Leucine imparts cardioprotective effects by enhancing mTOR activity and mitochondrial fusion in a myocardial ischemia/reperfusion injury murine model

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Abstract

Background: Coronary artery disease is a leading cause of morbidity and mortality among patients with diabetes. Previously, we demonstrated that branched-chain amino acids (BCAAs) showed cardioprotective effects against cardiac ischemia/reperfusion (I/R) injury. A recent study suggested that leucine (Leu), a BCAA, is a key amino acid involved in mammalian target of rapamycin (mTOR) activity and mitochondrial function. However, whether Leu has cardioprotective effects on diabetic hearts is unclear. In this study, we examined the preconditioning effect of Leu treatment on high-fat diet (HFD)-induced obese mouse which simulate prediabetic heart.

Methods: In vivo mice models of I/R injury were divided into the following groups: control, mTOR+/−, and high-fat diet (HFD)-induced obese groups. Mice were randomly administered with Leu, the mTOR inhibitor rapamycin (Rap), or Leu with Rap. Isolated rat cardiomyocytes were subjected to simulated I/R injury. Biochemical and mitochondrial functional assays were performed to evaluate the changes in mTOR activity and mitochondrial dynamics caused by Leu treatment.

Results: Leu-treated mice showed a significant reduction in infarct size when compared with the control group (34.8% ± 3.8% vs. 43.1% ± 2.4%, n = 7, p < 0.05), whereas Rap-treated mice did not show the protective effects of Leu. This preconditioning effect of Leu was attenuated in mTOR+/− mice. Additionally, Leu increased the percentage of fused mitochondria and the mitochondrial volume, and decreased the number of mitochondria per cell in isolated cardiomyocytes. In HFD-induced obese mice, Leu treatment significantly reduced infarct size (41.0% ± 1.1% vs. 51.0% ± 1.4%, n = 7, p < 0.05), which was not induced by ischemic preconditioning, and this effect was inhibited by Rap. Furthermore, we observed enhanced mTOR protein expression and mitochondrial fusion with decreased reactive oxygen species production with Leu treatment in HFD-induced obese mice, but not in mTOR+/− mice.

Conclusions: Leu treatment improved the damage caused by myocardial I/R injury by promoting mTOR activity and mitochondrial fusion on prediabetic hearts in mice.

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**Introduction**

Coronary artery disease (CAD) is a leading cause of morbidity and mortality among patients with diabetes, which increases the risk of developing CAD by two- to fourfold [1, 2]. Both type 1 and type 2 diabetic individuals are prone to developing ischemic heart disease, including acute myocardial infarction (AMI) and post-infarct complications [3]. Mortality from AMI is approximately twice among diabetic patients compared with that among non-diabetic individuals [4, 5]. Despite the burden of ischemic heart disease among patients with diabetes, effective treatment is currently unavailable [6].

Myocardial ischemia/reperfusion (I/R) injury is a condition wherein the damage is caused by the occlusion of coronary arteries and restoration of blood flow to the ischemic myocardium [7, 8]. Several studies have reported the preconditioning effect of pharmacological agents through signaling pathways regulating cellular processes such as necrosis, apoptosis, and autophagy [9–11].

Recently, we showed the cardiac preconditioning effect of branched-chain amino acid (BCAA) treatment on I/R injury in mice, resulting from increased mammalian target of rapamycin (mTOR) activity and improved mitochondrial function [12]. Among BCAAs, leucine (Leu) can activate mTOR kinase, thereby leading to the phosphorylation of p70S6 kinase and increasing the phosphorylation of serine residues in insulin receptor substrate-1 [13], which inhibits insulin signaling and insulin-stimulated glucose transport in muscles and fats. However, whether Leu has cardioprotective effects on diabetic hearts is unclear.

In this study, we examined the changes in mTOR activity and mitochondrial dynamics caused by Leu administered during I/R injury of the heart in a high-fat diet (HFD)-induced obese mouse model.

