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Leucine induces cardioprotection in vitro by promoting mitochondrial function via mTOR and Opa-1 signaling

Atsushi Morio [a,1], Rie Tsutsumi [b,1], Takashi Kondo [a], Hirotsugu Miyoshi [a], Takahiro Kato [a], Soshi Narasaki [a], Shiho Satomi [a], Erika Nakaya [b], Masashi Kuroda [b], Hiroshi Sakaue [b], Tadahiro Kitamura [c], Yasuo M. Tsutsumi [a,*]

[a] *Department of Anesthesiology and Critical Care, Hiroshima University, Hiroshima, Japan*
[b] *Department of Nutrition and Metabolism, Institute of Biomedical Sciences, Tokushima University, Tokushima, Japan*
[c] *Laboratory of Metabolic Signal, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan*

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Abstract *Background and aims:* Coronary heart disease is a major global health concern. Further, severity of this condition is greatly influenced by myocardial ischemia/reperfusion (I/R) injury. Branched-chain amino acids (BCAAs) have cardioprotective effects against I/R via mammalian target of rapamycin (mTOR) activity, wherein Leu is considered to particularly regulate mTOR activation. However, the mechanism underlying cardioprotective effects of Leu via mTOR activity is not fully elucidated. Here, we aimed to study the signaling pathway of cardioprotection and mitochondrial function induced by Leu treatment.
Methods and results: Cardiac myocytes isolated from adult male Wistar rats were incubated and exposed to simulated I/R (SI/R) injury by replacing the air content. Cardiac myocytes were treated with Leu and subsequently, their survival rate was calculated. To elucidate the signaling pathway and mitochondrial function, immunoblots and mitochondrial permeability transition pore were examined. Cell survival rate was decreased with SI/R but improved by 160 μM Leu (38.5 ± 3.6% vs. 64.5 ± 4.2%, respectively, $p < 0.001$). Although rapamycin (mTOR inhibitor) prevented this cardioprotective effect induced by Leu, wortmannin (PI3K inhibitor) did not interfere with this effect. In addition, we indicated that overexpression of Opa-1 and mitochondrial function are ameliorated via Leu-induced mitochondrial biogenesis. In contrast, knockdown of Opa-1 suppressed Leu-induced cardioprotection.
Conclusion: Leu treatment is critical in rendering a cardioprotective effect exhibited by BCAAs via mTOR signaling. Furthermore, Leu improved mitochondrial function.

Acronyms: BCAAs, branched-chain amino acids; CAD, coronary artery disease; I/R, ischemia/reperfusion; SI/R, simulated ischemia/reperfusion; mTOR, mammalian target of rapamycin; Ψmt, mitochondrial membrane potential.

* Corresponding author. Department of Anesthesiology and Critical Care, Hiroshima University, 1-2-3 Kasumi, Minami, Hiroshima, 734-8551, Japan. Fax: +81 82 257 5269.
E-mail address: yasuo223@hiroshima-u.ac.jp (Y.M. Tsutsumi).
[1] Morio A and Tsutsumi R contributed equally to this work.

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Introduction

Incidences of coronary artery disease (CAD) have significantly increased in the world, rendering it being as a leading cause of morbidity and mortality. It is well known that the severity of CAD is usually influenced by myocardial ischemia/reperfusion (I/R) injury. Thus, it is necessary to elucidate novel strategies to prevent myocardial I/R injury and improve clinical outcomes in patients with CAD. To prevent myocardial I/R injury, various methods have been reported. In addition to transient ischemia, several pharmacologic agents, such as anti-inflammatory, antioxidants, fatty acids, opioids, and organic nitrate esters, provide myocardial protective effect [1–5]. With respect to signal transduction pathways, several studies have shown that cardiac protection against I/R injury frequently involves activation of survival kinases (i.e., phosphoinositide 3-kinase (PI3K), protein kinase B (PKB/Akt), and/or extracellular signal-regulated kinase (ERK) [6]. However, the exact mechanism underlying cardioprotective action of these signal transduction pathways against I/R injury remains poorly understood.

