Doctorial Thesis

Regulation of Branched-Chain Amino Acid Catabolism

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Abstract

Leucine is an essential branched-chain amino acid and plays a key role in protein synthesis through mammalian target of rapamycin (mTOR). Recently, it has been reported that an oral administration of leucine has been revealed to influence the plasma concentrations of other amino acids not only isoleucine and valine but also phenylalanine, tyrosine and methionine in humans. However, the regulatory mechanisms responsible for the leucine action remained to be clarified. In this study, to investigate whether the effect of leucine administration on plasma amino acid profiles is through system L amino acid transporter and/or mTOR complex 1 (mTORC1), mice were treated with and without 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) or rapamycin, respectively. The elevation of plasma leucine concentration after leucine administration was associated with a significant decrease in the plasma concentrations of isoleucine, valine, methionine, phenylalanine, and tyrosine, and BCH treatment almost completely blocked the leucine-induced decreases in plasma amino acid concentrations. Rapamycin treatment had much less effects on the actions of leucine than BCH treatment. These results suggest that leucine regulates the plasma concentrations of branched-chain amino acids (BCAAs), methionine, phenylalanine, and tyrosine, and system L amino acid transporters are involved in the leucine action.

Leucine administration also activates the branched-chain α-keto acid dehydrogenase (BCKDH) complex that regulates BCAA catabolism by

controlling the second step of the BCAA catabolic pathway. In the present study, to evaluate whether mTOR is involved in the action of leucine, the effect of mTORC1 inhibitor, rapamycin, on cardiac BCKDH complex activity in mice were examined. Oral administration of leucine in control mice significantly activated the cardiac BCKDH complex with an increase in cardiac concentrations of leucine and α-ketoisocaproate. However, rapamycin treatment significantly suppressed the leucine-induced activation of the complex despite similar increases in cardiac leucine and α-ketoisocaproate levels. Rapamycin treatment fully inhibited mTORC1 activity, measured by the phosphorylation state of ribosomal protein S6 kinase 1. These results suggest that mTORC1 is involved in the regulation of cardiac BCAA catabolism.

Abbreviations

4EBP-1	4E-eukaryotic initiation factor bindingprotein-1
4F2hc	4F2 cell-surface antigen heavy chain
AMPK	AMP-activated protein kinase
ASCT2	Na+ -neutral AA exchanger 2
BCAA	branched-chain amino acid
BCAT	branched-chain aminotransferase
BCH	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
BCKA	branched-chain α-keto acid
BCKDH	branched-chain α-keto acid dehydrogenase
BDK	BCKDH kinase
BDP	BCKDH phosphatase
BW	body weight
CoA	coenzyme A
DEPTOR	DEF domain-containing mTOR-interacting protein
DMB	1,2-diamino-4,5- methylene-dioxybenzene
DTT	dithiothreitol
ECL	enhanced chemiluminescence
eIF4E	eukaryotic initiation factor 4E
FKBP12	12kDa FK506-binding protein
HPLC	high-performance liquid chromatography
i.p.	intraperitoneal
IRS-1	insulin receptor substrate 1
KIC	α-ketoisocaproate

KIV	α-ketoisovalerate
KMV	α-keto-ß-methylvalerate
LAT	system L amino acid transporter
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3
mLST8	mammalian lethal with SEC13 protein 8
mSIN1	mammalian stress-activated map kinase-interacting protein1
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PI3K	phosphoinositide 3-kinase
PIKK	phosphoinositide 3-kinase-related protein kinases
PPAR α	peroxisome proliferator-activated receptor α
PRAS40	40 kb proline-rich kinase B substrates
PROTOR	protein observed with RICTOR
PVDF	polyvinylidene difluoride
Rags	Ras-related GTPases
RAPTOR	regulatory-associated proteins of mTOR
RICTOR	rapamycin-insensitive companion of mTOR
S6K1	ribosomal protein S6 kinase 1
TCA	tricarboxylic acid cycle
TOR	target of rapamycin
TPCK	phenylalanyl chloromethyl ketone
TPP	thiamine pyrophosphate
Vps34	vacuolar protein sorting 34

Chapter 1 General introduction

1. The importance of branched-chain amino acids

The branched-chain amino acids (BCAAs) leucine, isoleucine and valine are among the most hydrophobic amino acids (Creighton, 1993). The hydrophobicity of the BCAAs, as well as the hydrophobicity of phenylalanine and methionine, is important for the structure and function of globular proteins, membrane proteins, and the formation of coiled-coils because it affects the folding and stability of the final structure (Dill, 1990). For example, BCAAs comprise 37% of the amino acids in lung surface protein B (Hawgood et al., 1998), and help drive the interaction between the transmembrane domains of membrane proteins with the phospholipid bilayer (Tomita et al., 1978; Hawgood et al., 1998). Leucine and its hydrophobic associates happen in coiled-coiled α-helices in proteins as myosin, fibrinogen, and various transcription factors (Glover and Harrison, 1995; Walshaw and Woolfson, 2003). Several papers have also suggested that increased hydrophobic interactions due to a higher number of leucine residues are important for the thermostability of proteins in thermophilic organisms (Baldwin, 1986; Zhou et al., 2007).

