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Title	Branched-chain amino acids-induced cardiac protection against ischemia/reperfusion injury
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Citation	Life Sciences , 245 : 117368
Issue Date	2020-03-15
DOI	<a href="https://doi.org/10.1016/j.lfs.2020.117368">10.1016/j.lfs.2020.117368</a>
Self DOI	
URL	<a href="https://ir.lib.hiroshima-u.ac.jp/00050462">https://ir.lib.hiroshima-u.ac.jp/00050462</a>
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Relation	



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

2

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16

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18 **Acknowledgements**

19 This work was supported by JSPS KAKENHI [grant numbers 19K09353].

20

21 **Declaration of Interest statement**

22 The authors declare that there are no conflicts of interest.

23

24 **Word count** 2,602 words

25 **Figure / table count** 4 figures / 2 supplementary figures / no tables

26 **ABSTRACT**

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

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

35 Key findings: Mice treated with BCAAs had a significant reduction in infarct size as a percentage  
36 of the area at risk compared to controls ( $34.1 \pm 3.9\%$  vs.  $44.7 \pm 2.6\%$ ,  $P = 0.001$ ), whereas mice  
37 treated with the mTOR inhibitor rapamycin were not protected by BCAA administration ( $42.2 \pm$   
38  $6.5\%$ , vs. control,  $P = 0.015$ ). This protection was not detected in our hetero knockout mice of  
39 mTOR. Western blot analysis revealed no change in AKT signaling whereas activation of mTOR  
40 was identified. Furthermore, BCAAs prevented swelling which was reversed by the addition of  
41 rapamycin. In myocytes undergoing simulated I/R, BCAA treatment significantly preserved cell  
42 viability ( $71.7 \pm 2.7\%$  vs.  $34.5 \pm 1.6\%$ , respectively,  $p < 0.0001$ ), whereas rapamycin prevented this

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

44 Significance: BCAA treatment exhibits a protective effect in myocardial I/R injury and that mTOR

45 plays an important role in this preconditioning effect.

46 **Keywords**

47 Amino acid, Ischemia, Reperfusion, mTOR, Mitochondria

48 **1. Introduction**

49 Ischemia/reperfusion (I/R) injury in the myocardium significantly affects morbidity and  
50 mortality. Various preconditioning methods have been discovered that prevent cardiac I/R injury.  
51 Murry et al. first reported that brief ischemic episodes provide cardioprotective effects against  
52 subsequent ischemic injury [1]. In addition to ischemia, several pharmacologic agents such as  
53 volatile anesthetics, opioids, and organic nitrate esters provide myocardial preconditioning effects  
54 [2-6]. Signal transduction pathways involved in cardiac preconditioning are believed to include the  
55 connection of G proteins and several mediators including adenosine. This causes the activation of  
56 protein kinase C via activation of phospholipase C and phospholipase D and initiates a downstream  
57 signaling cascade involving the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, release of  
58 reactive oxygen species, and activation of endothelial and inducible nitric oxide synthase. It also  
59 inhibits the opening of the mitochondrial permeability transition pore (mPTP) or the activation of  
60 mitochondrial ATP-sensitive potassium channels [7].

61 Recent advances in our understanding of the translation mechanism and its control have  
62 facilitated studies at the molecular level into the regulation of protein synthesis by nutrients. Amino  
63 acids, which belong to one class of nutrients [8], have important regulatory roles in protein  
64 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.