Branched-chain amino acids (BCAAs), namely, valine, leucine (Leu), and isoleucine, are known to have protective effects against I/R injury in various organs, including the kidney and liver [7–9]. Furthermore, We have previously reported the cardioprotective effects of BCAAs against cardiac I/R injury via mammalian target of rapamycin (mTOR) activity in an *in vivo* rodent study [9]. Among the BCAAs, Leu is of particular importance as it is involved in the regulation of mTOR activation, leading to phosphorylation of p70S6 kinase (p70S6K) and increased serine phosphorylation of IR substrate-1 [10]. However, whether Leu can confer cardioprotective effects all by itself remains to be elucidated. Previous studies have demonstrated that mitochondria play a significant role in molecular events, leading to tissue damage after pathological stimulation such as ischemia [11,12]. Further, mTOR is known to control mitochondrial function [13].

In the present study, we hypothesized that Leu is associated with both cardioprotective effects and mitochondrial function. We aimed to examine the cardioprotective effects of Leu treatment via mTOR activity and improvement of mitochondrial function in the setting of simulated I/R (SI/R) injury in cardiomyocytes collected from Wistar rats.

Methods

Antibodies

Antibodies were purchased from the following sources: monoclonal antibody to OPA-1 (1E81D9, ab119685, Abcam, Cambridge, MA, USA), polyclonal antibody to MFN-1 (H-65, sc-50330, Santa Cruz Biotechnology, Santa Cruz, CA, USA), MFN-2, (H-68, sc-50331, Santa Cruz Biotechnology), and DRP-1 (H-300, sc-32898, Santa Cruz Biotechnology).

Isolation and maintenance of cardiac myocytes

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, Japan). The protocols were in compliance with the ARRIVE guidelines [14] and approved by the Animal Care and Use Committee of Tokushima university.

Cardiac myocytes were isolated from adult male Wistar rats (Japan SLC, Shizuoka, Japan). Briefly, animals were heparinized (1.0 IU/g, i.p.) 30 min before being anesthetized with pentobarbital (80 mg/kg, i.p.). Myocytes were obtained by enzymatic (210 U/mg collagenase II; Worthington, Lakewood, NJ, USA) digestion of the heart using Langendorff apparatus. The enzymatic dissociation method was similar to that previously published [15,16]. Isolated myocytes were cultured in laminin (2 $\mu g/cm^2$)-coated plates using 4% fetal bovine serum for 1 h. Plating/maintenance media were changed to a serum-free medium [1% bovine serum albumin +0.1% penicillin/streptomycin in Medium 199 (Invitrogen, Carlsbad, CA, USA)] to remove all non-myocytes. Cardiac myocytes were incubated at 37 °C in the presence of 5% CO_2 for 24 h. Simulated ischemia was induced by replacing the air content with a gas mixture of 95% N_2 and 5% CO_2 at the rate of 2 L/min in a chamber and by replacing media with glucose-free media for 60 min. This was followed by 60 min of "reperfusion" by replacing the media with amino acid-free DMEM and incubating the cells in the presence of 21% O_2 and 5% CO_2 [17,18].

Mitochondrial function

Mitochondrial membrane potential (Ψmt) was measured during reperfusion injury using the membrane potential-sensitive dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetratheylbenzimidazoly-carbocyamine iodine (JC-1). Images of cardiomyocytes stained with JC-1 were captured at 1 min intervals using a perfusion chamber system following an ROS stress induced by H_2O_2.

Leu treatment

Stock solution (175 mM) of L-Leu (Sigma Aldrich, St Louis, MO, USA) was prepared by dissolving 2.3 g in 100 mL distilled water. This solution was used to treat cardiomyocytes in a dose-dependent manner with final concentrations ranging between 0 and 160 μM. Six hours prior to experimentation, all media were replaced with amino acid-free DMEM to wash out any amino acids within Medium 199. Stimulation with Leu was conducted at 0, 40, 80, 160 μM and for 0, 0.5, 1, 2, or 4 h before SI/R.

Immunoblotting

Lysates were separated by SDS-PAGE on 10% polyacrylamide precast gels (Invitrogen) and transferred to

polyvinylidene difluoride membranes by electroelution. Membranes were blocked with 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) and visualized using an enhanced chemiluminescence reagent (GE Healthcare, Waukesha, WI, USA) [19,20].

