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ORIGINAL RESEARCH

Dietary Branched-Chain Amino Acids Modify Postinfarct Cardiac Remodeling and Function in the Murine Heart

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BACKGROUND: Branched-chain amino acids (BCAAs), which are derived from the diet, are markedly elevated in cardiac tissue following myocardial infarction (MI). Nevertheless, it remains unclear whether dietary BCAA levels influence post-MI remodeling.

METHODS: To investigate the impact of dietary BCAAs on cardiac remodeling and function after MI, we fed mice a low or a high BCAA diet for 2 weeks before MI and for 4 weeks after MI. Cardiac structural and functional changes were evaluated by echocardiography, gravimetry, and histopathological analyses. Immunoblotting was used to evaluate the effects of BCAAs on isolated cardiac myofibroblast differentiation.

RESULTS: The low BCAA diet decreased circulating BCAA concentrations by >2-fold when compared with the high BCAA diet. Although neither body weights nor heart masses were different in female mice fed the custom diets, male mice fed the high BCAA diet had significantly higher body and heart masses than those on the low BCAA diet. The low BCAA diet preserved stroke volume and cardiac output after MI, whereas the high BCAA diet promoted progressive decreases in cardiac function. Although BCAAs were required for myofibroblast differentiation in vitro, cardiac fibrosis, scar collagen topography, and cardiomyocyte cross-sectional area were not different between the dietary groups; however, male mice fed the high BCAA diet had longer cardiomyocytes and higher capillary density compared with the low BCAA group.

CONCLUSIONS: A low BCAA diet mitigates eccentric cardiomyocyte remodeling and loss of cardiac function after MI in mice, with dietary effects more prominent in males.

Key Words: branched-chain amino acids ■ fibrosis ■ heart failure ■ hypertrophy ■ myocardial infarction

The cardiac response to injury involves coordination of several fundamental processes. Particularly important is the ability of the metabolic network to maintain sufficient energy to fulfill work demands and contribute to anabolic responses that facilitate limited cardiac repair. Several metabolic pathways and metabolites are implicated in cardiac remodeling after myocardial infarction (MI), with metabolic pathways such as glycolysis, fatty acid oxidation, and ketone body oxidation contributing to changes in the phenotype and function of both parenchymal and mesenchymal cells of the heart.[1–5] Interestingly, recent studies have shown that branched-chain amino acids (BCAAs) are not only linked with cardiovascular disease risk in humans (reviewed in[6]) but are elevated substantially in the context of heart failure in both clinical[7] and preclinical[8,9]

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RESEARCH PERSPECTIVE

What Is New?

- This study provides proof-of-principle using a preclinical mouse model that dietary constituents can have marked effects on cardiac remodeling after myocardial infarction.
- We show that, compared with a diet high in branched-chain amino acids, a diet low in branched-chain amino acids improves cardiac structure and function after myocardial infarction.

What Are the Clinical Implications?

- Modifications to the diet after myocardial infarction may be a readily translatable intervention to improve cardiac remodeling and function.

Nonstandard Abbreviations and Acronyms

BCAA	branched-chain amino acids
TGF-β	transforming growth factor beta

scenarios. Nevertheless, it remains unclear whether elevations in BCAAs are deleterious to cardiac healing and function after infarction and whether modulating BCAA levels could be an actionable strategy for improving outcomes.

BCAAs, as essential amino acids, are primarily obtained from the diet and play crucial roles in protein synthesis and energy homeostasis. Although BCAAs are thought to be required for anabolic responses that contribute to repair of tissue in response to physiological stress (eg, exercise),[10] chronically high levels of circulating BCAAs are associated with insulin resistance and cardiometabolic disease[11] and could act as mediators of heart failure pathogenesis.[6,12] Indeed, lowering dietary BCAA intake has been shown to regulate metabolic health, leading to improvements in glucose tolerance and body composition.[13] However, their role in cardiac remodeling remains unclear. Some studies have reported that consumption of an essential amino acid-enriched diet and a high-leucine diet can attenuate pathology in models of cardiac injury.[14,15] Conversely, other studies have found that, in the context of pressure overload, a BCAA-enriched diet accelerates cardiac dysfunction, whereas a BCAA-free diet may mitigate maladaptive hypertrophy and fibrosis.[16,17] Notably, few efforts have been made to interrogate directly whether dietary BCAAs influence remodeling following acute MI. To address this issue, we examined in mice whether dietary alterations in BCAA levels influence cardiac remodeling and function after MI. Our findings lend support to the idea that careful modulation of the diet after MI could be an actionable strategy to optimize cardiac recovery.

METHODS

Experimental Animals and Diets

Experimental protocols were reviewed and approved by the University of Louisville Institutional Animal Care and Use Committee, and all animal studies were completed in compliance with the *Guide for the Care and Use of Laboratory Animals*. Male and female C57BL/6J mice were purchased from the Jackson Laboratory. The low (Cat: TD.150662) and high (Cat: TD.170323) BCAA custom diets were purchased from Envigo Teklad (see Tables S1, S2 for diet formulations). The standard chow diet was purchased from LabDiet (Cat: 5010, see Tables S1, S3 for diet formulations). All animals were housed in a pathogen-free facility under controlled conditions (24 °C, 44%–65% relative humidity, 12-hour light/dark cycle).

Rigor, Transparency, and Reproducibility

The study was conducted in accordance with our recently published guidelines on scientific rigor.[18] Based on prior experiences, 11 female and 13 male mice were used for each diet group with the expectation of post-MI attrition being different between sexes. Echocardiography confirmation of infarction was required for inclusion in subsequent analysis. One female mouse in the low BCAA diet group was excluded due to lack of MI. To maintain a rigorous experimental design, in vivo experiments included blinding of surgeons, sonographers, and microscopists to group identity. Tissue sample processing and analysis were performed in a blinded manner as well. The primary end point of this study was defined by cardiac functional parameters at follow-up, measured by echocardiography. We used the Animal Research: Reporting of In Vivo Experiments checklist when writing our report.[19] The authors declare that all supporting data are available within the article and its online supplementary files.

Nonreperfused Myocardial Infarction and Echocardiography

In a dedicated laboratory space, adult, 12-week-old, male and female mice were acclimated for at least 1 hour before nonreperfused MI, as described.[20,21] Nonreperfused MI was chosen because approximately 25% of patients with MI are not reperfused and because the nonreperfused MI model can be a more desirable model for assessing interventions to prevent heart failure.[22] Briefly, mice were first anesthetized

through intraperitoneal injections of (pentobarbital, 50 mg/kg; ketamine, 50 mg/kg) before surgery. Subsequently, they were orally intubated and ventilated with oxygen. Using a 7–0 silk suture, the left coronary artery was permanently ligated and then the chest wall was sutured closed. Mice were extubated only after the recovery of spontaneous breathing, and analgesia (meloxicam, 10 mg/kg) was provided before the recovery as well as twice a day at 24 and 48 hours post surgery. Research team and veterinary staff monitored the animals twice daily during the first week post MI and then twice weekly throughout the duration of the study. Health was gauged by temperature, body weight, food and water intake, and general assessment of respiratory status, ambulation, arousal, posture and surgical site healing. Indications for euthanasia include loss of body weight (≥15%), respiratory stress, suture site dehiscence, hunched posture, surgical site infections, vocalization to touch, or other clinical signs of distress as listed in the University of Louisville Institutional Animal Care and Use Committee Humane Endpoints Policy. Cardiac structure and function were assessed through transthoracic echocardiography (Vevo 3100, Visual Sonics) while under anesthetic (isoflurane) at the designated times.