**Materials and methods**

**Animals**

All animals were treated in compliance with the guidelines for proper conduct of animal experiments and related activities (Ministry of Education, Culture, Sports, Science, and Technology of Japan). Additionally, the protocols, which follow the ARRIVE guidelines [14], were approved by the Animal Care and Use Committee at the University of Tokushima. Adult male Wistar rats and male C57BL/6 mice at 4 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan). mTOR+/− mice were created as previously reported [15]. Mice were housed under temperature- (23±3 °C) and humidity-controlled conditions with a 12 h light/12 h dark cycle. Mice had free access to water and a control diet (14% of calories from fat; Oriental Yeast Co., Ltd., Tokyo, Japan) or an HFD (60% of calories from fat; Oriental Yeast Co., Ltd.). Mice were randomly assigned to 6 weeks of HFD or control diets, and the experiments are performed on them at 10 weeks of age that is considered a juvenile. At the time of 10 weeks, body weight of mice was wild-type mice; 26.4±0.6 g, HFD-induced obese mice; 32.1±1.1 g, mTOR+/− mice; 26.3±0.8 g (mean±SD).

**In vivo myocardial I/R experiments**

The surgical methods used were similar to those described previously [16–18]. Mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and mechanically ventilated with 100% oxygen using a pressure-controlled ventilator (TOPO ventilator, Kent Scientific Co., Torrington, CT, USA). The core body temperature was maintained with a heating pad, and electrocardiogram leads were placed to record the heart rate. An intercostal thoracotomy was performed to expose the heart. Ischemia was induced by occluding the left coronary artery (LCA) with a 7–0 silk suture for 30 min, after which the ligature was released, and the heart was perfused for 2 h. Mice were randomly assigned to each experimental group. Saline (0.9%) or Leu (200 mg/kg, IV) was administered 20 min before the occlusion. In the ischemic preconditioning (IPC) group, IPC was induced by occluding the LCA for 5 min, followed by 15 min of reperfusion immediately before the I/R procedure. After reperfusion, mice were heparinized, and the LCA was again occluded. The area at risk (AAR) was determined by staining with 1% Evans blue (1.0 mL, Sigma). The heart was immediately excised and cut into 1.0-mm slices (McIlwain tissue chopper; Brinkmann Instruments). Each slice of left ventricle was then counterstained with 2,3,5-triphenyltetrazolium chloride (Sigma). After overnight storage in 10% formaldehyde, slices were weighed and visualized under a microscope (SZ61-TR, Olympus) equipped with a charge coupled device camera (DXM 1200F, Nikon). The images were analyzed (Image-Pro Plus, Media Cybernetics), and AAR and infarct size were determined by planimetry as previously described [8].

**Isolation and maintenance of rat cardiomyocytes**

Cardiomyocytes were isolated from adult male Wistar rats. Rats were heparinized (1.0 IU/g, i.p.) 30 min before anesthetizing them with pentobarbital (80 mg/kg, i.p.).
Myocytes were obtained via enzymatic (210 U/mg collagenase II; Worthington, Lakewood, NJ, USA) digestion of the heart using a Langendorff apparatus. Enzymatic digestion was performed as previously described [19, 20]. Isolated myocytes were then cultured in 4% fetal bovine serum on laminin (2 μg/cm²)-coated plates for 1 h. Culturing/maintenance media were changed to serum-free media [1% bovine serum albumin + 0.1% penicillin/streptomycin M199 media (Invitrogen, Carlsbad, CA, USA)] to eliminate all non-myocytes, and cardiac myocytes were incubated at 37 °C in 5% CO₂ for 24 h.

Mitochondrial dynamics analysis in isolated rat cardiomyocytes
Six hours prior to the pretreatment, all media were replaced with amino acid-free Dulbecco’s Modified Eagle’s medium to wash out any amino acids in M199 media. L-Leu (2.3 g; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 100 mL distilled water, creating a 175 mM stock solution. Cells were pretreated with Leu (160 μM) or control [phosphate-buffered saline (PBS)] for 2 h. We evaluated the changes in mitochondrial dynamics of each group for 4 h at 30 min intervals after the administration of Leu or PBS.

After pretreatment with Leu or PBS, simulated ischemia/reperfusion was performed. Simulated ischemia was induced by replacing the air content with a 95% N₂ and 5% CO₂ gas mixture at 2 L/min in a chamber and the media with glucose-free media. This was performed for 60 min, followed by 60 min of “reperfusion” by replacing the media with normal maintenance media and by incubating the cells with 21% O₂ and 5% CO₂. Finally, the cells were maintained in Krebs solution and fixed with 4% paraformaldehyde after incubating them for 30 min with MitoTracker Green FM (400 nmol/L; Molecular Probes, Invitrogen). Confocal image stacks were captured using a Leica laser microscope (Leica, Tokyo, Japan), as described previously [21]. Mitochondrial density was quantified using the ImageJ software (NIH, Bethesda, MD, USA). The number and volume of each mitochondrion were quantified using the Image J 3D Object Counter plug-in. The percentage of cells with a fusion pattern was determined based on the criteria that evaluated mitochondrial fusion with mitochondrial volume and a decrease in the number of mitochondria [22, 23].