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cardiomyocytes using RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Extracted RNA was amplified using CellAmp Whole Transcriptome Amplification Kit Version 2 (Takara Bio Inc., Shiga, Japan). PCR was performed in a final volume of 10 μL containing 50 ng of the cDNA template, SYBR green (Life Technologies, Carlsbad, CA, USA), and primers on StepOnePlus Real-Time PCR System (Life Technologies) [21,22]. Following are the primer sequences: Opa-1 rat forward 5′-TGACAAACTTAAGGAGGCTGTG-3′; Opa-1 rat reverse 5′-CATTGTGCTGAATAACCCTCAA-3′; Mfn1 rat forward 5′-GCAGACAGCACATGGAAAGA; Mfn1 rat reverse 5′-CTTGCCTGAAATCCTTCTGC-3′; Mfn2 rat forward 5′-CAGCGTCCTCTCCCTCTGAC-3′; Mfn2 rat reverse 5′-GGTCCAGGTCAGTCGCTCAT-3′; Drp1 rat forward 5′-AGGCTCAGCAGGTCACTCAT-3′; Drp1 rat reverse 5′-GAAAGCAAGCCCATAGCAAG-3′.

Small interfering RNA (siRNA) knockdown

Opa-1 siRNA and controls were transfected using Neon transfection system (Invitrogen). Conditions for electroporation were as follows: pulse voltage = 1700 V, pulse width = 20 ms, pulse number = 1, cell density = 1×10^7 cells/mL. Cell viability was 93% and transfection efficiency was 57%. After

