates remain interesting topics for future investigations. The observed increase in glucose oxidation in the $BCATm^{-/-}$ hearts caused a slight increase in the total ATP production along with a slight increase in cardiac work; both were not statistically significant. This increase in cardiac insulin sensitivity occurs despite the presence of high levels of cardiac BCAAs in the $BCATm^{-/-}$ hearts, ruling out a major involvement of BCAAs in manipulating cardiac insulin sensitivity. This enhancement in insulin-stimulated glucose oxidation rates in $BCATm^{-/-}$ hearts seems unlikely to be due to enhanced glucose uptake since GLUT4 expression did not change. In addition, our direct measurements of glycolysis rates in the $BCATm^{-/-}$ hearts showed no significant difference compared to the WT^{Cre+} hearts, suggesting that this increase in glucose oxidation is not secondary to an increase in glycolysis. Importantly, there was a significant increase in the activity of AKT, a key component in the insulin signaling pathway, in the $BCATm^{-/-}$ hearts, evidence of

enhanced activity of the insulin signaling pathway in these hearts. Although there were less dramatic changes in the downstream effectors of AKT in the BCATm$^{-/-}$ hearts, a number of studies have shown that insulin can directly stimulate mitochondrial glucose oxidation, independent of the cytosolic downstream effector of AKT [41–45]. Direct insulin stimulation of glucose oxidation is associated with dephosphorylation and activation of PDH, the main regulatory enzyme of mitochondrial glucose oxidation [41–45]. This stimulation of PDH by insulin is also associated with mitochondrial translocation of AKT following insulin stimulation [45–47]. Indeed, we have recently shown that insulin-stimulation of mitochondrial AKT is a prerequisite to mediate the direct insulin stimulation of PDH and glucose oxidation, independent of glucose uptake and glycolysis [45]. We also showed that inhibition of insulin-stimulated mitochondrial AKT completely abrogated the direct insulin stimulation of glucose oxidation with no significant effect on either glucose uptake of glucolysis [45]. Therefore, it seems plausible to suggest that enhanced glucose oxidation in the BCATm$^{-/-}$ hearts could be due to enhanced activity of mitochondrial AKT that can activate PDH, independent of significant changes in either IRS1, GSK-3β or p70S6K. At least in the heart, these findings also suggest that BCKAs negatively regulates glucose oxidation through inhibiting AKT. In further support of this, we found that, exposing hearts to high levels of BCKAs, levels that are seen in diabetes and obesity, markedly abrogated insulin-stimulated cardiac glucose oxidation rates. BCKAs-induced inhibition of glucose oxidation was accompanied by marked inhibition of AKT activity, suggesting an inhibitory effect of BCKAs, not BCAAs, on cardiac insulin signaling. The impact of these high levels of BCKAs on glycolysis, lactate production and the activity of PDH and PDK warrant further investigation. In addition, investigating the impact of enhancing BCKAs levels on the basal rates of cardiac glucose oxidation would be an interesting scope for future investigations. The exact molecular mechanism through which BCKAs inhibit AKT activity warrants further investigation. Our data showed that enhancing BCKA levels inhibits the stimulatory effect of insulin on glucose oxidation, Akt, and PDH. However, it is still not clear how BCKAs exactly mediates this inhibitory effect, and that is an interesting topic for future investigations. Of importance is that insulin's action on Akt, PDH and glucose oxidation can be mimicked by other insulin signaling kinases in the absence of insulin. For example, we have recently shown that inhibition of protein kinase delta (PKC-δ) enhances cardiac glucose oxidation rates in the absence of insulin [45]. This enhancement of glucose oxidation by PKC-δ inhibition was associated with the activation of Akt and PDH. Whether BCKAs interact directly with Akt and/or PDH or with other kinases to inhibit the stimulatory effect of insulin on glucose oxidation is yet to be determined. It would also be important to investigate whether mitochondrial Akt can interact with other kinase(s) or mitochondrial compartment(s) to modulate glucose oxidation, independent of PDH.