70 **2. Material and methods**

71

72 *2.1. Animals*

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

81

82 *2.2. Antibodies and BCAAs*

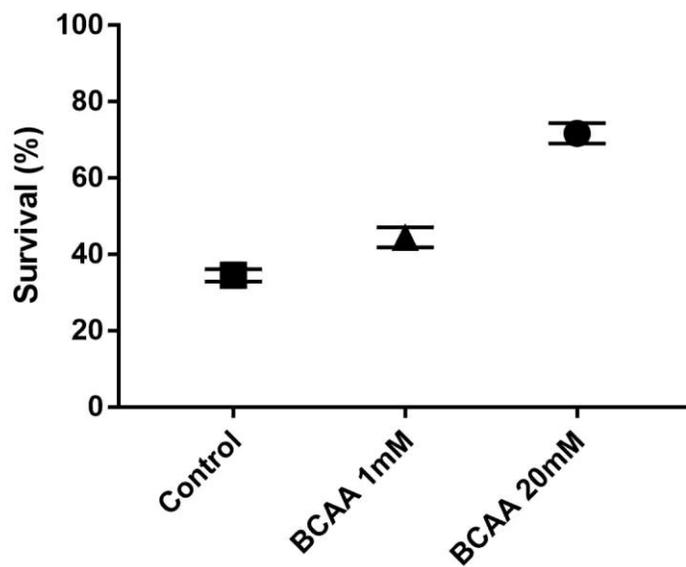
83 The following primary antibodies were used in this study in a 1:1000 dilution: polyclonal  
84 antibodies to Akt, phospho-Akt (Ser473), GSK3 $\beta$ , phospho-GSK3 $\beta$  (Ser9), mTOR, phospho-mTOR  
85 (Ser2448), Cell Signaling Technology (Danvers, MA); and glyceraldehyde 3-phosphate  
86 dehydrogenase (GAPDH), Santa Cruz Biotechnology (Dallas, TX). BCAAs were purchased from

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

89

90

Supplementary Figure 1



91

92

93 **Supplementary Figure 1.**

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

95 *2.3. Genotyping of mTOR kinase domain knockout mice by polymerase chain reaction (PCR)*

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

106

107 *2.4. In vivo ischemia/reperfusion experiments*

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

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

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

122

### 123 *2.5. Serum cardiac troponins*

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

126

### 127 *2.6. Mitochondrial isolation and swelling assay*

128           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<sub>2</sub> (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<sub>2</sub>-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

146 hours prior to experimentation to washout any residual amino acids found in the maintenance  
147 medium. Simulated ischemia was induced in metabolic chamber by replacing the air with a 95% N<sub>2</sub>  
148 and 5% CO<sub>2</sub> gas mixture at 2 L/min and the media with glucose-free media (glucose-free DMEM,  
149 Invitrogen) for 60 min. This was followed by 60 min of simulated reperfusion by replacing the  
150 media with amino acid-free DMEM and incubating the cells with 21% O<sub>2</sub> and 5% CO<sub>2</sub>. Before the  
151 simulated ischemia/reperfusion (SI/R), cardiac myocytes were exposed with or without rapamycin  
152 (20 nM). This was followed by exposure to media with or without BCAA dissolved in PBS (2 mM)  
153 for 30 min prior to SI/R. Cell death was quantified by counting trypan blue-stained cells with the  
154 results expressed as a percentage of total survival [20].