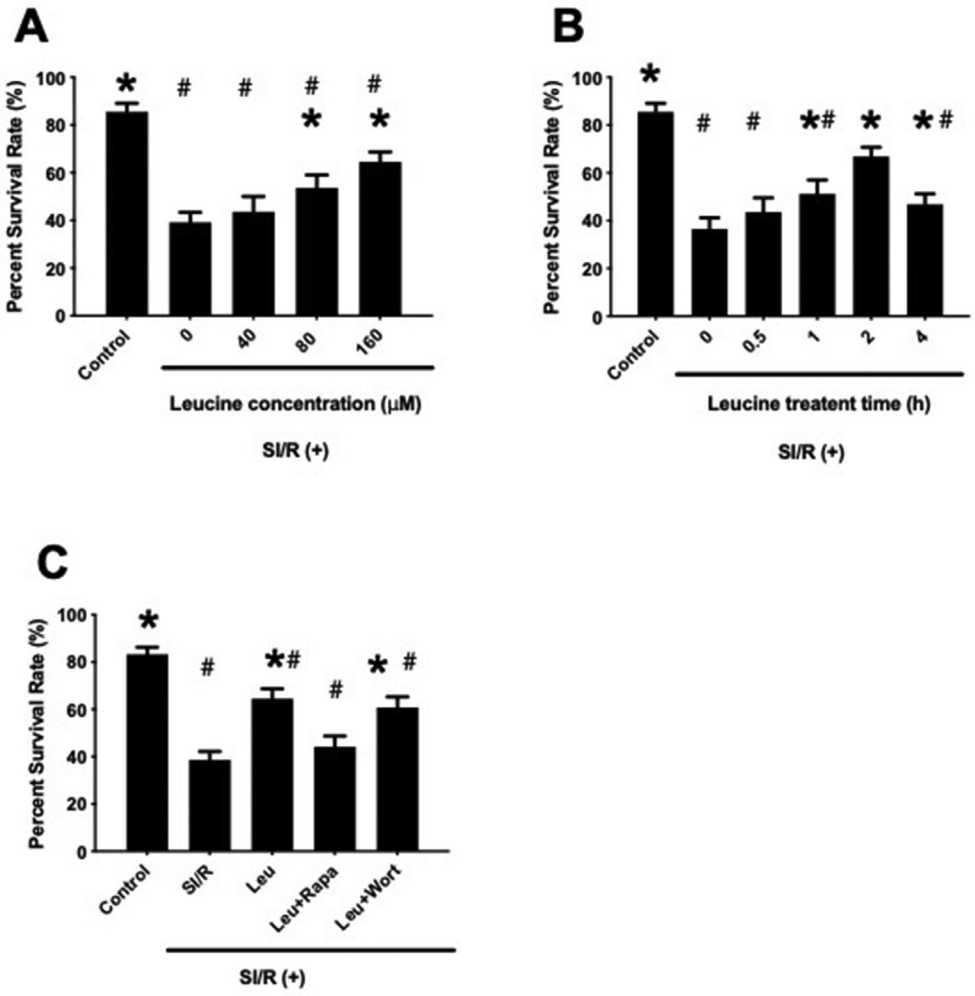

Figure 1 Leucine improves survival rate of myocytes exposed to simulated ischemia/reperfusion (SI/R). (A) Survival rate of adult cardiac myocytes exposed to SI/R. Rat cardiomyocytes were stimulated with Leu at 0, 40, 80, and 160 μM for 2 h before SI/R. n = 6 per group. Data are shown as the mean ± SD. $^*p < 0.05$ vs. SI/R. (B) Rat cardiomyocytes were stimulated with Leu at 160 μM for 0, 0.5, 1, 2, or 4 h before SI/R. Data are shown as mean ± SD. n = 6 per group. $^*p < 0.05$ vs. control. (C) Rat cardiomyocytes were stimulated with Pre-treatment with Leu, Leu + rapamycin, and Leu + wortmannin. Data are shown as mean ± SD. n = 6 per group. $^*p < 0.001$ vs. SI/R.

24 h of transfection, cells were lysed and RNA was extracted and analyzed.

Mitochondrial permeability transition

To assess mitochondrial permeability transition pore function, MitoPT JC1 assay kit (Immunochemistry Technologies, Bloomington, MN, USA) was used. Cardiomyocytes were incubated with MitoPT JC-1 (1 g/mL) in Medium 199 for 30 min and washed with Krebs solution. Images were captured at 570 nm and 535 nm every min for 15 min. The mPTP opening was measured by incubating the cardiomyocytes at room temperature for 15 min in the dark in Medium 199 containing 1 μM calcein-AM (Invitrogen) and 2 mM $CoCl_2$ using Mitochondrial PT Pore Assay kit (Cayman chemical, Ann Arbor, MI, USA). Cells were analyzed by flow cytometry (Life technologies, Carlsbad, CA, USA) to quantify green fluorescence.

Statistical analyses

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

Results

Leu improves cell survival after SI/R

We examined the cardioprotective effects of Leu in isolated rat cardiac myocytes in response to SI/R to accurately assess myocyte survival under controlled experimental conditions. Adult cardiac myocytes under

