Branched-chain amino acids-induced cardiac protection against ischemia/reperfusion injury

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Declaration of Interest statement

The authors declare that there are no conflicts of interest.

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ABSTRACT

Aims: Amino acids, especially branched chain amino acids (BCAAs), have important regulatory roles in protein synthesis. Recently studies revealed that BCAAs protect against ischemia/reperfusion (I/R) injury. We studied the signaling pathway and mitochondrial function affecting a cardiac preconditioning of BCAAs.

Main methods: An *in vivo* model of I/R injury was tested in control, $mTOR^{+/+}$, and $mTOR^{+/-}$. Mice were randomly assigned to receive BCAAs, rapamycin, or BCAAs + rapamycin. Furthermore, isolated cardiomyocytes were subjected to simulated ischemia and cell death was quantified. Biochemical and mitochondrial swelling assays were also performed.

Key findings: Mice treated with BCAAs had a significant reduction in infarct size as a percentage of the area at risk compared to controls (34.1 ± 3.9% vs. 44.7 ± 2.6%, P = 0.001), whereas mice treated with the mTOR inhibitor rapamycin were not protected by BCAA administration (42.2 ± 6.5%, vs. control, P = 0.015). This protection was not detected in our hetero knockout mice of mTOR. Western blot analysis revealed no change in AKT signaling whereas activation of mTOR was identified. Furthermore, BCAAs prevented swelling which was reversed by the addition of rapamycin. In myocytes undergoing simulated I/R, BCAA treatment significantly preserved cell viability (71.7 ± 2.7% vs. 34.5 ± 1.6%, respectively, $p < 0.0001$), whereas rapamycin prevented this

BCAA-induced cardioprotective effect (43.5 ± 3.4% vs. BCAA, $p < 0.0001$).

Significance: BCAA treatment exhibits a protective effect in myocardial I/R injury and that mTOR plays an important role in this preconditioning effect.

Keywords

Amino acid, Ischemia, Reperfusion, mTOR, Mitochondria

1. Introduction

Ischemia/reperfusion (I/R) injury in the myocardium significantly affects morbidity and mortality. Various preconditioning methods have been discovered that prevent cardiac I/R injury. Murry et al. first reported that brief ischemic episodes provide cardioprotective effects against subsequent ischemic injury [1]. In addition to ischemia, several pharmacologic agents such as volatile anesthetics, opioids, and organic nitrate esters provide myocardial preconditioning effects [2-6]. Signal transduction pathways involved in cardiac preconditioning are believed to include the connection of G proteins and several mediators including adenosine. This causes the activation of protein kinase C via activation of phospholipase C and phospholipase D and initiates a downstream signaling cascade involving the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, release of reactive oxygen species, and activation of endothelial and inducible nitric oxide synthase. It also inhibits the opening of the mitochondrial permeability transition pore (mPTP) or the activation of mitochondrial ATP-sensitive potassium channels [7].

Recent advances in our understanding of the translation mechanism and its control have facilitated studies at the molecular level into the regulation of protein synthesis by nutrients. Amino acids, which belong to one class of nutrients [8], have important regulatory roles in protein synthesis. Of all amino acids, the branched-chain amino acids (BCAAs), a group of essential amino

65 acids comprised of valine, leucine, and isoleucine, have a unique role in this process [9]. Previous
66 studies in rats demonstrated that BCAAs have protective effects against I/R injury in various
67 organs, including the kidney and the liver [10, 11]. However, the effects of BCAAs in the ischemic
68 myocardium are still unclear. In this study, we examined the signaling pathways and mitochondrial
69 functions related to cardioprotective effects of BCAAs in cardiac I/R injury.

2. Material and methods

2.1. Animals

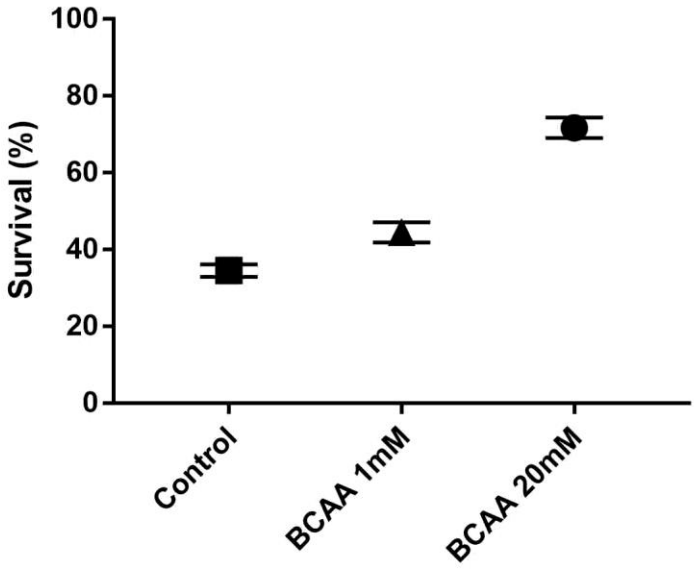
All animals were treated in compliance with the Guidelines for Proper Conduct of Animal Experiments and Related Activities and the Guideline for Care and Use of Lab Animals at Tokushima University (Tokushima, Japan). Animal use protocols were approved by the Animal Care and Use Committee, Tokushima University (Tokushima, Japan). Male C57BL/6 mice (21-26 g) and Wistar rats (250-300 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan), and $mTOR^{+/-}$ mice were created as reported previously [12]. The animals were kept on a 12-hour light-dark cycle in a temperature-controlled room and randomly assigned to treatment groups by an independent observer.