Echocardiography Analysis

Ventricular wall thickness was obtained with M-mode images through parasternal long axis view. Reported measurements included left ventricular (LV) interior diameter, LV mass (corrected), LV posterior wall thickness, and LV anterior wall thickness near the border zone. Each parameter was the cumulative average of 4 measurements and LV mass (corrected) was calculated from the formula: (1.053×[(LV interior diameter;d+LV posterior wall;d+intravenous septum;d)^3–LV interior diameter;d^3])×0.8 (d = diastole). Additionally, Simpson's method B-mode with parasternal long axis and apical view was used to obtain images for measuring LV end-diastolic volume, LV end-systolic volume, stroke volume, ejection fraction, cardiac output, fractional shortening, and heart rate with 5 measurements taken at 3 different ventricular planes and averaged. An apical 4-chamber view in pulsed wave Doppler was used for measurement of isovolumic relaxation time; reported values were averaged from 9 individual measurements.

Histopathology

At the conclusion of the study, infarcted hearts were excised and arrested in diastole with ice-cold 2% potassium chloride. Each heart was then sectioned into 1-mm cross-sectional segments and fixed in 10% formalin before being embedded in paraffin, sectioned (4 μm), and mounted. Slides were then deparaffinized and rehydrated before staining. Picrosirius red staining (Picric Acid, Sigma, P6744; Direct Red 80, Sigma, 365548) was used to detect collagen in intact sections. Cardiomyocyte size was assessed by staining with wheat germ agglutinin (Vector Labs, RL-1022) to demarcate cell membranes followed by assessment of cardiomyocyte cross-sectional area[23] and length.[24,25] Capillary density was measured by staining with Isolectin B4 (Vector Labs, FL-1201). DAPI (Invitrogen, D3571) was used to detect nuclei. Sections were visualized using the Keyence Imaging System. Acquired images were then analyzed using the Keyence BZ-X800 analyzer.

Second Harmonic Generation Imaging

Transverse myocardial tissue sections were deparaffinized by heating at 80 °C for 30 minutes, followed by rinsing in xylene. The sections were then rehydrated stepwise in decreasing concentrations of ethanol (100%, 96%, 90%, 80%), with a final submersion in deionized water for 3 minutes. After a brief placement in 1× PBS, the sections underwent second harmonic generation imaging using a Nikon A1R MP+ multiphoton microscope with an Apo LWD 25× immersion objective. The excitation laser was tuned to 920 nm, and the second harmonic generation signal was collected through a DAPI bandpass filter with an emission wavelength of 446 nm. Images were acquired with a resolution of 3595×3598 pixels at a scan speed of 15 seconds. Over 20 regions of interest (256×256 pixels) were selected per heart, focusing on the longitudinal aspect of collagen fibers within infarct scar regions. The regions of interest were subject to computational analyses using MATLAB software packages CurveAlign and CT-FIRE,[26] as described,[27] to quantify macrostructural attributes of the collagen fiber network (collagen fiber alignment, width, and straightness).

Measurement of BCAAs

To measure circulating levels of BCAAs, blood samples were collected via a right ventricular puncture using a 23-gauge needle and an EDTA-coated syringe. Samples were centrifuged at 3500*g* for 20 minutes, followed by separation of the plasma fraction. Plasma BCAA levels were then measured using a commercially available BCAA assay kit (Abcam; ab83374), according to manufacturer's protocol.

To measure intracardiac levels of BCAAs, a reverse-phase liquid chromatography tandem mass spectrometry custom assay[28,29] was used to measure BCAA levels in 30 to 40 mg tissue from powdered post-MI hearts (The Metabolomics Innovation Centre, Edmonton, AB, Canada). Tissue samples were homogenized in a 3-fold volume of tissue extraction buffer (extraction buffer was prepared by mixing 85 mL methanol and 15 mL of 10 mM phosphate buffer). After

homogenization, samples were centrifuged at 20000g for 20 minutes and the supernatants were transferred into new Eppendorf tubes. After derivatization and extraction of analytes, a selective mass-spectrometric detection method using multiple reaction monitoring pairs was used. Isotope-labeled internal standards were used for metabolite quantification. Mass spectrometric analysis was performed on an ABSciex 4000 Qtrap tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA). The samples were delivered to the mass spectrometer after liquid chromatography separation followed by direct injection. Data analysis was performed using Analyst 1.6.3.

Primary Cardiac Fibroblast Isolation and Culture

Cardiac fibroblasts were isolated from naïve, adult mice. Briefly, following euthanasia, hearts were excised, and the aorta was cannulated with a 23-gauge needle. Ice-cold PBS was flushed through the aorta, and excess aorta and adipose tissue were trimmed from the heart before mincing with a razor blade under sterile conditions. Heart tissue was then digested in collagenase solution for 45 minutes at 37 °C with gentle agitation. The collagenase solution contained type II collagenase (Worthington, 46H16739) prepared in sterile PBS at 5000 units per 7.5 mL per heart. Subsequently, culture medium (ie, DMEM/F12 GlutaMax [DMEM, Gibco 10565018] containing 10% fetal bovine serum [VWR 1500-500H], 1% penicillin/streptomycin/amphotericin B [Sigma, A5955], 1% insulin transferrin selenium [ITS-G, Gibco 41400045], and 20 ng/mL human bFGF [basic fibroblast growth factor; Peprotech 100-18B]) was added to quench collagenase activity, and the mixture was centrifuged at 500*g* for 10 minutes. Supernatants were aspirated, and cell pellets were resuspended in culture media before being passed through a 70 μm cell strainer. Cells were then plated in a T-75 cell culture flask and incubated at 37 °C in 5% CO_2. Media was changed after 2 hours and again after 24 hours.

For in vitro studies, fibroblasts were used at passage 1 and serum-starved in custom BCAA-free DMEM/F12 GlutaMax media (ThermoFisher) without any supplementation for 24 hours before treatment with TGF-β (transforming growth factor beta; 10 ng/mL, PeproTech, 100–21) for 48 hours. To modify individual BCAA concentrations, L-leucine (Sigma-Aldrich, L8912), L-isoleucine (Sigma-Aldrich, I7403), and L-valine (Sigma-Aldrich, V0513) were added individually, as indicated. The concentrations used (100, 200, 400 μM) included 45.9% valine, 35.3% leucine, and 18.8% isoleucine, similar to the ratio occurring in the murine heart.[8]

Protein and Gene Expression Analyses

Total protein was harvested after washing cells with ice-cold PBS. Cells were scraped with a rubber policeman in lysis buffer (20 mM HEPES, 110 mM KCl, 1 mM EDTA, 1% IGE-PAL, and 0.1% SDS, pH 7.0) containing protease inhibitor cocktail (Sigma-Aldrich, P8340). The cell lysates were incubated on ice for 30 minutes and then centrifuged at 20000*g* for 20 minutes. The supernatants were then carefully removed and saved for total protein measurements (Lowry assay) and Western blotting.