155

## 156 *2.8. Immunoblots*

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

163 2.9. *Statistics*

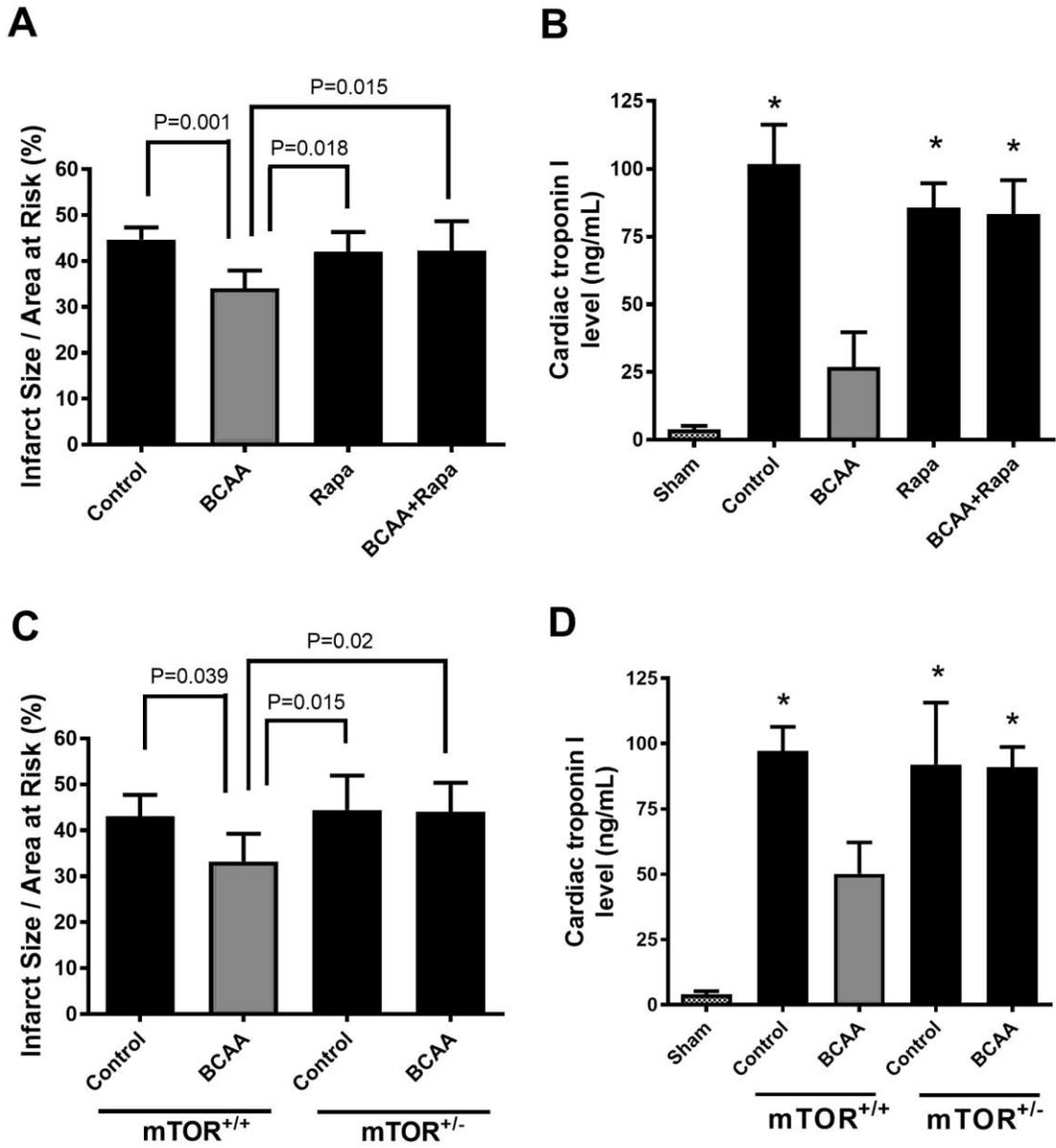
164 All results were analyzed by observers blinded to the experimental conditions. Data are  
165 presented as the means  $\pm$  standard deviation. Differences between treatment groups were tested for  
166 statistical significance by one-way analysis of variance followed by Bonferroni's post hoc test.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

168 **3. Results**

169 *3.1. Involvement of mTOR in BCAA-induced cardiac protection*

170           Among the treatment group, there were no differences in the baseline hemodynamics  
171 (heart rate, arterial blood pressure, or rate pressure product) before the occlusion (data not shown).  
172 No differences were observed in the area at risk as a percentage of the left ventricular area between  
173 the groups (data not shown). Mice treated with BCAAs had a significant reduction in infarct size as  
174 a percentage of the area at risk compared to controls ( $34.1 \pm 3.9\%$  vs.  $44.7 \pm 2.6\%$ ,  $n = 7/\text{group}$ ,  $P =$   
175  $0.001$ ). Pretreatment with the mTOR inhibitor rapamycin prevented in mice this protection by  
176 BCAAs ( $42.2 \pm 6.5\%$ ,  $n = 7$ ,  $P = 0.015$  vs. control, Fig. 1A and Supplementary Figure 2). We  
177 confirmed these effects by measuring serum troponin I levels, a marker of cardiac myocyte damage  
178 (Fig. 1B). Additionally, the protection produced by BCAA treatment was also eliminated in  
179 mTOR<sup>+/-</sup> mice ( $44.1 \pm 6.3\%$ ,  $n = 7$ , Fig. 1C and D). These results strongly suggest that the  
180 cardioprotective effects of BCAA depend on intact mTOR signaling. Of note, troponin I levels in  
181 sham mice with BCAAs and with and without rapamycin showed no significant differences (Data  
182 not shown).