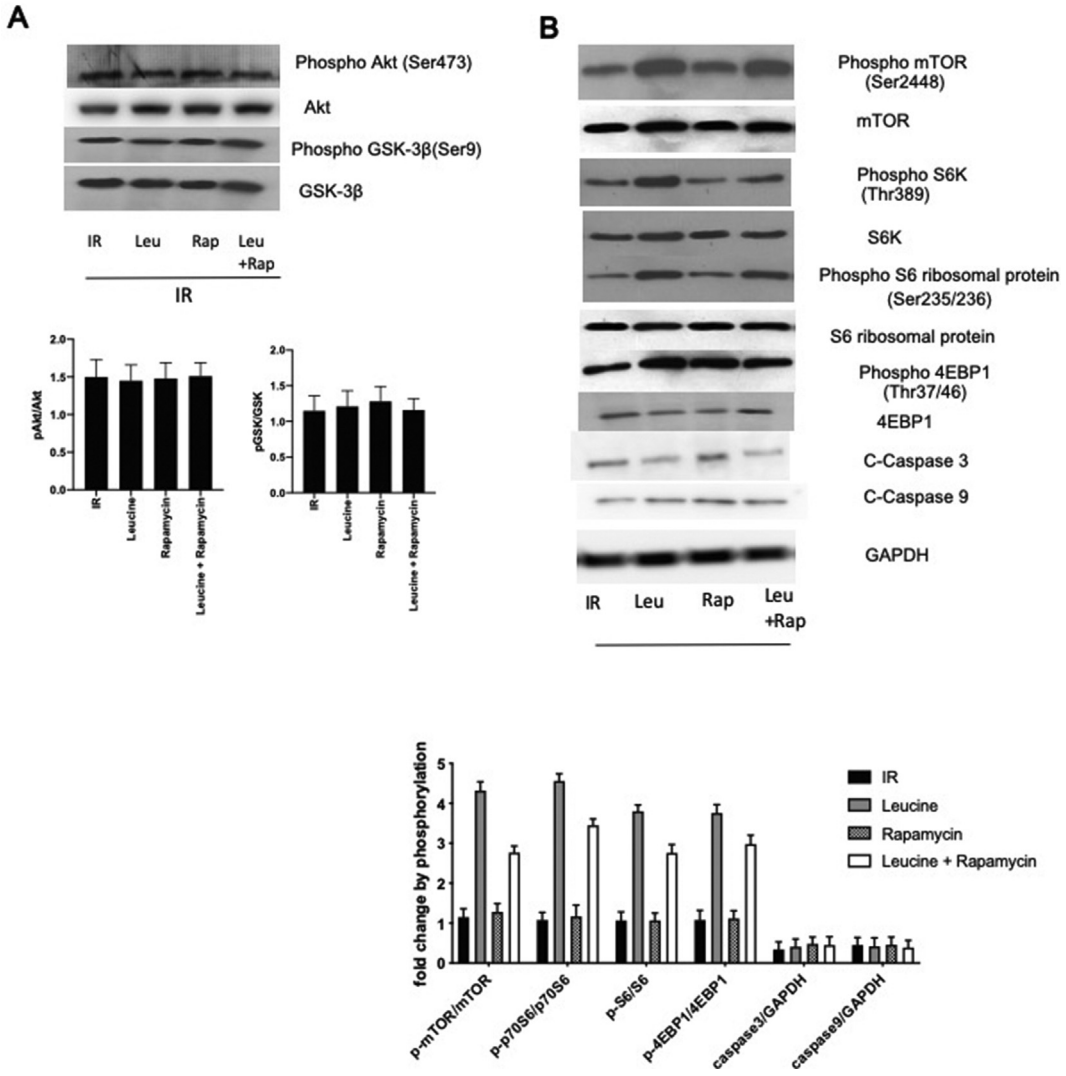

Figure 2 Analysis of molecules downstream of mTOR after treatment with Leu or Leu and rapamycin. (A) Immunoblot analysis and relative expression levels of Akt, phospho-Akt, GSK3β, phospho-GSK3β. Data are shown as mean ± SD. n = 3 per group. (B) Immunoblot analysis and relative expression levels of mTOR, phospho-mTOR, S6K, phospho-S6K, S6 ribosomal protein, phospho-S6 ribosomal protein, EBP1, phospho-4EBP1, C-Cas 3, C-Cas 9. Data are shown as mean ± SD. n = 3 per group. $^{*}p < 0.05$ vs. control.

control conditions had minimal cell death. Survival rate decreased with SI/R but improved in the presence of 160 μM Leu (38.5 ± 3.6% vs. 64.5 ± 4.2%, respectively, $p < 0.001$) (Fig. 1A). To assess the importance of concentration and duration of Leu treatment, we compared different concentrations and treatment time of Leu. Because Leu is soluble up to 2.3 g/100 mL (175 μM in water), we used 0, 40, 80, and 160 μM of Leu. Leu showed a dose-dependent effect on cell survival (Fig. 1A). As shown in Fig. 1B, cell survival significantly increased with 2 h of Leu treatment but was lost after 4 h of treatment (Fig. 1B). Therefore, 160 μM Leu for 2 h was used in all further experiments involving cardiac myocytes. Rapamycin, an mTOR inhibitor, blocked the Leu-induced cardioprotective effects (44.2 ± 4.5%, $p < 0.001$). However, the PI3K inhibitor, wortmannin, did not alter the cardiac protection induced by Leu (60.8 ± 4.5%) (Fig. 1C). Although Leu stimulation did not induce phosphorylation of Akt or GSK3β, which are downstream of the insulin receptor signaling (Fig. 2A), it did induce phosphorylation of mTOR, S6K, S6 ribosomal protein, and 4EBP-1, which are downstream of mTOR, and were inhibited by rapamycin (Fig. 2B).

Leu promotes mitochondrial fusion in cardiomyocytes via Opa-1

To evaluate Leu-dependent mitochondrial function, changes in expression levels of Opa-1, Mfn-1, and Mfn-2 (regulate mitochondrial fusion) and Drp-1 (regulates mitochondrial fission) were assessed. As shown in Fig. 3A–B, total protein expression of Opa-1 was increased after 2 h of Leu treatment, whereas expression of Mfn-1 and Mfn-2 remained unchanged.