2.2. Antibodies and BCAAs

The following primary antibodies were used in this study in a 1:1000 dilution: polyclonal antibodies to Akt, phospho-Akt (Ser473), GSK3β, phospho-GSK3β (Ser9), mTOR, phospho-mTOR (Ser2448), Cell Signaling Technology (Danvers, MA); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Santa Cruz Biotechnology (Dallas, TX). BCAAs were purchased from

Sigma Aldrich (St Louis, MO). Cell survival was investigated at 1mM and 20mM doses to identify optimal dosing (Supplementary Figure 1).

Supplementary Figure 1



Supplementary Figure 1.

Cell survival was investigated at 1mM and 20mM doses to identify optimal dosing

2.3. Genotyping of mTOR kinase domain knockout mice by polymerase chain reaction (PCR)

Mouse genomic DNA was extracted from tail tips. The concentration of cDNA was determined and adjusted for real-time PCR analysis, which was performed on an MJ Research Opticon 2 (Bio-Rad, Hercules, CA) in triplicate with the iQ SYBR Green Supermix (Bio-Rad). A sense primer, finTOR-k-tailu 6671 (5'-GCG GCA GGA TGA ACG AGT GAT GC-3'), was designed from exon 47 to amplify both the wild-type and targeted loci. An antisense primer, βgeo-screening 1 (5'-AAT GGG CTG ACC GCT TCC TCG TGC TT-3'), was designed from the βgeo cassette to amplify the targeted locus. Another antisense primer, TOR-kin-tail-L 20636 (5'-GTG ATC CGC CTG CCT CTG CCT CCT GT-3'), was designed from intron 47 to amplify the wild-type locus. Amplification with these three primers produced an 803-bp band from the wild-type locus and a 468-bp band from the targeted locus.

2.4. In vivo ischemia/reperfusion experiments

Surgery was performed as previously described [4]. Briefly, mice were anesthetized with pentobarbital sodium (80 mg/kg ip) were mechanically ventilated with oxygen. Cardiac catheterization via the right carotid artery was performed with a Microtip pressure transducer (Millar Instruments Inc., Houston, TX) to examine hemodynamical change, and ischemia was

produced by occluding the coronary artery. After 30 min of occlusion, the ligature was released, and the heart was reperfused for 2 h [13]. Mice were randomly assigned to receive either a BCAA cocktail in saline (0.14 g/kg iv) or vehicle 30 minutes before the ischemic injury. Some mice were treated with rapamycin (mTOR inhibitor; 5.0 mg/kg iv) 45 min before the ischemia.

After reperfusion, the coronary artery was again occluded, and the area at risk (AAR) was determined by staining with 1% Evans blue. The heart was immediately excised and cut into 1-mm slices. The left ventricle was counterstained with 1% 2,3,5-triphenyltetrazolium chloride. After overnight storage in 10% formaldehyde, slices were weighed and visualized under a microscope equipped with a digital camera (D90, Nikon Imaging, Japan). The images were analyzed, and the area at risk and the infarct size were determined by planimetry as previously described [14].

2.5. Serum cardiac troponins

Cardiac troponin I levels in the serum were measured using a High Sensitivity Mouse Cardiac Troponin-I ELISA Kit (Life Diagnostics, West Chester, PA) as described before [15].

2.6. Mitochondrial isolation and swelling assay

C57Bl/6 mice were injected with vehicle, BCAAs, and with rapamycin. Hearts were then

129 harvested after various treatments and I/R experiment. Hearts containing 4 mL sucrose buffer A
130 (300 mM sucrose, 10 mM Tris-HCl, 2 mM EGTA and 5 mg/mL bovine serum albumin, pH 7.4)
131 were homogenized, and the homogenate was centrifuged at 2000×*g* for 2 min at 4°C to remove cell
132 debris. The supernatant was further centrifuged at 10 000×*g* for 30 min at 4°C to sediment impure
133 mitochondria. The mitochondrial pellet was purified and washed as described previously [16]. 200
134 μL of mitochondria in sucrose buffer B (300 mM sucrose, 10 mM Tris-HCl, pH 7.4) was loaded in
135 to a 96-well plate and challenged with 100 μM $CaCl_2$ (2 mg/mL protein concentration). The
136 absorbance was measured 600 times every 2 s at 520 nm using a VarioSkan Flash
137 spectrophotometer (Thermo Scientific, Japan). In some experiments, mitochondria were pretreated
138 with 250 nM cyclosporine A to inhibit $CaCl_2$-induced mitochondrial swelling to confirm the mPTP
139 dependence of the calcium-induced swelling [17, 18].
140
141 *2.7. Isolation and treatment of adult rat cardiac myocytes*
142 Cardiac myocytes were isolated by cardiac retrograde aortic perfusion and collagenase
143 treatment as described previously [19]. Cardiac myocytes were plated on laminin-coated 12-well
144 plates, allowed to incubate for 24 h, and then subjected to various experimental conditions at 37 °C.
145 Culture medium was changed to amino acid-free Dulbecco's modified Eagle's medium (DMEM) 6