Protein samples were mixed with Laemmli sample buffer (125 mM Tris–HCl, 10% SDS, 50% glycerol, 0.05% Bromophenol Blue, pH 6.8) and incubated at 95 °C for 5 minutes. Each sample was then loaded on a 7.5% acrylamide/bis SDS-PAGE gel for electrophoresis at 120 V. SDS-PAGE-resolved proteins were then transferred overnight onto a PVDF membrane (Cytiva, 10600021) at 4 °C at 20 V. Subsequently, membranes were blocked for 1 hour at room temperature with Tris-buffered saline containing Tween-20 and 5% BSA. Membranes were probed with primary antibodies overnight at 4°C. The following day, membranes were washed three times using Tris-buffered saline containing Tween-20 and then incubated with secondary antibody for 1.5 hours. Membranes were then washed 3 times with Tris-buffered saline containing Tween-20 before exposure to Pierce ECL Plus Western Blotting Substrate (Thermo Scientific, 32132) and imaging on a ChemiDoc imager (BioRad). Protein was normalized to total lane protein with an amido black stain, and parallel blots were normalized to an anchor protein. The primary antibodies used included anti-COL1A1 (1:20000; Thermo Fisher, PA5-29569), antiperiostin (1:5000; Millipore, ABT253), and anti-α-SMA (α-smooth muscle actin; 1:30000; Cell Signaling, 19245S). Secondary antibody included anti-rabbit IgG HRP-linked (1:2500; Cell Signaling, 7074).

Expression of markers of pathological remodeling and BCAA catabolism were evaluated through real-time polymerase chain reaction. Total RNA was extracted from flash-frozen cardiac tissue using the RNeasy plus universal mini kit (Qiagen, 73404) and cDNA was made from 600ng RNA per sample via Superscript IV VILO Master Mix with ezDNase Enzyme (Thermo Scientific, 11766050). The relative levels of mRNA transcripts were quantified by real-time polymerase chain reaction using SYBR Green (VWR, 101414–278) on a real-time polymerase chain reaction system (QuantStudio5). The data were normalized to mouse Rpl13a threshold cycle values and reported using the ΔΔ threshold cycle

comparative method. All primer sequences are listed in Table S4.

Statistical Analysis

Data are expressed as mean±SEM. Normality was assessed via Shapiro–Wilk test. Mann–Whitney *U* test, unpaired 2-tailed *t* test, Kruskal–Wallis test with Dunn's post hoc, 1-way ANOVA with Tukey's post hoc test, or 2-way repeated measurement ANOVA with Šidák post hoc test was performed for between-group comparisons, as appropriate and as indicated in the figure legends. Comparisons were considered significant when $P<0.05$. Detailed statistical information is shown in Tables S5–S10. Graphpad Prism 10 was used to perform statistical analyses.

RESULTS

A Diet Low in BCAAs Decreases Circulating BCAA Concentrations in Mice

To investigate whether altering dietary BCAA levels is sufficient to influence blood levels of BCAAs, we fed male and female C57BL/6J mice a low or high BCAA custom diet over a 7-day period (Figure 1A); these diets have 1/3× or 2× the level of BCAAs, respectively, than the typical "normal chow" diet, which was included as a comparator (see Tables S1–S3 for diet compositions).

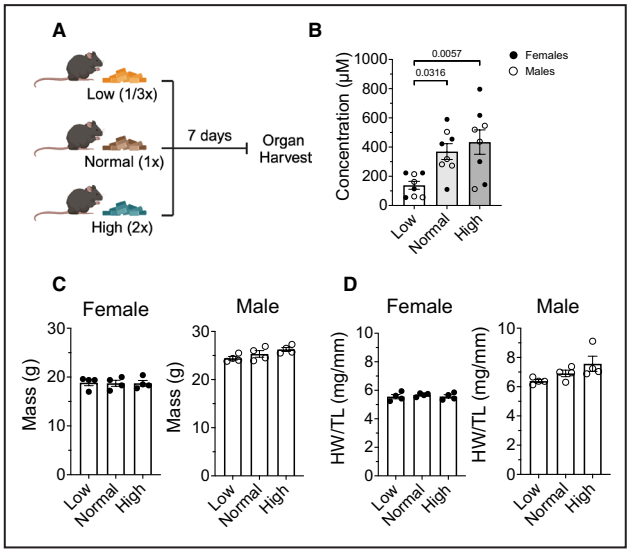

Figure 1. Dietary BCAA levels modulate circulating BCAAs. **A**, Mice were fed a low (1/3×), normal (1×), or high (2×) BCAA diet for 7 days before measurement of (**B**) plasma BCAA concentrations; (**C**) body mass; and (**D**) heart weight (normalized to tibial length). n=4 male and 4 female mice per group. Panel **B** shows pooled male and female mice. Data are mean±SEM. Normality was assessed via Shapiro–Wilk test, Panel **B–D**, 1-way ANOVA with Tukey's post hoc or Kruskal–Wallis with Dunn's post hoc. BCAA indicates branched-chain amino acid; HW, heart weight, and TL, tibia length.

Plasma BCAA concentrations measured at the conclusion of the pilot study revealed that the high BCAA diet did not elevate BCAAs beyond that of the normal chow diet; however, the low BCAA diet decreased circulating BCAAs by more than 2-fold (Figure 1B). Neither male nor female mice showed significant differences in body mass or heart weight normalized to tibial length between BCAA feeding groups (Figure 1C,D). These findings demonstrate that providing a diet that is low in BCAAs effectively reduces circulating BCAA levels in mice.

BCAAs Influence Body Mass and Cardiac Size in a Sex-Dependent Manner

To assess the chronic effects of modified BCAA diets in a cardiac injury model, male and female mice were fed either a low BCAA diet or a high BCAA diet for 2 weeks before, and for 4 weeks after, MI (Figure 2A). We did not include a normal chow dietary group because the normal and high BCAA diets led to similar circulating BCAA concentrations (Figure 1B) and because the composition of the normal chow is vastly different from the BCAA custom diets, which were otherwise matched for nitrogen, carbohydrate, fat, and calorific content (Tables S1–S3). To determine whether chronic BCAA dietary modifications would alter intracardiac abundance after MI, BCAA concentrations in post-MI hearts were measured at the conclusion of the study by liquid chromatography tandem mass spectrometry, using powdered cardiac tissue for analysis. Although the myocardial levels of each BCAA were consistent with previously reported post-MI values,[8] the concentrations of leucine, isoleucine, and valine were not significantly different between low and high BCAA diet groups (Figure S1A,B). Baseline body weights were measured 1 week before surgery, and at 2 and 4 weeks after MI. Consistent with our previous findings in the acute feeding pilot study, the female cohort did not display differences in body weight (Figure 2B). Conversely, male mice fed a high BCAA diet consistently demonstrated higher body weights compared with mice fed a low BCAA diet (Figure 2C). Gravimetric assessments 4 weeks post MI revealed that male mice fed the low BCAA diet had significantly lower heart mass (heart weight, heart weight normalized to tibial length) and wet/dry lung weight ratios (Figure 2D,E). Notably, these effects were not observed among the female cohort, suggesting that dietary BCAA levels significantly influence body weight, heart mass, and wet/dry lung weight ratio in a sex-dependent manner. Analysis of cardiac BCAA transporter (*Slc25a44*) and catabolic gene (*Bcat2, Bckdk, Bckdha, Bckdhb*) expression and genes known to be altered in heart failure (*Pgc1a, Ppara, Nppa, Nppb, Myh6, Myh7*) were not significantly different in either