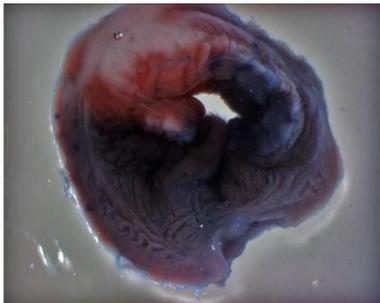
**Figure 1**



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

186 Branched chain amino acids (BCAAs) protects the mouse myocardium from ischemic injury. (A)  
187 Infarct size (IS) expressed as a percentage of area at risk (AAR). The IS was reduced by BCAA  
188 treatment; however, additional rapamycin pretreatment abolished the BCAA-induced protection in  
189 mice. (B) Cardiac troponin I, a serum marker of myocardial damage, was significantly decreased in  
190 BCAA-treated mice, but this cardioprotective effect was eliminated by rapamycin. (C) The IS was  
191 reduced in BCAA-treated mTOR<sup>+/+</sup>, but not in mTOR<sup>+/-</sup> mice. (D) BCAAs induced a decrease in  
192 cardiac troponin I in mTOR<sup>+/+</sup> mice whereas no effect was observed in mTOR<sup>+/-</sup> mice. \* represents P  
193 < 0.05.

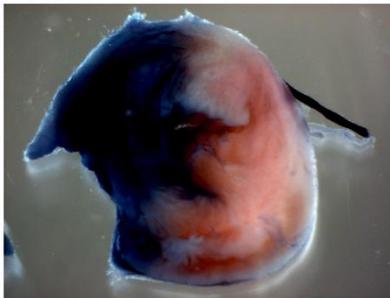
**Supplementary Figure 2**



**Control**



**BCAA**



**Rapa**



**BCAA + Rapa**

195 **Supplementary Figure 2.**

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

198

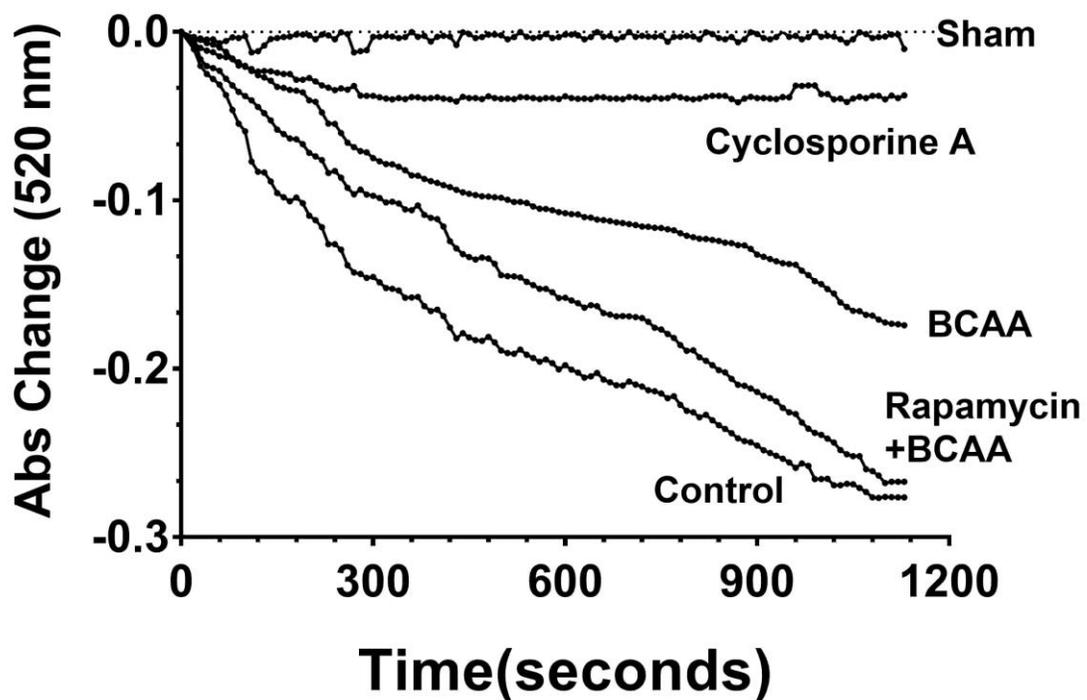
199

200

201 *3.2. Mitochondrial permeability transition pore*

202 The effects of BCAA on Ca<sup>2+</sup>-induced swelling in isolated mouse heart mitochondria are  
203 shown in Fig. 2. The addition of 100 μM Ca<sup>2+</sup> caused a significant decrease in absorbance,  
204 indicating mitochondrial swelling. The Ca<sup>2+</sup>-induced swelling was inhibited by cyclosporine A, an  
205 mPTP inhibitor. Under these conditions, BCAA significantly attenuated the Ca<sup>2+</sup>-induced swelling  
206 compared with the control. Rapamycin was effective in inhibiting BCAA induced protection. These  
207 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<sup>2+</sup>-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