hours prior to experimentation to washout any residual amino acids found in the maintenance medium. Simulated ischemia was induced in metabolic chamber by replacing the air with a 95% N_2 and 5% CO_2 gas mixture at 2 L/min and the media with glucose-free media (glucose-free DMEM, Invitrogen) for 60 min. This was followed by 60 min of simulated reperfusion by replacing the media with amino acid-free DMEM and incubating the cells with 21% O_2 and 5% CO_2. Before the simulated ischemia/reperfusion (SI/R), cardiac myocytes were exposed with or without rapamycin (20 nM). This was followed by exposure to media with or without BCAA dissolved in PBS (2 mM) for 30 min prior to SI/R. Cell death was quantified by counting trypan blue-stained cells with the results expressed as a percentage of total survival [20].

2.8. Immunoblots

Lysates were separated by SDS-PAGE on 10% polyacrylamide precast gels (Invitrogen) and transferred to polyvinylidene difluoride membranes by electroelution. Membranes were blocked in 20 mM TBS-Tween (1%) containing 5% skim milk and incubated with primary antibodies overnight at 4°C. Immunolabeled blots were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX) in a 1:2000 dilution and visualized by enhanced chemiluminescence reagent (GE Healthcare, Waukesha, WI) [21, 22].

163 *2.9. Statistics*

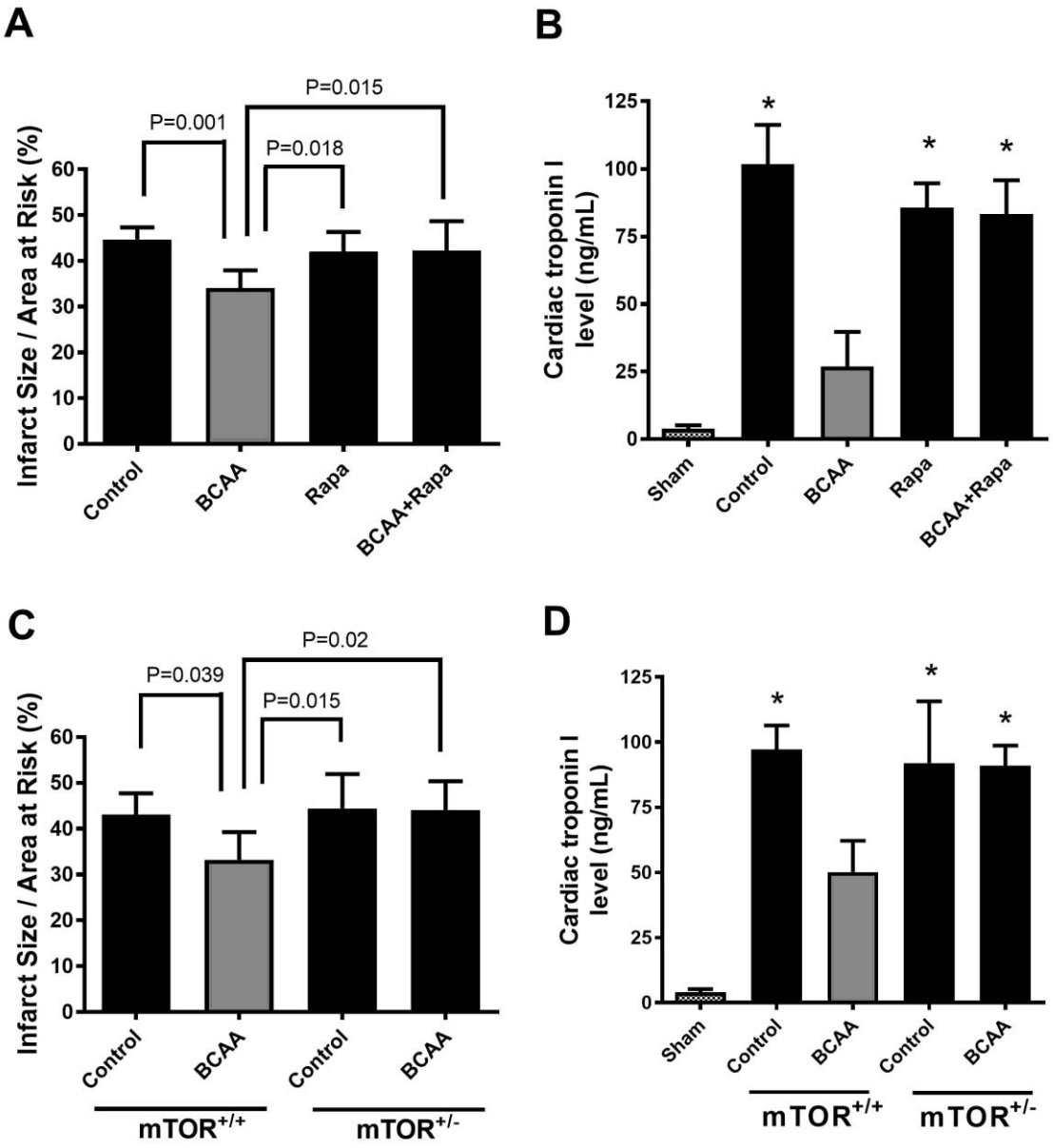
164 All results were analyzed by observers blinded to the experimental conditions. Data are
165 presented as the means ± standard deviation. Differences between treatment groups were tested for
166 statistical significance by one-way analysis of variance followed by Bonferroni's post hoc test. A
167 difference was considered significant if the probability value was <0.05.