Reactive oxygen species (ROS)
To measure ROS production in the myocardium, the OxiSelect™ in vitro ROS/RNS Assay kit (Cell Biolabs, San Diego, CA, USA) was used. Before the I/R procedure, mice were injected with 200 μL of saline, Leu, or Leu and rapamycin (Rap) into the right atrium. Hearts were excised immediately after the I/R procedure, and the measurement of ROS were performed according to the manufacturer’s instructions. The ROS content was determined using the predetermined dichlorodihydrofluorescein standard curve, and mean fluorescence units were recorded.

Western blotting
Lysates were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% polyacrylamide precast gels (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes through electrophoresis. Membranes were blocked in 20 mM Tris-buffered saline with 1% Tween containing 5% skimmed milk and incubated with primary antibodies overnight at 4 °C. Immunolabeled blots were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and enhanced chemiluminescence reagent (GE Healthcare, Waukesha, WI, USA) [24].

Electron microscopy
Whole hearts or cardiomyocytes were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at room temperature, post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature, and embedded as monolayers using LX-112 embedding kits (Ladd Research, Williston, VT, USA). Sections were stained with uranyl acetate and lead citrate, and observed under an electron microscope (EM). Random sections were taken by an EM technician who was blinded to the treatments [25].

Statistical analyses
All results were analyzed by observers who were blinded to the animal treatment history. Data are presented as mean ± standard deviation. Differences between the treatment groups were tested for statistical significance by one-way analysis of variance, followed by Bonferroni’s post hoc test. Differences were considered significant at p < 0.05.

Results
Leu reduces infarct size in wild-type but not in mTOR+/− mice
The area at risk was calculated as a percentage of infarct size, and was found to be similar between the groups. In mice treated with Leu, a significant reduction in myocardial I/R injury was observed when compared with wild-type mice treated with vehicle control (34.8% ± 3.8% vs. 43.1% ± 2.4%, n = 7, p < 0.05), but not in those treated with Leu and Rap. In addition, the effect of Leu was inhibited in mTOR+/− mice (Fig. 1).
Leu promotes mitochondrial fusion in isolated rat cardiomyocytes

In cardiomyocytes treated with Leu, mitochondria appeared to be interconnected. The percentage of cells displaying fused mitochondria was significantly increased at 2 h after Leu pretreatment when compared with the percentage of cells without Leu pretreatment (20% ± 8.5% vs. 78% ± 6.2%, n = 5, p < 0.01) (Fig. 2a). The volume of individual mitochondria was measured using the three-dimensional reconstitution of confocal stacks. Leu caused a 71% increase in the volume of individual mitochondria at 2 h after Leu treatment (Fig. 2b). In addition, Leu stimulated mitochondrial fusion with an increase in mitochondrial size, whereas the number of mitochondria per cell was found to be decreased (Fig. 2c, d).

Leu reduces infarct size in HFD-induced obese mice through mTOR activity

In HFD-induced obese mice, IPC did not affect the infarct size caused by I/R injury (47.0 ± 1.4% vs. 47.0 ± 1.5%, n = 7, p > 0.999) (Fig. 3a). However, Leu treatment reduced the infarct size (41.0 ± 1.1% vs. 47.0 ± 1.5%, n = 7, p < 0.05), whereas this effect was not observed in the group administered with Leu and Rap (Fig. 3a).

Leu enhanced mTOR protein expression

mTOR expression was enhanced by Leu administration in wild-type and HFD-induced obese mice, but not in mTOR+/− mice (Fig. 3b). However, Rap inhibited the increase in mTOR expression in wild-type and HFD-induced obese mice.

Mitochondrial dynamics under in vivo conditions

Mitochondrial dynamics were examined upon Leu administration in the heart tissue of HFD-induced obese mice (Fig. 3c) and we found that Leu administration enhanced mitochondrial fusion in the myocardium of HFD-induced obese mice.