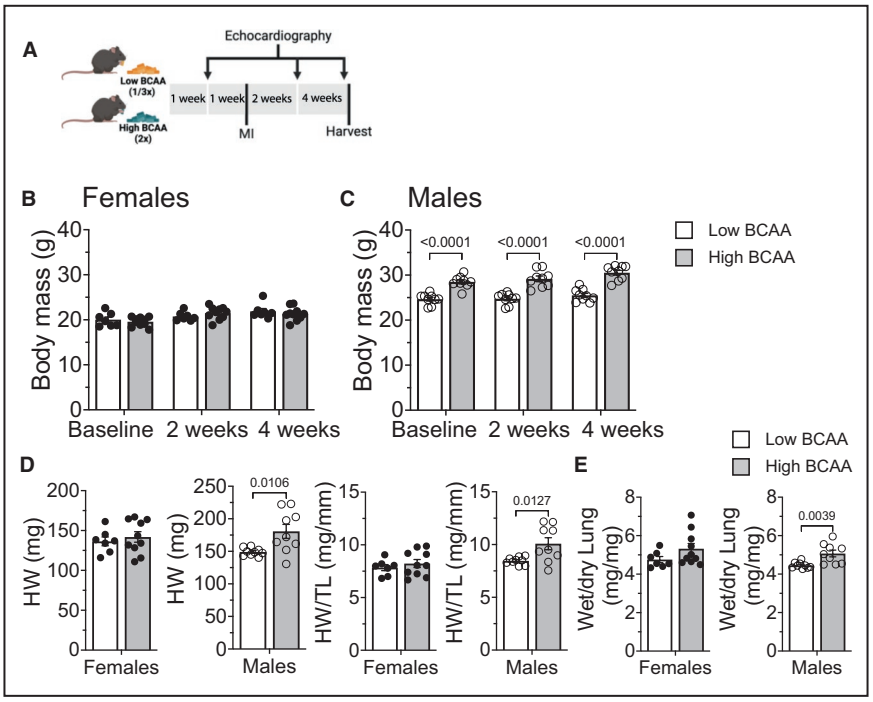

Figure 2. Low dietary BCAA levels prevent cardiac hypertrophy and MI-induced congestive heart failure.
Gravimetric measurements: **A**, Schematic of study design: mice were fed a low or high BCAA diet for 7 days before nonreperfused MI and continued on the respective diet for 28 days after MI; **B**, Female and (**C**) male body mass recorded at baseline and 2 and 4 weeks post MI; **D**, Heart weight and heart weight normalized to tibia length measured 4 weeks post MI; **E**, Wet/dry lung mass ratio measured 4 weeks post MI; n=7–10/group. Data are mean±SEM. Normality was assessed via Shapiro–Wilk test Panel **B**, **C**, 2-way repeated measures ANOVA with Šidák post hoc; Panel **D**, **E**, Unpaired 2-tailed *t* test; BCAA indicates branched-chain amino acid; HW, heart weight; HW/TL, heart weight normalized to tibia length; MI, myocardial infarction; and TL, tibia length.

males or females between low and high BCAA diets (Figure S2A–D).

Lower Dietary BCAA Consumption Prevents Worsening of Cardiac Function Post Infarction, With a More Pronounced Effect on Cardiac Function and Structure in Male Mice

To study the effects of dietary BCAAs on MI-induced cardiac dysfunction and remodeling, mice were subjected to echocardiographic assessment. Serial echocardiograms were collected immediately before (baseline), and at 2 and 4 weeks after MI (see Figure 2A). Although we found that dietary BCAAs did not significantly influence heart rate (Figure 3A) or volumetric and functional parameters (eg, ventricular volumes (LV end-diastolic volume and LV end-systolic volume), ejection fraction, fractional shortening, isovolumetric relaxation time; Figure 3B–F), we found that they had more prominent effects on parameters that are calculated from aggregate volumetric indices (i.e. stroke volume and cardiac output). After MI, both groups demonstrated lower stroke volume and cardiac output in the high BCAA group compared with the low BCAA group, with more significant changes in the male cohort (Figure 3G,H). Because body weights differed in male mice with high BCAA feeding, we also normalized cardiac output values to body weight to obtain cardiac index values. As shown in Figure 3I, mice fed the high BCAA diet showed progressively declining cardiac index values after MI; however, provision of the low BCAA diet completely preserved cardiac index.

Stratified analysis of the structural parameters indicates that male mice on the high BCAA diet experienced greater levels of cardiac hypertrophy, whereas this effect was relatively mild in female mice (Figure 4A). Interestingly, there were no appreciable differences observed in LV internal diameters (Figure 4B,C), suggesting