3. Results

3.1. Involvement of mTOR in BCAA-induced cardiac protection

Among the treatment group, there were no differences in the baseline hemodynamics (heart rate, arterial blood pressure, or rate pressure product) before the occlusion (data not shown). No differences were observed in the area at risk as a percentage of the left ventricular area between the groups (data not shown). Mice treated with BCAAs had a significant reduction in infarct size as a percentage of the area at risk compared to controls (34.1 ± 3.9% vs. 44.7 ± 2.6%, n = 7/group, P = 0.001). Pretreatment with the mTOR inhibitor rapamycin prevented in mice this protection by BCAAs (42.2 ± 6.5%, n = 7, P = 0.015 vs. control, Fig. 1A and Supplementary Figure 2). We confirmed these effects by measuring serum troponin I levels, a marker of cardiac myocyte damage (Fig. 1B). Additionally, the protection produced by BCAA treatment was also eliminated in $mTOR^{+/-}$ mice (44.1 ± 6.3%, n = 7, Fig. 1C and D). These results strongly suggest that the cardioprotective effects of BCAA depend on intact mTOR signaling. Of note, troponin I levels in sham mice with BCAAs and with and without rapamycin showed no significant differences (Data not shown).

Figure 1

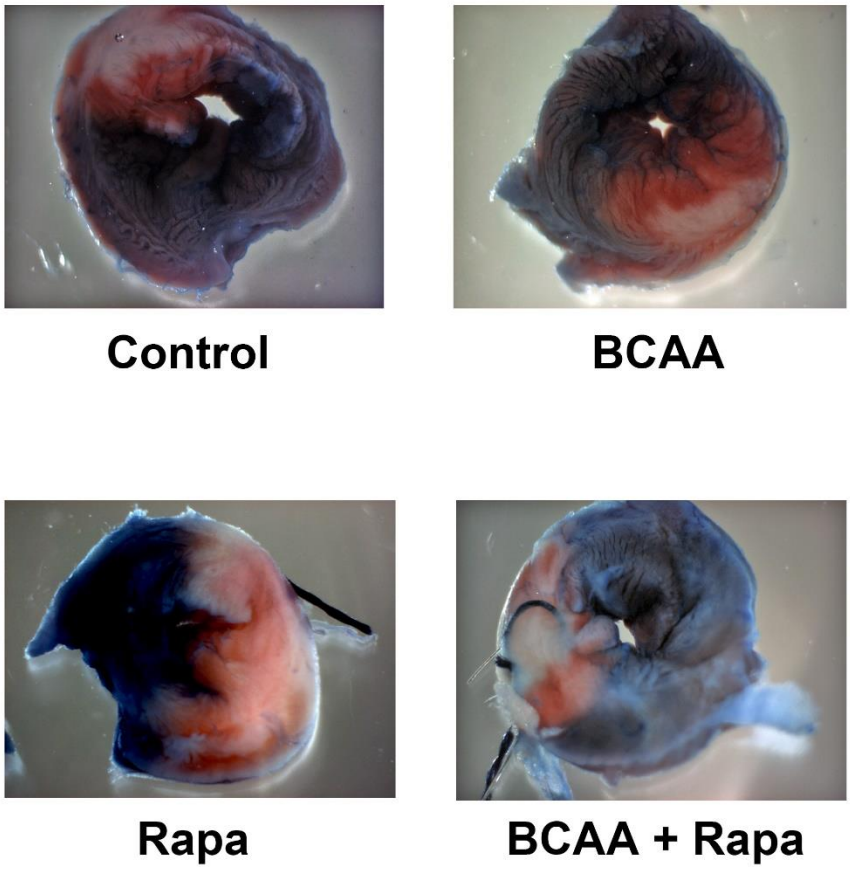

A
P=0.001
P=0.015
P=0.018
Infarct Size / Area at Risk (%)
0
10
20
30
40
50
60
Control
BCAA
Rapa
BCAA+Rapa
B
Cardiac troponin I level (ng/mL)
0
25
50
75
100
125
*
*
*
Sham
Control
BCAA
Rapa
BCAA+Rapa
C
P=0.039
P=0.02
P=0.015
Infarct Size / Area at Risk (%)
0
10
20
30
40
50
60
Control
BCAA
Control
BCAA
mTOR+/+
mTOR+/-
D
Cardiac troponin I level (ng/mL)
0
25
50
75
100
125
*
*
*
Sham
Control
BCAA
Control
BCAA
mTOR+/+
mTOR+/-

183

Figure 1. Branched-chain amino acids (BCAAs) protects the mouse myocardium from ischemic injury.