Leu decreased ROS production in wild-type and HFD-induced obese mice but not in mTOR+/− mice

After the I/R procedure, ROS production was decreased by Leu treatment in wild-type and HFD-induced obese mice but not in mTOR+/− mice. Moreover, Rap inhibited the effect of Leu in wild-type and HFD-induced obese mice (Fig. 4).

Discussion

Through several experimental approaches, we have provided new evidence suggesting that Leu reduces the infarct size caused by myocardial I/R injury in wild-type and HFD-induced obese mouse models. Additionally, Leu enhanced mitochondrial fusion after simulated I/R in rat cardiomyocytes. In mTOR+/− mice, Leu did not affect infarct size, thereby suggesting that the effect of Leu was mediated through the mTOR signaling pathway. As observed in wild-type mice, Leu could impart protective effects on an HFD-induced obese mouse model by preconditioning treatment, which is not provided by IPC. Furthermore, Leu treatment led to an increase in mitochondrial fusion and a decrease in ROS production in prediabetic hearts.

Leu is a BCAA that plays a significant role in protein synthesis [26]. Previously, we showed that BCAAs have cardioprotective effects via the mTOR signaling pathway [12]. In present study, we found that Leu reduced infarct size in wild-type mice, but not in mTOR+/− mice. Therefore, this result indicates that Leu is the key amino acid among BCAAs protects the heart from I/R injury through mTOR activity.

In this study, our finding indicates that Leu regulates the mTOR signaling pathway and improve mitochondrial dynamics, which means the increase in mitochondrial fusion. Mitochondrial dynamics and coordinated fission and fusion cycles are important for maintaining the shape, distribution, and size of mitochondria [27]. The Disintegration of the reticular form of mitochondria into fragments has been considered as a physiological indicator of mitochondrial dysfunction [28]. Several studies have shown that mTOR activity contributes to mitochondrial function [29]. Szabo et al. showed...
that mitochondrial fusion with mTOR phosphorylation had a preventive effect on mitochondrial fragmentation [30]. Further studies are, however, needed to identify the detailed signaling pathways involved between mTOR activity and mitochondrial dynamics in cardiac preconditioning induced by Leu treatment.

Mitochondria have been implicated as a major source of I/R-induced ROS production in a variety of organs [31]. Damage to mitochondria can change mitochondrial structure and function. These ultrastructural and functional defects partially recover upon reperfusion, and are accompanied by increased superoxide anion production.
generation, resulting in a burst of ROS production upon reperfusion [32]. In our study, the decrease in ROS production was observed with Leu treatment, suggesting that Leu can prevent enhanced ROS production followed by the loss of mitochondrial function due to I/R injury in wild-type and HFD-induced obese mice.

In HFD-induced obese mice, we demonstrated that Leu reduces the infarct size, which is not observed by IPC. Cardiac preconditioning is attenuated by diabetes [33], and the loss of cardioprotective effects may be caused by the inhibition of insulin signaling whereas the effects of Leu seem not to largely depend on the insulin-related...
mechanisms [34]. Our previously study revealed that the effects of BCAA preconditioning are mediated via the mTOR pathway and not the phosphoinositide 3-kinase pathway, which is downstream of insulin receptor signaling [12]. Our results suggest that Leu is a key protein that recovers the effect of preconditioning, which was attenuated in case of IPC, through mTOR activity in prediabetic hearts.

This study has several limitations. First, we did not conduct experiments to investigate the molecular mechanism of enhanced mitochondrial fusion caused by Lue treatment for myocardial I/R injury. There are some proteins that relate to the process of mitochondrial fusion [35]. Identifying the specific protein could lead to the detail of pharmacological preconditioning mechanism by Leu via mTOR pathway in the future study. Second, we measured total ROS production in mice hearts. ROS is mainly produced by mitochondria but there are other ways to generate through several enzymatic reactions [31]. It might be more appropriate to measure mitochondrial ROS production to investigate the interaction between mitochondrial dynamics and ROS production.

**Conclusion**

Leu treatment resulted in cardiac preconditioning with increased mTOR activity and mitochondrial fusion in both wild-type and HFD-induced obese mice, thereby suggesting that Leu could be potentially used for myocardial I/R injury treatment in patients with diabetes.
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