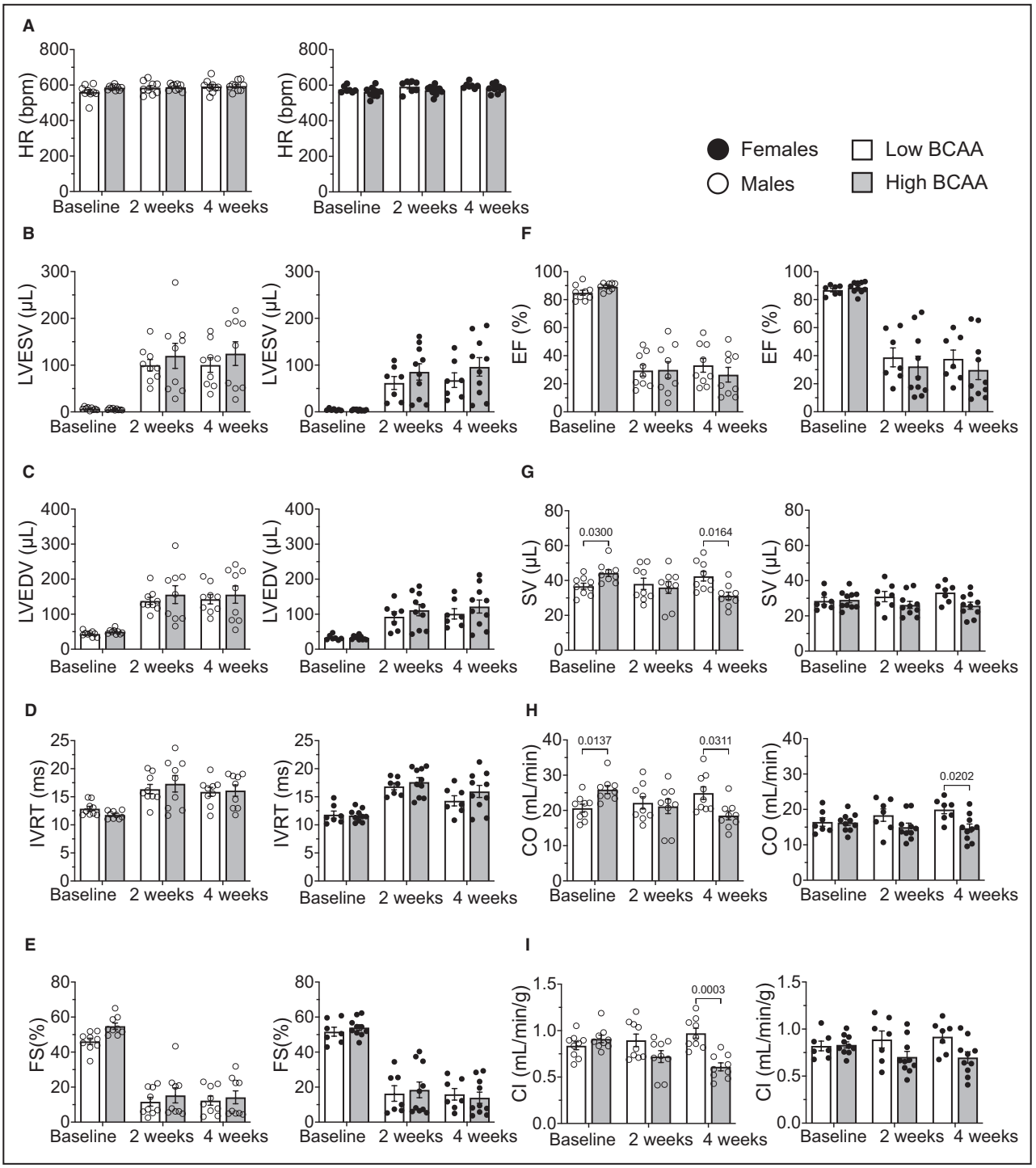

Figure 3. Low dietary BCAA levels prevent MI-induced decreases in stroke volume and cardiac output.
Echocardiographic measurements for (**A**) heart rate; (**B**) end-systolic volume; (**C**) end-diastolic volume; (**D**) isovolumetric relaxation time; (**E**) fractional shortening; (**F**) ejection fraction; (**G**) stroke volume; (**H**) cardiac output; (**I**) cardiac index; n=7 to 10/group. Data are mean±SEM. Two-way repeated measures ANOVA with Šidák post hoc. BCAA indicates branched-chain amino acid; CO, cardiac output; EF, ejection fraction; FS, fractional shortening; HR, heart rate; IVRT, isovolumetric relaxation time; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; MI, myocardial infarction; and SV, stroke volume.

no overt changes in LV dilatation. Assessment of LV wall thickness further revealed that male mice had significantly larger anterior and posterior wall diameters during diastole, which did not reach significance in female mice (Figure 4D–G). These results suggest that biological sex governs responses to dietary BCAAs,

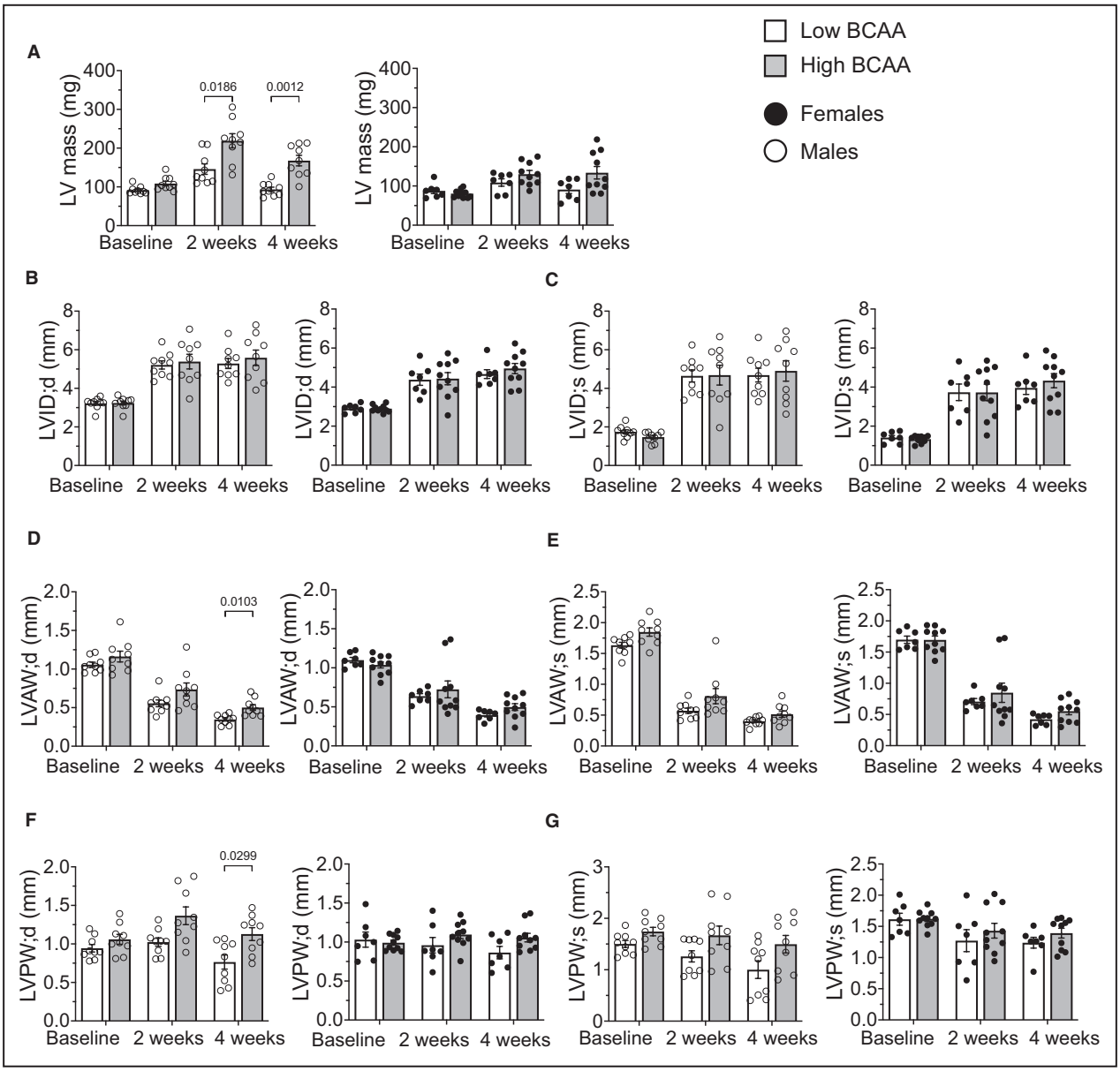

Figure 4. Low dietary BCAA results in smaller LV mass and wall thickness post MI.
Echocardiographic measurements of structural features: (**A**) calculated left ventricular mass (corrected); (**B, C**) left ventricular internal diameter in systole and diastole; (**D, E**) left ventricular anterior wall thicknesses in systole and diastole; (**F, G**) left ventricular posterior wall thicknesses in systole and diastole. 7–10/group. Data are mean±SEM. Two-way repeated measures ANOVA with Šidák post hoc. BCAA indicates branched-chain amino acid; LV, left ventricular; LVAW;d, left ventricular anterior wall diastole; LVAW;s, left ventricular anterior wall systole; LVID;d, left ventricular interior diameter diastole; LVID;s, left ventricular interior diameter systole; LVPW;d, left ventricular posterior wall diastole; LVPW;s, left ventricular posterior wall systole; and MI, myocardial infarction.

with male mice showing a more pronounced response in structural and functional remodeling.