Branched chain amino acids (BCAAs) protects the mouse myocardium from ischemic injury. (A) Infarct size (IS) expressed as a percentage of area at risk (AAR). The IS was reduced by BCAA treatment; however, additional rapamycin pretreatment abolished the BCAA-induced protection in mice. (B) Cardiac troponin I, a serum marker of myocardial damage, was significantly decreased in BCAA-treated mice, but this cardioprotective effect was eliminated by rapamycin. (C) The IS was reduced in BCAA-treated $mTOR^{+/+}$, but not in $mTOR^{+/-}$ mice. (D) BCAAs induced a decrease in cardiac troponin I in $mTOR^{+/+}$ mice whereas no effect was observed in $mTOR^{+/-}$ mice. * represents $P < 0.05$.

Supplementary Figure 2



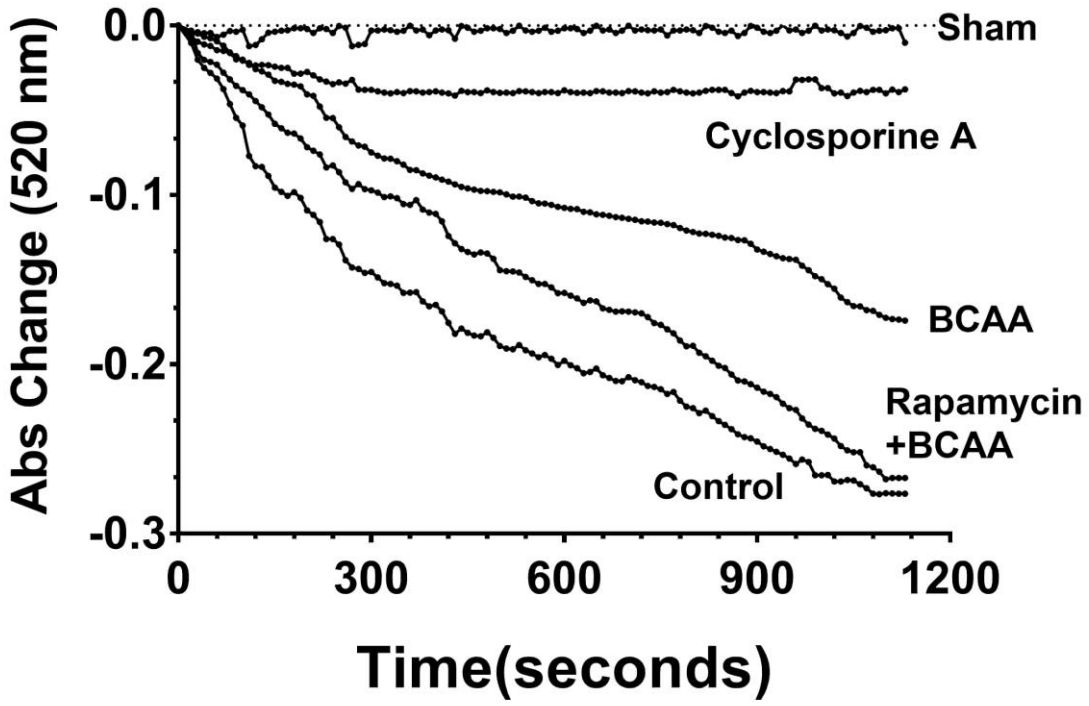
Supplementary Figure 2.

Representative photos of infarct size with BCAA, Rapa, or BCAA+Rapa. White – infarct size, Blue – intact tissue, Red – Area at risk (AAR).

3.2. Mitochondrial permeability transition pore

The effects of BCAA on Ca^{2+}-induced swelling in isolated mouse heart mitochondria are shown in Fig. 2. The addition of 100 μM Ca^{2+} caused a significant decrease in absorbance, indicating mitochondrial swelling. The Ca^{2+}-induced swelling was inhibited by cyclosporine A, an mPTP inhibitor. Under these conditions, BCAA significantly attenuated the Ca^{2+}-induced swelling compared with the control. Rapamycin was effective in inhibiting BCAA induced protection. These experiments were repeated with similar results three times.