Leu improves mitochondrial function by promoting mitochondrial biogenesis

We next examined whether Leu-induced promotion of mitochondrial fusion improves mitochondrial function. Subsequently, we measured Ψmt to assess whether Leu modified this parameter. As shown in Fig. 4A, Leu treatment for 2 h prevented the reduction of Ψmt that occurred in the reoxygenated cells undergoing either the control or Leu treatment. Moreover, we observed that Leu allowed maintenance of Ψmt during H_2O_2 stimulation (Fig. 4B). Mito PTP opening experiments using calcein-AM were also measured as shown in Fig. 4C. Mitochondrial permeability was impaired by hypoxia whereas Leu ameliorated hypoxia induced mitochondoria dysfunction. In conclusion, mitochondrial function is ameliorated via Leu-induced mitochondrial biogenesis.

Downregulation of Opa-1 suppresses Leu-induced cardiac protection and mitochondrial function

To examine the direct role of Opa-1 in Leu-induced cardioprotection, we knocked down the expression of Opa-1 (Fig. 5A–B) and assessed the resulting mitochondrial function. Adult cardiac myocytes were transfected with Opa-1 siRNA or control siRNA. In the presence of Leu, myocytes treated with control siRNA showed improved cell viability, whereas myocytes treated with Opa-1 siRNA showed decreased cell survival (64.5 ± 4.2% vs. 44.5 ± 1.6%, respectively, $p < 0.05$) (Fig. 5C). From these data, we conclude that impairment of mitochondrial fusion by Opa-1 knockdown suppresses Leu-induced cardiac protection.

Discussion

In the current study, Leu significantly improved the survival rate of ischemic cardiomyocytes, whereas the rapamycin, an mTOR inhibitor, prevented this protective effect. However, the PI3K inhibitor wortmannin did not interfere with the cardioprotective effect induced by Leu. Moreover, immunoblot analysis demonstrated that Leu induced mTOR phosphorylation that was prevented by the addition of rapamycin, although phosphorylation of Akt or GSK3β was not observed after Leu pretreatment. These results indicate that PI3K/Akt/GSK3β pathways do not but mTOR

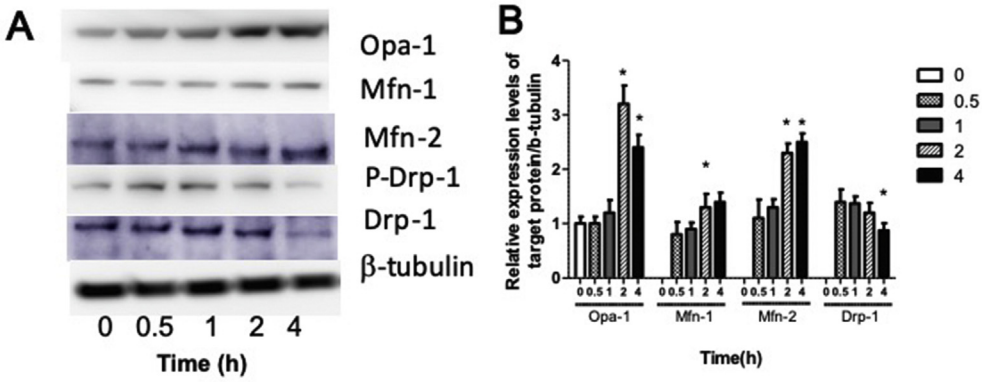

Figure 3 Protein expression of mitochondria fusion and fission related protein. (A) Representative western blots of OPA-1, MFN1, MFN2, DRP-1, and β-tubulin after incubation with Leu at 160 μM for 0, 0.5, 1, 2, or 4 h n = 4 per group. (B) Densitometric analysis of normalized OPA-1 levels from total extracts. Data are shown as. mean ± SD. n = 4 per group. $^{**}p < 0.01$ vs. 0 h.