BCAA Levels Influence Myofibroblast Differentiation In Vitro

Although BCAAs are known to promote cardiomyocyte hypertrophy,[16] it is less clear whether they influence fibroblasts in the heart. Therefore, we examined whether BCAAs influence cardiac fibroblast activation in vitro. Isolated primary cardiac fibroblasts were treated with either bFGF (control) or TGF-β in medium containing 0 to 400 μM BCAAs for 48 hours, followed by analysis of Col1a1, periostin, and α-SMA protein abundance by immunoblotting (Figure 5A). Interestingly, the absence of BCAAs in the culture medium prevented TGF-β-induced upregulation of Col1a1 and periostin (Figure 5B,C), but did not prevent upregulation of α-SMA (Figure 5D). Furthermore, intermediate levels of upregulation of Col1a1 and periostin were apparent

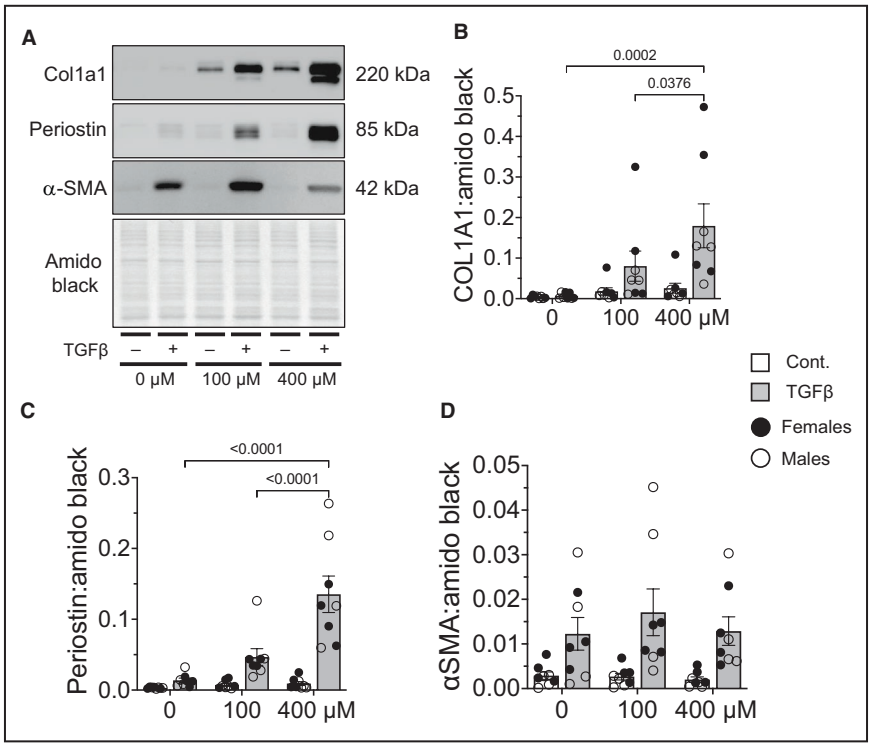

Figure 5. Cardiac fibroblast activation in vitro is dependent on BCAA availability. Isolated murine cardiac fibroblasts were treated with TGF-β (or vehicle control) for 48 hours in medium containing different concentrations of BCAAs (0, 100, 400 μM) and were assessed for Col1a1, periostin, and α-smooth muscle Actin abundance by immunoblotting. **A**, Representative image of immunoblots; **B**, quantification of Col1a1 abundance; **C**, quantification of periostin abundance; and **D**, quantification of α-SMA abundance. Proteins were normalized to amido black signal from stained membranes. n=8/group, with males and females pooled. Data are shown as mean±SEM. Statistics: Two-way ANOVA with Tukey's post hoc test. α-SMA indicates α-smooth muscle actin; BCAA, branched-chain amino acid; and TGF-β, transforming growth factor beta.

in fibroblasts cultured in medium containing 100 μM BCAAs, which indicates concentration dependence in the response. Collectively, these findings indicate that BCAA levels modulate levels of secreted extracellular matrix proteins but do not affect gross indices of the contractile myofibroblast phenotype.

High Dietary BCAA Consumption Is Associated With Eccentric Cardiomyocyte Hypertrophy and Increases in Capillary Density Post MI in Male Mice

To determine if circulating BCAA levels influence cardiac fibrosis, we stained cardiac sections with picrosirius red; however, in contrast to our expectations based on in vitro results, we found no difference in total picrosirius red staining between the groups (Figure 6A,B). Assessments of transmural scar thickness showed modest differences, with greater thickness in male mice fed the high BCAA diet (Figure 6C), which closely match the left ventricular anterior wall thickness measurements in Figure 4E. To investigate potential macrostructural differences in the collagenous scar, infarct regions were imaged via second harmonic generation microscopy and subjected to computational enumeration of collagen macrostructural attributes; however, we observed no measurable differences in collagen fiber alignment, width, or straightness between the groups (Figure 6D–G). These results suggest that our low BCAA diet may not deplete BCAAs to levels that impede collagen production and scar formation/maturation. Indeed, in a separate study, we performed experiments in isolated cardiac fibroblasts using 0, 200, or 400 μM levels of BCAAs, and although complete lack of BCAAs inhibited TGF-β-induced collagen and periostin upregulation, there was no difference between experimental groups that had 200 and 400 μM levels of BCAAs present in the medium. These findings are consistent with the interpretation that the low BCAA diet does not decrease BCAAs below the threshold that impedes extracellular matrix production (Figure S3). Further consistent with this interpretation, survival after MI was not different between the low and high BCAA diet groups when sexes were