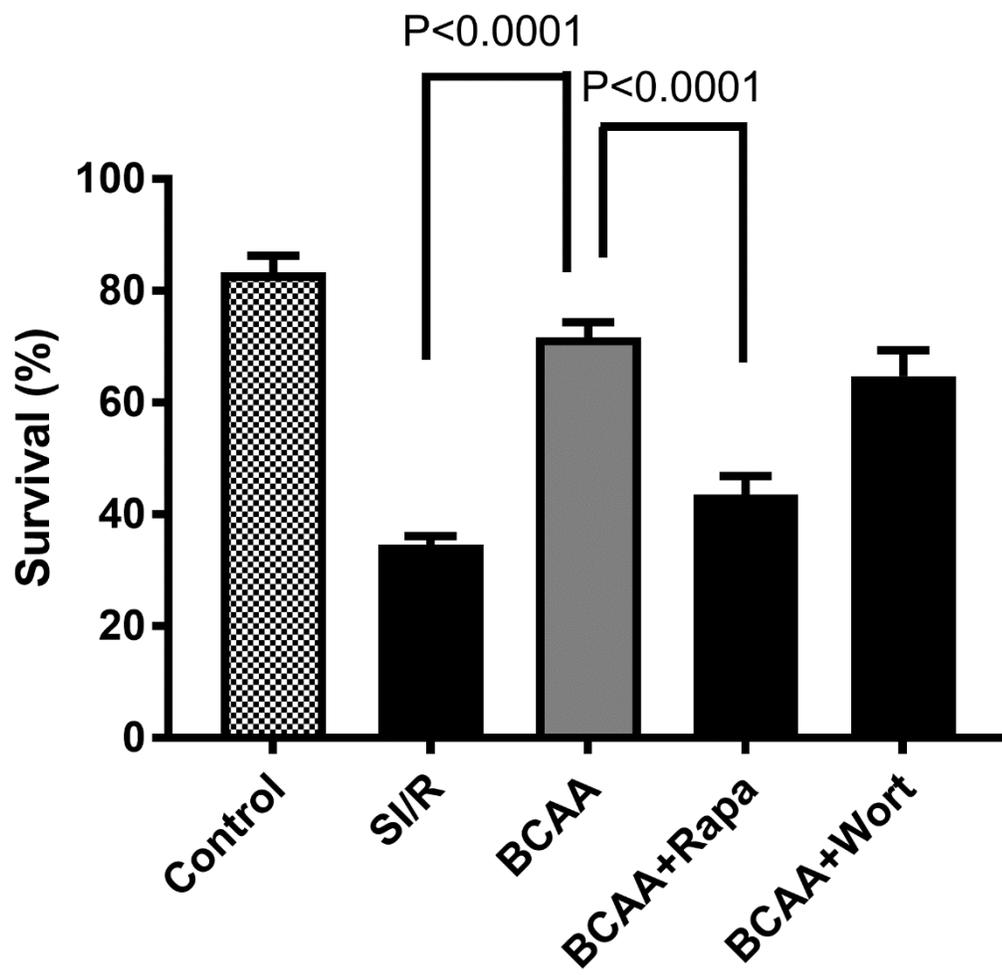
214 exposed to calcium chloride. Cyclosporine A was used as a control experiment to inhibit CaCl<sub>2</sub>-  
215 induced mitochondrial swelling, confirming the dependence of the calcium-induced swelling on the  
216 activity of the mitochondrial permeability transition pore.

217 *3.3. BCAA improves the cell survival after simulated ischemia/reperfusion*

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

225

Figure 3



227 **Figure 3. The survival rate of adult cardiac myocytes exposed to simulated**

228 **ischemia/reperfusion.**

229 Branched-chain amino acids (BCAAs) improve the survival rate of adult cardiac myocytes exposed

230 to simulated ischemia/reperfusion, but rapamycin inhibited this preventive effect. Wortmannin, a

231 phosphatidylinositol-3-kinase (PI3K) inhibitor, does not affect the cardiac protection induced by

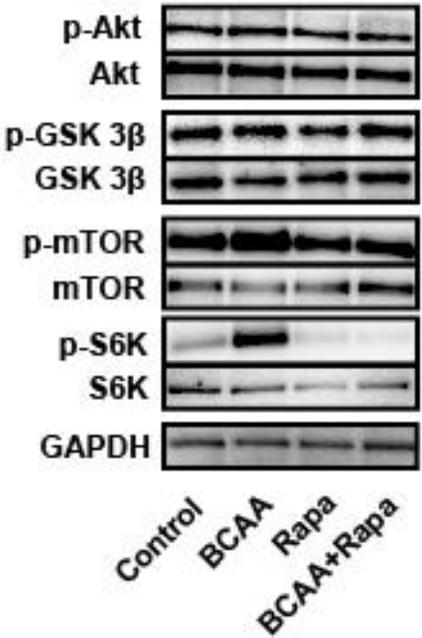
232 BCAAs.