Figure 2



208

209 **Figure 2. Sensitivity to mitochondrial permeability transition pore formation according to**

210 **Ca^{2+}-induced mitochondrial swelling.**

211 Branched-chain amino acids (BCAAs) inhibited mitochondrial swelling caused by ischemia/

212 reperfusion injury. BCAA-treated mitochondria (BCAA) presented substantially less swelling

213 compared to untreated (Control) and rapamycin-treated (Rapamycin + BCAA) mitochondria when

214 exposed to calcium chloride. Cyclosporine A was used as a control experiment to inhibit $CaCl_2$-

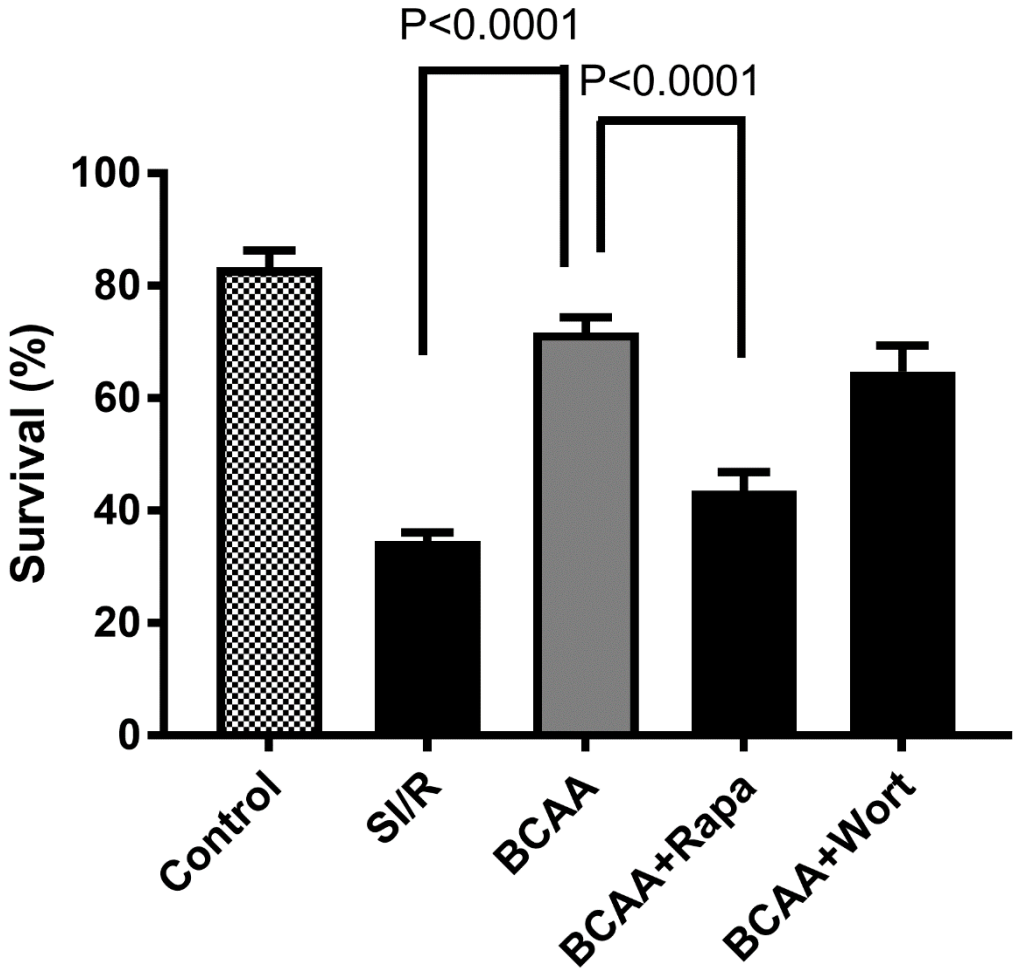
215 induced mitochondrial swelling, confirming the dependence of the calcium-induced swelling on the

216 activity of the mitochondrial permeability transition pore.

3.3. BCAA improves the cell survival after simulated ischemia/reperfusion

To more accurately assess myocyte survival under controlled experimental conditions, we next examined the cardioprotective effects of BCAA in isolated rat cardiac myocytes in response to SI/R (Fig. 3). Adult cardiac myocytes under control conditions exhibited no substantial signs of cell death. In myocytes undergoing SI/R, cells pretreated with BCAA significantly retained viability compared to cells without pretreatment (71.7 ± 2.7% vs. 34.5 ± 1.6%, respectively, $P < 0.0001$), whereas the addition of rapamycin to the pretreatment prevented this BCAA-induced cardioprotective effect (43.5 ± 3.4% vs. BCAA, $P < 0.0001$).

Figure 3


P<0.0001
P<0.0001
Survival (%)
100
80
60
40
20
0
Control
SI/R
BCAA
BCAA+Rapa
BCAA+Wort