Curiously, there was no significant change in glycolytic rates in the BCATm$^{-/-}$ hearts compared to the WT^{Cre+} mice hearts. This could be, at least in part, due to the fact that glycolysis rates are very high and maximally stimulated in the mouse heart [48]. Indeed, in previous studies, we have shown that, unlike glucose oxidation, insulin does not have major effects on glycolysis in the mouse heart [10,48]. Cardiac work is a major determinant of mitochondrial oxidative rates and it influences cardiac preference for oxidative substrates [49]. We also did not observe any difference in cardiac work between BCATm$^{-/-}$ hearts and the WT^{Cre+} mice hearts. There is a slight trend toward an increase in TCA cycle activity in the KO hearts. However, this trend was not statistically significant. We should point out that there was also a slight trend toward an increase in cardiac work, although it was not statistically significant, that could account for this slight trend of increase in TCA cycle activity. Considering the absence of any change in glycolytic rates and cardiac work following BCATm deletion, our data confirms that the effects of cardiac BCATm deletion on insulin stimulation of glucose and inhibition of fatty acid oxidation are not occurring due to changes in cardiac function or alterations in glycolytic rates. In addition, we also did not see any transcriptional or posttranscriptional changes in any of the major glucose or fatty acid metabolic enzymes. It is important to note here that BCATm deletion was associated with significantly increased expression of PP2CM (downstream) and KLF15 (upstream) in the BCATm$^{-/-}$ hearts, which suggests a possible feedback response due to BCATm deletion. Taken together, these data confirm that the observed changes in cardiac insulin signaling and hypertrophy are, at least in part, due to alterations in the levels of BCKAs and BCAAs, respectively.

While it is hard to unequivocally confirm whether impaired cardiac BCAA oxidation is a cause or consequence of cardiac insulin resistance, it seems plausible to suggest that impaired cardiac BCAA oxidation contributes to the development of cardiac insulin resistance. First, unlike glucose and fatty acid, BCAAs are not regulated by insulin in the heart, which makes it highly unlikely that impaired cardiac BCAA oxidation is a consequence of impaired cardiac insulin signaling [5,10]. Second, we and others have shown that circulating as well as cardiac levels of BCAAs and BCKAs increase, evidence of impaired BCAA oxidation, in different models of cardiac insulin resistance, including diabetes/obesity [10,33], myocardial ischemia/reperfusion injury [6,50,51] and heart failure [4,5]. Moreover, disruption of cardiac BCAA oxidation aggravates cardiac insulin resistance and contractile dysfunction in murine models of myocardial ischemia/reperfusion [50] and aortic constriction [4]. In line with that, BCAAs supplementation further aggravates cardiac dysfunction in a mouse model of myocardial infarction [51]. In contrast, enhancing cardiac BCAA oxidation lessens the severity of cardiac insulin resistance [4,5,33,50,52]. It needs to be emphasized that alterations in cardiac BCAA oxidation are less likely to mediate cardiac insulin resistance by altering the contribution of glucose and/or fatty acid to the TCA cycle activity since BCAAs only contribute to 1–2% of the total ATP production in the heart. Therefore, it seems more likely that BCAAs and/or BCKAs are acting as signaling molecules in the heart to mediate insulin resistance. Our findings in this study further support this proposal. We showed that a selective decrease in BCKA levels in the BCATm$^{-/-}$ hearts enhances the insulin signaling pathway, while increased BCAA levels in these heart is associated with an increased mTOR signaling. Furthermore, we also showed that selectively increasing cardiac BCKAs levels inhibits cardiac insulin signaling, without any significant effect on mTOR signaling, confirming that BCKA and BCCA have divergent effects on the insulin signaling and the mTOR signaling, respectively, in the heart.

To conclude, this study is the first to report that the impact of cardiac-specific deletion of BCATm of cardiac energy metabolism and function (Fig. 7). We showed that BCATm deletion reduces cardiac BCAA oxidation and selectively increases cardiac BCAA levels, while decreasing BCKA levels. We also demonstrate that BCAA accumulation in BCATm$^{-/-}$ hearts does not influence cardiac insulin signaling. Instead, BCAA accumulation results in activation of mTOR and an increase in LV mass in BCATm$^{-/-}$ hearts. This crosstalk between BCAAs and mTOR was further confirmed by the absence of mTOR activation when a working mouse heart was perfused with high levels of BCKAs, not BCAAs, demonstrating that BCAAs play a critical role in triggering the hypertrophic signal in the heart. Despite BCAA accumulation in the BCATm$^{-/-}$ heart, we show that reduced levels of BCKAs in our BCATm$^{-/-}$ mouse hearts enhance insulin-stimulated cardiac glucose oxidation and its contribution to cardiac ATP production via enhancing AKT activity. This distinct inhibitory effect of BCKAs, not BCAAs, on cardiac insulin signaling was further supported by the marked inhibition of insulin-stimulated glucose oxidation and inhibition of AKT when mice hearts were perfused with high levels of BCKAs (Fig. 7). Therefore, manipulating BCKAs represent an attractive therapeutic approach to improve insulin signaling, energy metabolism and contractile function in the heart in the setting of obesity, diabetes and heart failure.