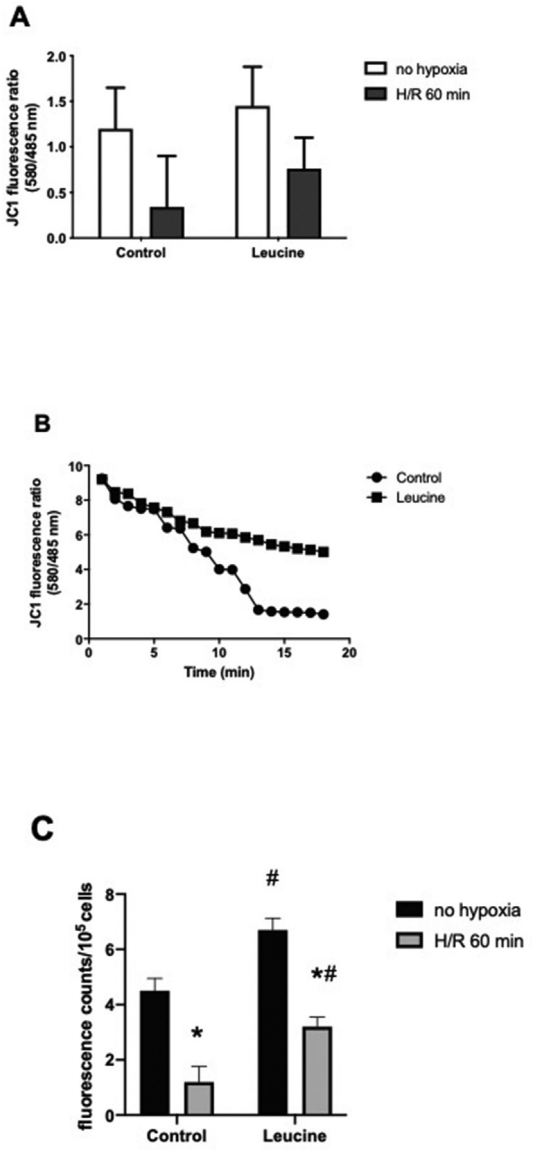

Figure 4 Maintenance of mitochondrial membrane potential by Leu treatment following SI/R. (A) Mitochondrial membrane potential measured in cells treated with Leu during reoxygenation injury using the membrane potential-sensitive dye JC-1 and TMRM after SI/R. Excitation ratio (red/green) indicates the mitochondrial membrane potential Data are shown as mean ± SD. * $p < 0.05$ vs. control. n = 4 per group. (B) Leu protects form shifts in mitochondrial membrane potential as assessed in cells by imaging JC-1 dye in perfusion chamber following induction of apoptosis by H2O2. Data are shown as mean. * $p < 0.05$ vs. control. n = 4 per group. (C) Mito PTP opening assay in cultured cardiomyocytes using calcein-AM in the presence of cobalt chloride. Data are shown as mean ± SD. * $p < 0.05$ vs. control. n = 4 per group.

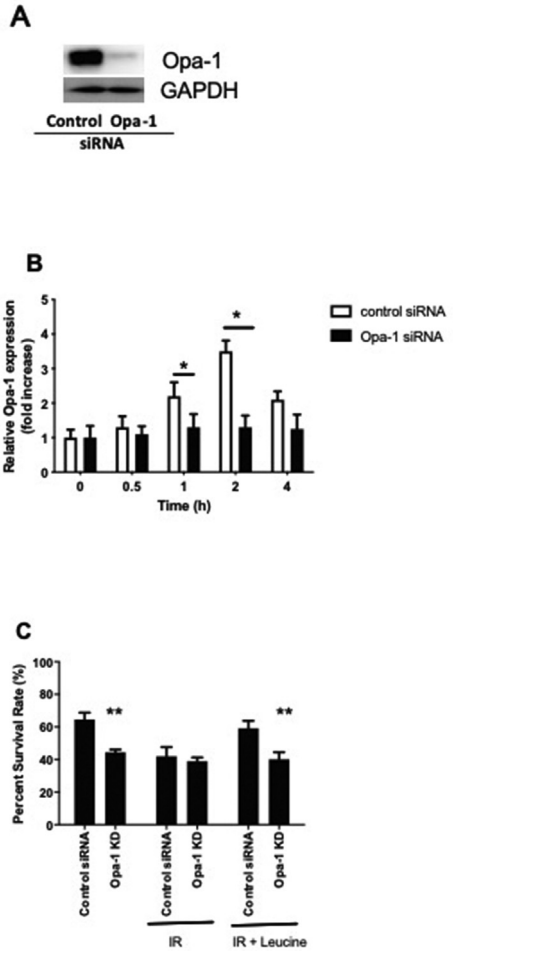

Figure 5 Effect of Opa-1 knockdown by siRNA in cardiomyocytes with IR. (A) Immunoblot analysis of knocked down Opa-1. (B) Relative Opa-1 expression (fold increase) is knocked down by siRNA. Data are shown as mean ± SD. n = 5 per group. (C) Effect of Opa-1 knockdown and Leu treatment (160 μM) on cell survival following hypoxia. Data are shown as mean ± SD. n = 5 per group. ** $p < 0.01$ vs. control siRNA.