233 *3.4. Signaling pathways involved in BCAA-induced cardiac protection*

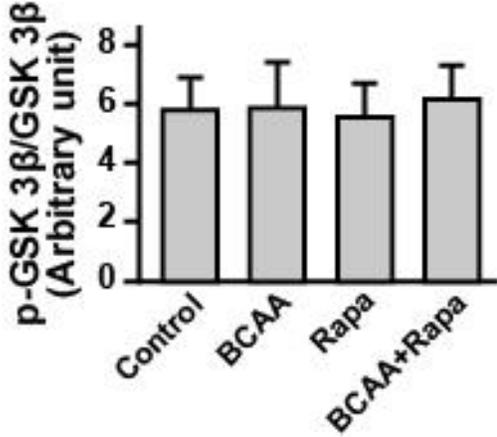
234 To investigate the mechanism for cardiac protection induced by BCAA, we examined the effect of  
235 BCAA on the phosphorylation of the cytoprotective kinase Akt and its downstream substrate  
236 GSK3 $\beta$  as well as on the phosphorylation of mTOR (Fig. 4). BCAA treatment caused neither Akt  
237 nor GSK3 $\beta$  phosphorylation. By contrast, mTOR was phosphorylated after BCAA pretreatment but  
238 not after pretreatment with BCAA in the presence of rapamycin. Thus, the cytoprotective effects of  
239 BCAA likely depend on mTOR activity but not on Akt/GSK3 $\beta$  signaling. Additionally, following  
240 I/R injury we noted no changes in mTOR expression similar to previous reports (Data not shown)  
241 [23].

**Figure 4**

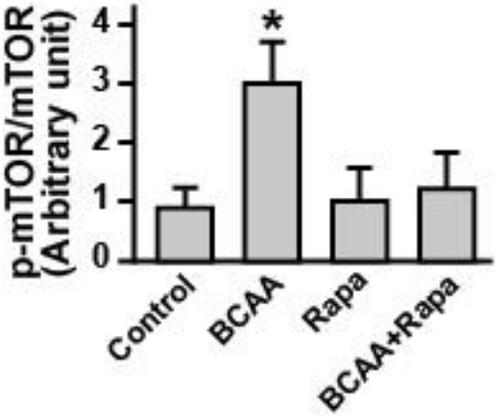
**A**



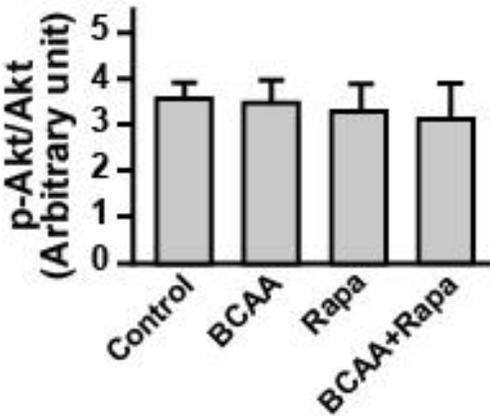
**C**



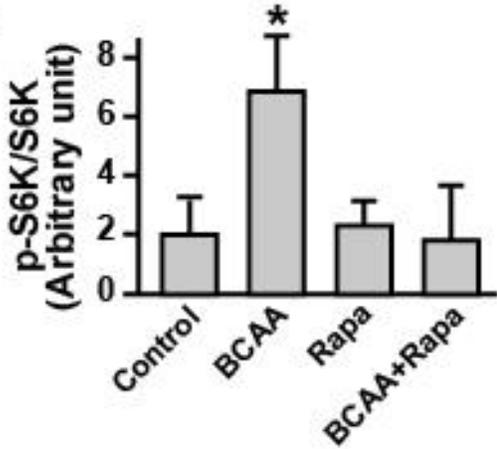
**D**



**B**



**E**



243 **Figure 4. Immunoblots for Akt, phospho-Akt, GSK3 $\beta$ , phospho-GSK3 $\beta$ , mTOR, phospho-**  
244 **mTOR, phospho-S6K and S6K.**