CRediT authorship contribution statement

GMU wrote the manuscript. GMU planned the experimental design, generated the BCATm knockout animals, performed the animal experiments and biochemical assays and performed the data analysis for the

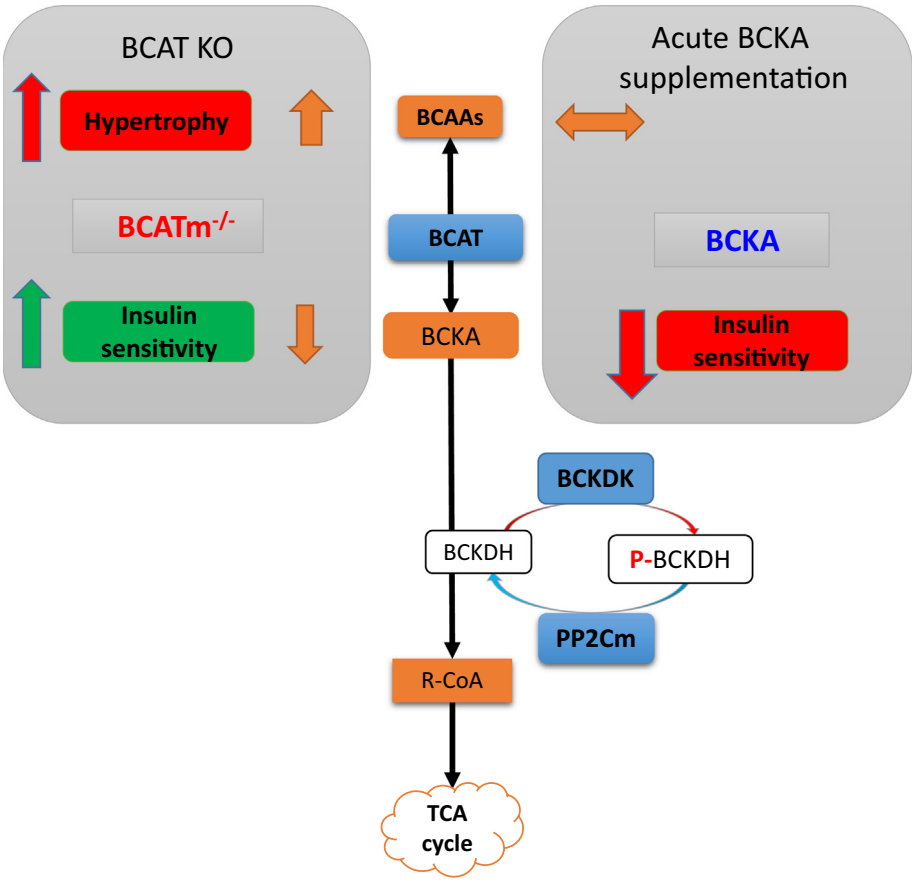

Fig. 7. Proposed model of how BCKAs and BCAAs mediate cardiac insulin sensitivity and cardiac hypertrophy: Cardiac specific deletion of the first BCAA catabolic enzyme ($BCATm^{-/-}$) in the mouse, results in elevated levels of BCAAs and reduced levels of BCKAs. This is associated with an increased insulin sensitivity and hypertrophy. We propose that the reduction of BCKAs in the heart is responsible for the increased insulin signaling, while the increase in BCAAs is responsible for the increased hypertrophy. In support of this, addition of BCKAs to the heart results in insulin rresistance. We propose that lowering cardiac BCKAs levels, not BCAAs, enhances cardiac insulin sensitivity.

BCATm knockout studies. QGK designed the BCKAs studies, performed the immunoprecipitation for the BCATm studies, and executed all the biochemical assays and data analysis for the BCKAs studies. SP performed biochemical analysis and genotyping. KG and JRU provided technical support for the genetically modified mice line and performed the echocardiograph analysis. GW and MA performed biochemical analysis in muscle and kidney tissue. CSW performed the isolated working mouse heart perfusions. DB and TP measured the BCAAs and its metabolites in cardiac samples. YW, KLH, and LZ collected and processed the tissues. GDL designed the experiments, coordinated the study and edited the manuscript as the corresponding author.

Declaration of competing interest

The authors declare no competing interests.

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Ethics approval

All animal protocols were approved by the University of Alberta Institutional Animal Care and Use Committee, which conform with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (eighth edition; revised 2011) and the guidelines of the Canadian Council on Animal Care.

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Data availibility

All data are available in the Lopaschuk Laboratory. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Gary D. Lopaschuk (glopasch@ualberta.ca).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2021.154871.

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