signaling indeed plays a critical role in cardiac protection via Leu. Previously, we have reported the cardioprotective effects of BCAAs against cardiac I/R injury via mTOR activity in an *in vivo* rodent study [9] and Leu is considered to mediate mTOR activation [10,23]. Therefore, we consider that Leu treatment is a key factor of cardioprotective effect via BCAAs.

Leu, an essential amino acid, has been shown to stimulate both muscle protein synthesis and increased oxidative metabolism [24]. Further, Leu shows a unique ability to stimulate both favorable anabolic and catabolic processes in highly metabolically active tissues [25]. Although Leu can be used to health supplement to aid muscle gain in athletes or as a pharmacologic agent in patients with decreased muscle mass, such as those with sarcopenia, dermatomyositis, or rheumatism, it sometimes leads to adverse effects by activating mTOR. Moreover, Leu is known to be the amino acid that most effectively activates mTOR [10,23]. However, genetic alterations that result in mTOR activation are frequent in human cancers [26]. In addition, rapamycin is used as antitumor agent Although Leu can be used as a health supplement or a pharmacologic agent, it can also have negative effects through its activation of mTORIn the present study, we have shown the effectiveness of Leu treatment in cardioprotection. However, further studies are needed to elucidate any adverse effects associated with this treatment strategy.

In the current study, we also evaluated the effects of Leu in improving mitochondrial functions. Mitochondrial dynamics are regulated by mitochondrial fusion and fission and prescribe mitochondrial biogenesis and degradation [27,28]. These processes maintain mitochondrial homeostasis and regulate mitochondrial form, volume and function. Mitochondrial dynamics vary based on the developmental stage and age, cell type, environmental factors, and genetic background [29]. Opa-1 is known to be a key regulator of mitochondrial fusion and plays a role in preventing apoptosis and maintaining mtDNA [30]. Olichon et al. have reported that Opa-1 controls cristae structure of the inner mitochondrial membrane and thereby, the release of cyt-c during apoptosis [30]. The increase in OPA-1 expression appears to control mitochondrial dynamics, although the detailed mechanisms remain unclear. In the present study, we demonstrated that Leu treatment promotes mitochondrial fusion in cardiomyocytes via Opa-1. Previous studies have shown that several mechanisms can be implicated in Opa-1 processing in response to cardiac IR injury. Rodríguez-Graciani et al. reported that IR-induced cardiac and mitochondrial dysfunctions are associated with OMA1 activation and L-Opa-1 cleavage. However, a mitochondria-targeting ROS scavenger, a permeability transition pore inhibitor and their combination do not prevent these changes despite improved heart and mitochondria [31]. This group also demonstrated that high Ca2+ (the main PTP inducer) induces OPA1 cleavage in vitro in isolated cardiac mito and OPA1 KD cells are less sensitive to Ca2+-induced swelling and display high mito ROS production [32]. OPA1 down-regulation/OMA1 activation could stimulate mito fragmentation and disturbs cristae morphology as well as induce apoptosis [33, 34]. Moreover, activating AMPK/mTOR signaling by leucine ameliorates myocardial ischemia-reperfusion injury via modulating apoptosis and autophagy. Our findings indicate that the impairment of mitochondrial fusion by Opa-1 knockdown suppresses Leu-induced cardiac protection. Furthermore, Ψmt during ROS stimulation was maintained by Leu treatment. Because the stability of Ψmt attenuates mitochondrial permeability transition pore, we consider that the cardioprotective effect of Leu may be associated with mitochondrial function as well. Further, our results indicate that mitochondrial function is ameliorated via Leu-induced mitochondrial biogenesis.

In conclusion, the present study suggests that Leu treatment plays a critical role in cardioprotection via BCAAs. Furthermore, Leu improves mitochondrial function.

Declaration of competing interest

The authors have nothing to disclose.

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