226

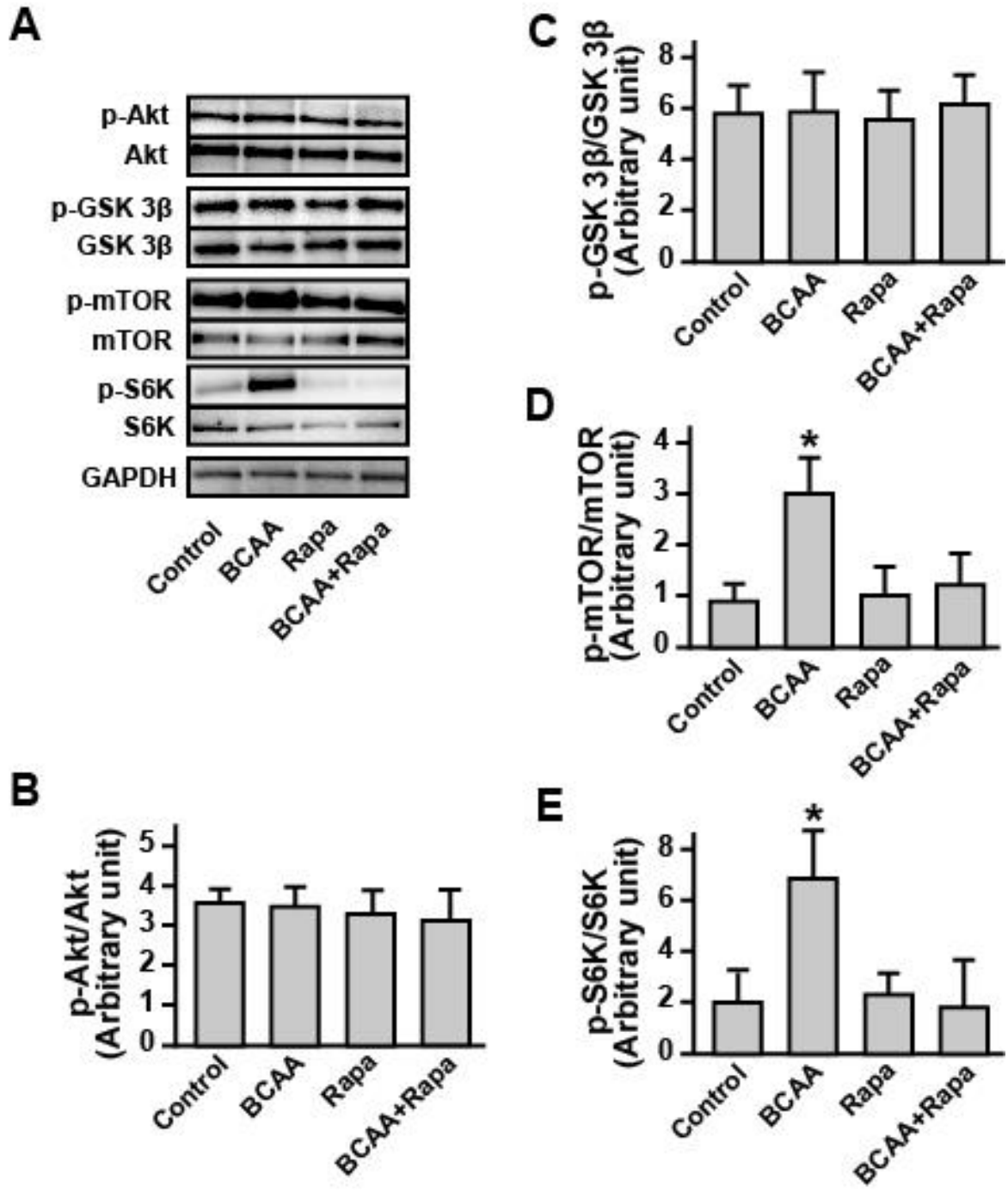
Figure 3. The survival rate of adult cardiac myocytes exposed to simulated ischemia/reperfusion.

Branched-chain amino acids (BCAAs) improve the survival rate of adult cardiac myocytes exposed to simulated ischemia/reperfusion, but rapamycin inhibited this preventive effect. Wortmannin, a phosphatidylinositol-3-kinase (PI3K) inhibitor, does not affect the cardiac protection induced by BCAAs.

3.4. Signaling pathways involved in BCAA-induced cardiac protection

To investigate the mechanism for cardiac protection induced by BCAA, we examined the effect of BCAA on the phosphorylation of the cytoprotective kinase Akt and its downstream substrate GSK3β as well as on the phosphorylation of mTOR (Fig. 4). BCAA treatment caused neither Akt nor GSK3β phosphorylation. By contrast, mTOR was phosphorylated after BCAA pretreatment but not after pretreatment with BCAA in the presence of rapamycin. Thus, the cytoprotective effects of BCAA likely depend on mTOR activity but not on Akt/GSK3β signaling. Additionally, following I/R injury we noted no changes in mTOR expression similar to previous reports (Data not shown) [23].

Figure 4


A
p-Akt
Akt
p-GSK 3β
GSK 3β
p-mTOR
mTOR
p-S6K
S6K
GAPDH
Control
BCAA
Rapa
BCAA+Rapa
B
p-Akt/Akt (Arbitrary unit)
C
p-GSK 3β/GSK 3β (Arbitrary unit)
D
p-mTOR/mTOR (Arbitrary unit)
E
p-S6K/S6K (Arbitrary unit)
*

242

Figure 4. Immunoblots for Akt, phospho-Akt, GSK3β, phospho-GSK3β, mTOR, phospho-mTOR, phospho-S6K and S6K.

Immunoblots for Akt, phospho-Akt, GSK3β, phospho-GSK3β, mTOR, and phospho-mTOR, pS6K and S6K. Branched-chain amino acids (BCAAs) significantly increased phosphorylation of mTOR and pS6K without altering the phosphorylation of Akt or GSK3β proteins expression in lysed hearts. Pretreatment with rapamycin blocked BCAA-mediated activation of mTOR. Values are expressed as mean ± standard deviation. * represents $P < 0.05$ vs. control.