245 Immunoblots for Akt, phospho-Akt, GSK3 $\beta$ , phospho-GSK3 $\beta$ , mTOR, and phospho-mTOR, pS6K  
246 and S6K. Branched-chain amino acids (BCAAs) significantly increased phosphorylation of mTOR  
247 and pS6K without altering the phosphorylation of Akt or GSK3 $\beta$  proteins expression in lysed hearts.  
248 Pretreatment with rapamycin blocked BCAA-mediated activation of mTOR. Values are expressed as  
249 mean  $\pm$  standard deviation. \* represents  $P < 0.05$  vs. control.

250 **4. Discussion**

251 In the current study, BCAAs significantly decreased the infarct size, whereas the mTOR  
252 inhibitor rapamycin prevented this protective effect using in vivo mouse model of regional  
253 myocardial ischemia and reperfusion. BCAA treatment also preserved cell viability after simulated  
254 I/R in cardiac myocytes. However, the PI3K inhibitor wortmannin did not interfere with the  
255 cardioprotective effect induced by BCAAs. Moreover, the immunoblot analysis demonstrated that  
256 BCAA led to mTOR phosphorylation, which was prevented by the addition of rapamycin. However,  
257 phosphorylation of Akt or GSK3 $\beta$  was not observed after BCAA pretreatment. These results suggest  
258 that mTOR signaling but not PI3K/Akt/GSK3 $\beta$  pathways may act as a key effector of myocardial  
259 protection by BCAA.

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

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

267 activity through the phosphorylation of insulin receptor substrate 1 and the stimulation of PI3K,  
268 which in turn leads to the activation of Akt [28]. Amino acids activate mTORC1 by recruitment to  
269 the surface of lysosome, which is caused by Regulator-Rag complex combining to raptor [29].  
270 Leucine, one of the three branched chain amino acids, is supposed to relate to the regulation of  
271 mTORC1 through cytosolic sensors such as leucyl-tRNA synthetase and Sestrin 2 [30]. Previous  
272 studies indicate that amino acids induced cytoprotective effects by reducing the inflammatory  
273 response [11]. BCAAs respond to several cells signaling pathways mainly through the activation of  
274 the mTOR axis and mTOR relates to myocardial I/R injury through multiple signaling pathways  
275 such as AMP-activated protein kinase (AMPK)/mTOR or PI3K/Akt/mTOR pathway associated  
276 with autophagy [31, 32]. In this study, there is no significant difference of infarct size as a  
277 percentage of the area at risk or cTnI in control of both mTOR<sup>+/+</sup> and mTOR<sup>+/-</sup> mice. This may  
278 result from some other signaling pathways known to show the protective effect on I/R injury in the  
279 heart. As one of the important intracellular signaling pathways of cardiac preconditioning, PI3K and  
280 its downstream target Akt, are also involved in the regulation of oxidation, inflammatory responses,  
281 and apoptosis. The PI3K/Akt/GSK3 $\beta$ -dependent signaling pathways have been demonstrated to  
282 result in the attenuation of myocardial I/R injury [33-37]. On the other hand, the present study  
283 suggests that mTOR signaling pathway, not PI3K/Akt/GSK3 $\beta$ -dependent signaling pathways may

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

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

292           Mitochondria play a central role in molecular events, leading to tissue damage after  
293 pathological stimulation such as ischemia [38, 39]. mTOR is known to control mitochondrial  
294 dynamics [40]. mTOR binds and regulates the voltage-dependent anion channel proteins [41],  
295 which are an important component of the mPTP in the outer mitochondrial membrane. Several  
296 studies showed inhibition of mTOR activity provoked a decrease in mPTP permeability [42].  
297 mTOR activation caused by BCAAs may preserve mitochondrial-mediated cell death triggered by  
298 unknown signaling pathways that are related to Ca<sup>2+</sup>-induced swelling in cardiac I/R injury. The  
299 mPTP is a large-conductance mega-channel found at the contact sites between the inner and outer  
300 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].

309 **5. Conclusions**

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

313 **Conflict of interest**

314           The authors declare that there are no conflicts of interest.

315

316 **Acknowledgements**

317           This work was supported by JSPS KAKENHI [grant numbers 19K09353].

318 **References**

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