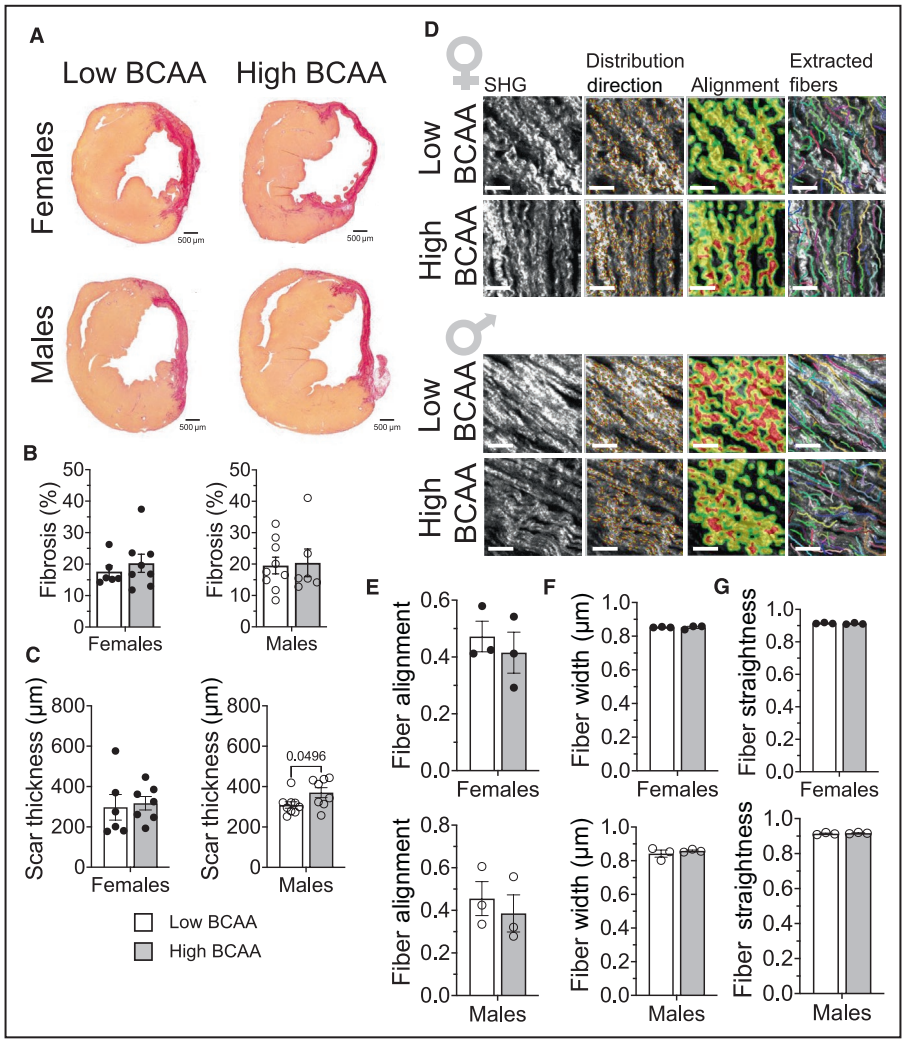

Figure 6. Modified dietary BCAA does not affect cardiac fibrosis and scar remodeling. Examination of cardiac collagen content and organization: **A**, Intact midpapillary sections stained with PSR; **B**, quantification of PSR signal (cardiac fibrosis) and (**C**) scar thickness (n=6–9 biological replicates); **D**, Computational assessment of scar collagen architecture in SHG-imaged infarct segments. Left to right, representative master SHG images from postinfarct scar regions (region of interest: 256×256 pixel), CurveAlign-generated images displaying the spatial distribution (red dots) and direction (green lines) of collagen fiber segments, corresponding CurveAlign sourced heatmaps depicting the relative alignment of collagen fibers across the region (red denotes regions of high alignment), and, lastly, CT-FIRE extracted fibers overlaid on master SHG images (individual fibers are separated by varying colored lines). From these computational analyses, macrostructural collagen attributes were enumerated and graphed, including (**E**) fiber alignment, (**F**) fiber width, and (**G**) fiber straightness (n=3 biological replicates per sex per group). Data are mean±SEM. Normality was assessed via Shapiro–Wilk test. Parametric unpaired Student's *t* test or nonparametric Mann–Whitney *U* test as appropriate. Scale bar=8.14 µm. BCAA indicates branched-chain amino acid; PSR, picrosirius red; and SHG, second harmonic generation.

either combined (Kaplan–Meier analysis, P=0.51, n=24 per group) or separated (Kaplan–Meier analysis, males P=0.95, n=13 per group; females P=0.26, n=11 per group), supporting the idea that the low BCAA diet did not negatively affect scar formation or promote cardiac rupture (Figure S4A–C).

To assess differences in cardiomyocyte size, we measured cardiomyocyte size and capillary density by staining sections with wheat germ agglutinin and isolectin B4. Surprisingly, we found no differences in cardiomyocyte cross-sectional area in hearts from low and high BCAA-fed mice (Figure 7A,B); however,

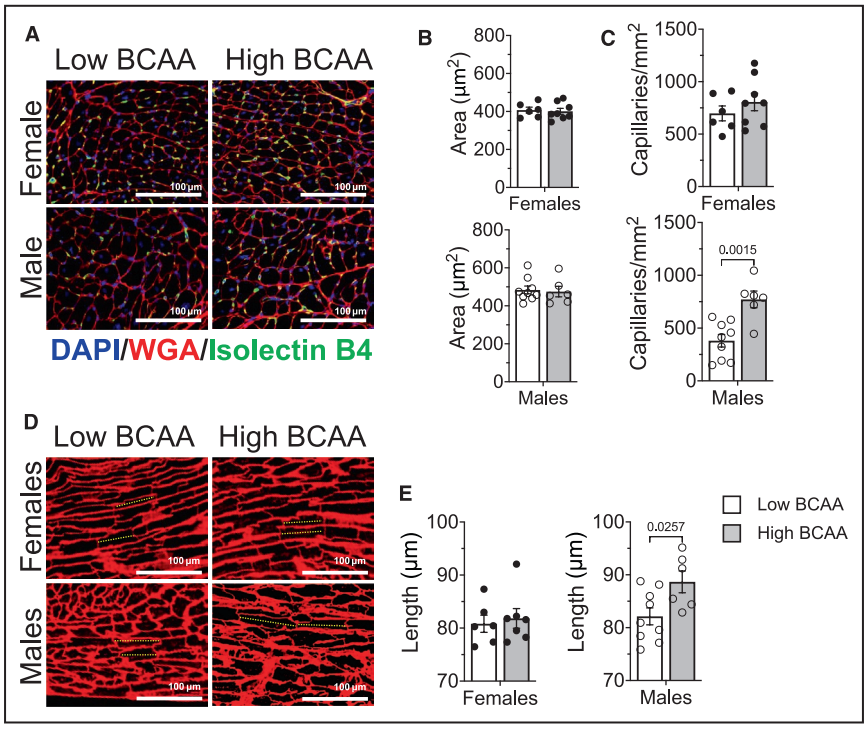

Figure 7. High dietary BCAA consumption is associated with eccentric cardiomyocyte hypertrophy and higher capillary density in male mice.
A–C, Cardiomyocyte cross-sectional area (wheat germ agglutin), and capillary density (Isolectin B4); **D, E**, Cardiomyocyte length measurements (averaged from 13 to 73 identified cells per section). Data are mean±SEM. n=6 to 9 biological replicates; Normality was assessed via Shapiro–Wilk test. Parametric unpaired Student's *t* test or nonparametric Mann–Whitney *U* test as appropriate. BCAA indicates branched-chain amino acid; and WGA, wheat germ agglutin.

wheat germ agglutinin assessments of cardiomyocyte length[24,25] demonstrated longer myocytes in mice fed the high BCAA diet compared with the low BCAA diet (Figure 7D,E). Of note, capillary density was also observed to be higher in the high BCAA-fed male group, which may suggest a compensatory increase in angiogenesis to support increases in cardiomyocyte volume (Figure 7C). These findings suggest that high dietary BCAA consumption in male mice contributes to eccentric myocyte hypertrophy with a compensatory increase in capillary density after MI.

DISCUSSION

Although numerous studies have highlighted a significant role for BCAAs in cardiovascular disease risk,[7,30] it remains unclear how levels of circulating BCAAs influence cardiac remodeling after MI. Accordingly, the current study was designed to understand whether modulating BCAA levels via the diet influences MI-induced remodeling. To address this question as simply as possible, we chose diets that are matched for calorific content and nitrogen balance, with the major difference being BCAA levels. Notably, the high BCAA diet and the typical normal chow diet led to similar levels of circulating BCAAs, with the low BCAA diet decreasing BCAA blood levels substantially. Our findings show that provision of a diet low in BCAAs ameliorates cardiac hypertrophy, lowers lung edema, and preserves stroke volume and cardiac output after MI. Moreover, our findings indicate that male mice are more responsive to BCAAs. Collectively, these findings provide support to the concept that simple modifications to the diet could have marked effects on cardiac remodeling after MI and that dietary BCAA levels, in particular, are impactful modifiers of cardiac recovery.

Although diet is a readily modifiable lifestyle factor that could improve cardiovascular outcomes, there remains a paucity of information about what components of the diet may affect cardiac remodeling and function after MI. Early investigations into dietary strategies for patients after MI revealed a lack of consensus on dietary guidelines,[31] with initial attempts to optimize dietary intervention leading to broad recommendations such as providing a liquid diet during the first 24 to 48 hours after MI, followed by adherence to low sodium and low cholesterol diets.[32,33] More recently, "heart healthy"

diets have been reported to be protective against adverse cardiovascular events.[34,35] These benefits may partially be attributed to the unique composition of these diets, which are often rich in fruits, vegetables, and legumes.[36,37] In particular, Mediterranean dietary patterns have been shown to be modestly protective against MI recurrence and composite cardiovascular disease outcomes.[38] Studies such as this have led to broad, helpful recommendations that provide general guidance, for example, implementation of plant-based and Mediterranean-based diets, minimization of saturated and trans fats, higher dietary fiber intake, and diminished consumption of simple carbohydrates.[39,40] Nevertheless, it remains unclear which specific components of the diet have deleterious or salutary effects after MI.