4. Discussion

In the current study, BCAAs significantly decreased the infarct size, whereas the mTOR inhibitor rapamycin prevented this protective effect using in vivo mouse model of regional myocardial ischemia and reperfusion. BCAA treatment also preserved cell viability after simulated I/R in cardiac myocytes. However, the PI3K inhibitor wortmannin did not interfere with the cardioprotective effect induced by BCAAs. Moreover, the immunoblot analysis demonstrated that BCAA led to mTOR phosphorylation, which was prevented by the addition or rapamycin. However, phosphorylation of Akt or GSK3β was not observed after BCAA pretreatment. These results suggest that mTOR signaling but not PI3K/Akt/GSK3β pathways may act as a key effector of myocardial protection by BCAA.

mTOR is a serine/threonine kinase in the PI3K-related kinase family that plays a vital role in cell growth, survival, and metabolism. mTOR and its downstream signaling networks regulate autophagy, protein synthesis, cell polarity, and cytoskeletal organization [24]. mTOR complex 1 (mTORC1) and 2 are known as the catalytic subunits of two distinct protein complexes. mTORC1 is defined by its three core components: mTOR, regulatory protein associated with mTOR (raptor), and mammalian lethal with Sec13 protein [25-27].

Over the last few years, studies have shown that growth factors modulate mTORC1

activity through the phosphorylation of insulin receptor substrate 1 and the stimulation of PI3K, which in turn leads to the activation of Akt [28]. Amino acids activate mTORC1 by recruitment to the surface of lysosome, which is caused by Regulator-Rag complex combining to raptor [29]. Leucine, one of the three branched chain amino acids, is supposed to relate to the regulation of mTORC1 through cytosolic sensors such as leucyl-tRNA synthetase and Sestrin 2 [30]. Previous studies indicate that amino acids induced cytoprotective effects by reducing the inflammatory response [11]. BCAAs respond to several cells signaling pathways mainly through the activation of the mTOR axis and mTOR relates to myocardial I/R injury through multiple signaling pathways such as AMP-activated protein kinase (AMPK)/mTOR or PI3K/Akt/mTOR pathway associated with autophagy [31, 32]. In this study, there is no significant difference of infarct size as a percentage of the area at risk or cTn1 in control of both $mTOR^{+/+}$ and $mTOR^{+/-}$ mice. This may result from some other signaling pathways known to show the protective effect on I/R injury in the heart. As one of the important intracellular signaling pathways of cardiac preconditioning, PI3K and its downstream target Akt, are also involved in the regulation of oxidation, inflammatory responses, and apoptosis. The PI3K/Akt/GSK3β-dependent signaling pathways have been demonstrated to result in the attenuation of myocardial I/R injury [33-37]. On the other hand, the present study suggests that mTOR signaling pathway, not PI3K/Akt/GSK3β-dependent signaling pathways may

be important in the cardioprotective effects of BCAA treatment. To identify the mechanisms involved in this protection in detail, further studies are needed.

In the current study, we also evaluated the effects of BCAAs on the improvement of mitochondrial functions. Cyclosporine A, an mPTP inhibitor, inhibited Ca^{2+}-induced swelling. The Ca^{2+}-induced swelling of mouse heart mitochondria was also abolished by BCAA. This result suggests that the opening of the mPTP was decreased by BCAA treatment, resulting in the prevention of mitochondrial-mediated cell death. In addition, our data demonstrated that rapamycin effectively attenuated this preventive effect.

Mitochondria play a central role in molecular events, leading to tissue damage after pathological stimulation such as ischemia [38, 39]. mTOR is known to control mitochondrial dynamics [40]. mTOR binds and regulates the voltage-dependent anion channel proteins [41], which are an important component of the mPTP in the outer mitochondrial membrane. Several studies showed inhibition of mTOR activity provoked a decrease in mPTP permeability [42]. mTOR activation caused by BCAAs may preserve mitochondrial-mediated cell death triggered by unknown signaling pathways that are related to Ca^{2+}-induced swelling in cardiac I/R injury. The mPTP is a large-conductance mega-channel found at the contact sites between the inner and outer mitochondrial membranes [39]. The long-term opening of this channel dissipates the inner

301 mitochondrial membrane potential, results in matrix swelling, rupture of the outer mitochondrial
302 membrane, and the release of cytochrome C from the intermembrane space into the cytosol where it
303 activates proteolytic processes and initiates cellular disintegration. Inner membrane depolarization,
304 high concentrations of inorganic phosphate, ROS, and reactive nitrogen species are all present
305 during myocardial ischemia and more importantly during reperfusion and facilitate mPTP opening
306 [43, 44]. In contrast to permanent mPTP opening, a transient channel activity may serve a
307 physiological function in ROS homeostasis and calcium release, and transient mPTP opening is
308 indeed cardioprotective during ischemic preconditioning [39].

5. Conclusions

We show that BCAA treatment reduces cardiac I/R injury and that mTOR activity plays a significant role in this preconditioning effect by BCAAs, which is separate from and acts in parallel to PI3K/Akt activation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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