Preclinical studies are helpful in shedding light on how particular nutrients, such as BCAAs, affect heart health after cardiac insult or injury. Although the diets used in our study had modified levels of all BCAAs (that is, leucine, isoleucine, and valine), a previous study in which only dietary leucine was elevated after MI suggested that leucine has generally beneficial effects, attenuating fibrosis and apoptosis and increasing survival.[15] These findings indicate that individual BCAAs may have different (patho)physiological effects—a claim further supported by recent studies showing that the adverse metabolic effects of BCAAs are mediated by isoleucine and valine, and not leucine.[41] Nevertheless, provision of a BCAA-free diet to mice with pressure overload significantly attenuated maladaptive fibrosis and cardiac hypertrophy.[17] Other studies that focused on the effects of BCAAs on the heart show that, in heart failure, cardiac BCAAs are substantially elevated in part due to their impaired catabolism (reviewed in[6]). For example, direct stimulation of BCAA catabolism with an inhibitor of branched-chain keto-acid dehydrogenase kinase decreased cardiac BCAA concentrations and had robust beneficial effects on chronic myocardial remodeling following MI[42] or pressure overload.[43] Our findings add to this growing body of literature and show that reducing circulating BCAA concentrations via their dietary restriction has the potential to be a safe and effective therapeutic approach to mitigate pathological cardiac remodeling.

Although the mechanisms by which lowering circulating BCAAs improves remodeling after MI remain unclear, our studies reveal that, compared with the high BCAA diet, the low BCAA diet prevents eccentric cardiomyocyte hypertrophy and improves stroke volume and cardiac output after MI. Previous studies demonstrate that even short-term feeding of the high BCAA diet, in the absence of MI, increases heart size and cardiomyocyte cross-sectional area.[16] Although we found no differences in cross-sectional area, we show that, after MI, the high BCAA diet significantly increased cardiomyocyte length and increased LV mass in male mice. These findings appear to suggest that a low BCAA diet may effectively prevent eccentric cardiac remodeling, which could in part underlie observed improvements in stroke volume and cardiac output. Interestingly, both stroke volume and cardiac output were increased at baseline (before MI) in male mice fed the high BCAA diet for 1 week, which suggests that worsened cardiac remodeling after MI in the high BCAA group is the amalgam of 2 separate hits—elevation of BCAAs and the pathological setting of MI. Although the mechanisms are likely to be multifaceted, they could include BCAA-induced changes in histone propionylation and gene expression,[17] modulation of mTOR (mechanistic target of rapamycin) signaling,[44] cardiac insulin resistance,[7] or extracardiac actions of BCAAs.[42] Interestingly, Murashige et al. demonstrated that a diet low in BCAAs increases systolic and diastolic blood pressure in mice[42]; hence, our findings showing improvement of post-MI remodeling in mice fed a low BCAA diet suggest that influences of BCAAs on blood pressure are unlikely to be responsible for the observed improvements in cardiac remodeling and function. Nevertheless, intracardiac levels of BCAAs were not different between post-MI mice fed the different BCAA diets, insinuating that extracardiac BCAA actions beyond blood pressure may be responsible for the observed salutary effects of the low BCAA diet.

Responses to dietary BCAAs were also conspicuously modified by biological sex, with male mice showing more robust responses, which adds another layer of complexity that makes defining a singular mechanism problematic. Notably, only in male mice did high BCAA feeding result in significantly higher body mass and heart weight compared with the low BCAA diet. Given that the diets were matched for calorific content (see Table S1) and that previous studies using the same diets and mouse strain found no differences in caloric intake between the 2 diets,[16] these findings suggest that BCAAs may provide a higher anabolic stimulus in male mice compared with female mice. Nevertheless, female mice fed the BCAA diets showed functional trends similar to males, with the low BCAA diet significantly preserving cardiac output. Because female mice have been previously reported to experience smaller infarct sizes, lower rates of cardiac rupture, and better LV function post MI compared with males, their threshold for developing heart failure may already be relatively high.[45,46] As a result, the protective effects of a low-BCAA diet on cardiac function may be less pronounced when compared with males. Lastly, the cardiac hypertrophic effects of BCAAs were markedly different between sexes, wherein male mice fed the high BCAA diet and subjected to MI showed higher LV mass, higher anterior and posterior wall thicknesses,

cardiomyocyte elongation, higher capillary density, and worsened lung edema; these effects of high dietary BCAAs were not apparent in female mice. Because increasing BCAA oxidation appears protective and beneficial with respect to cardiac remodeling,[6] it is tempting to speculate that the sex differences shown here might be due to a heightened capacity of the female heart to oxidize BCAAs, which could be addressed in future studies. Although the expression of genes known to be involved in heart failure and BCAA metabolism were not different in male or female mice or between dietary groups (Figure S2), it remains possible that the activity of these pathways could be influenced in a posttranscriptional manner.

Surprising to us was the finding that the low BCAA diet did not affect myocardial collagen formation or organization in vivo. Not only did previous studies demonstrate that a BCAA-free diet mitigates maladaptive fibrosis,[17] but also our isolated fibroblast studies showed clear evidence that BCAAs are required for collagen and periostin secretion. Interestingly, BCAA availability did not affect TGF-β-induced upregulation of α-SMA and acquisition of a contractile phenotype, but it did repress Col1a1 and periostin abundance in a dose-dependent manner, with notable differences discernable when BCAA levels in the medium were decreased to 100 μM. Nevertheless, this effect was lost when BCAA concentrations reached 200 μM (Figure S3). Given that the low BCAA diet decreased mean circulating BCAAs to concentrations slightly higher than 100 μM, it is likely that there were sufficient levels of BCAAs to allow for synthesis of collagen. Furthermore, evaluation of collagen fiber macrostructure through second harmonic generation imaging within the infarct region revealed no differences in attributes such as collagen fiber alignment, straightness, and width. We hypothesize that there is a threshold level of BCAAs, not reached by the low BCAA diet in this study, at which they may become limiting for synthesis of secreted extracellular matrix proteins.

Concusions

In summary, we show that sustained dietary modification of BCAA consumption can alter cardiac remodeling following MI. Specifically, our results indicate that a low BCAA diet mitigates the progressive decline in cardiac function observed with high BCAA diet feeding. This observation may be partially attributed to changes in LV mass and eccentric cardiomyocyte hypertrophy, which is more prominent in male mice. Although the absence of a clear mechanism is a limitation of this study, the discernment of a distinct, singular mechanism is unlikely given the multifaceted actions of BCAAs on anabolic signaling,[44] posttranslational modifications,[17] insulin sensitivity,[7] and blood pressure.[42] Furthermore, it could be conceived that a normal chow group should be included; however, the custom low and high BCAA diets were matched for total protein and nitrogen content as well as carbohydrate and fat content, and the normal chow diet was drastically different, obfuscating comparisons. Moreover, the normal chow diet and the high BCAA diet led to nearly identical levels of circulating BCAAs, which allowed us to address simply the question of whether or not BCAA levels are modifiers of post-MI remodeling. We conclude that dietary BCAAs influence cardiac remodeling post-MI and that a diet low in BCAAs prevents worsening of pathological remodeling after MI. These findings provide an exciting opportunity to further explore how dietary modulation of BCAA levels may be used to improve cardiac function and remodeling after MI.

ARTICLE INFORMATION

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Disclosures

None. Artificial intelligence was not used in this work.

Supplemental Material

Tables S1-S10
Figures S1-S4
ARRIVE Checklist

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