In addition to the roles of the building blocks for proteins (Harper et al.,

1984), BCAAs are substrates for energy production under caloric shortage conditions such as during endurance exercise (Odessey et al., 1974; Rennie et al., 2006), and are crucial for the biosynthesis of other molecules by acting as sources of nitrogen and carbon (Harper et al., 1984).

BCAAs also play an important signaling role in modulating the growth and metabolism of an organism. Leucine in particular promotes muscle protein synthesis by stimulating the initiation of mRNA translation (Buse and Reid, 1975; Hong and Layman, 1984; Anthony et al., 2000a), at least partially via protein kinase mammalian target of rapamycin (mTOR) (Anthony et al., 2001; Vary and Lynch, 2007). Furthermore, leucine suppresses protein (amino acid) deficiency-induced myofibrillar protein degradation by inhibiting autophagy-lysosomes (Sugawara et al., 2009). BCAAs favorably affect nutrient signaling and thus can impact obesity, insulin resistance, and type 2 diabetes (Lynch and Adams, 2014). BCAAs also regulate the release of hormones such as leptin and GLP-1, thus possibly influencing food intake and blood glucose levels (Lynch et al., 2006; Chen and Reimer, 2009; Potier et al., 2009; Guo et al., 2010; Torres-Leal et al., 2011). Furthermore, the BCAAs adjust the amino acid profile of the brain, and secondarily affect brain function by competing with neutral amino acids that are the precursors of neurotransmitters entering the brain (Fernstrom, 1983; Fernstrom, 2005).

The involvement of BCAAs in numerous biological pathways makes their continuous supply essential, yet excess BCAAs and their metabolites must be

eliminated quickly because of their harmful effects on mammalian cells (Silberman et al., 1961; Yudkoff et al., 2005). Consequently, the catabolism of BCAAs should be tightly controlled.

2. Catabolism of BCAAs

The first procedure of the catabolic breakdown of BCAAs, catalyzed by branched-chain aminotransferase (BCAT, EC 2.6.1.42), is a reversible reaction that produces the corresponding branched-chain α-keto acids (BCKAs). The BCKAs are then catalyzed by the branched-chain α-keto acid dehydrogenase (BCKDH) complex in an irreversible reaction that yields the corresponding branched-chain acyl-CoA esters that are ultimately oxidized by the TCA cycle to generate energy, or utilized as gluconeogenic or ketogenic precursors (Doering et al., 1998).

BCAT is highly expressed in pancreas, heart and kidney, somewhat expressed in skeletal muscle, and only slightly expressed in liver (Ichihara and Koyama, 1966). Although it is nutritionally important that this enzyme functions properly, no physiologically unique mechanism for regulation of BCAT has been identified. On the other hand, the function of BCKDH complex must be tightly governed.

The BCKDH complex is composed of three catalytic constituents: a branched-chain α-keto acid dehydrogenase (E1, EC 1.2.4.4), a dihydrolipoyl transacylase (E2, EC 2.3.1.168), and a dihydrolipoyl dehydrogenase (E3, EC

1.8.1.4) (Pettit et al., 1978; Heffelfinger et al., 1983). The BCKDH complex is modulated by two enzymes: BCKDH kinase (BDK, EC 2.7.11.4) and BCKDH phosphatase (BDP, EC 3.1.3.16). BDK and BDP control the activity state of the BCKDH complex through a phosphorylation-dephosphorylation cycle (Shimomura et al., 1990; Zhou et al., 2012).

BCKDH activity in tissue is regulated by three mechanisms: allosteric inhibition, reversible covalent phosphorylation, and expression control. The BCKDH complex is susceptible to end product inhibition, especially by NADH and CoA esters (Boyer and Odessey, 1990; Boyer and Odessey, 1991). BDK and BDP reversibly covalently phosphorylate and dephosphorylate the BCKDH complex, respectively. BDK is controlled both by α-ketoisocaproate (KIC), the transamination product of leucine which can allosterically inhibit BDK activity (Hutson and Harper, 1981), and by its association with the BCKDH complex (Shimomura et al., 2001). Animal studies on modified nutritional states caused by diets containing various protein levels or by treating with insulin have led to the proposal that an alteration in BDK subunit gene expression is related to altered activity of the BCKDH complex (Zhao et al., 1993; Costeas and Chinsky, 1996). The literature suggests that transient and persistent covalent modification of the BCKDH complex is the most effective approach for regulating the activity of the BCKDH complex.

BDK can be regulated by both allosteric inhibition and gene expression in tissue. Short term inhibition is usually accomplished by BDK inhibitors such as

BCKAs, thiamine pyrophosphate (TPP), clofibic acid, phenylpyruvate, and dichloroacetate (Harris et al., 2001). In contrast, a change in gene expression to alter the concentration of BDK involves nutrient status or hormones such as thyroid hormone, glucocorticoids, and ligands for peroxisome proliferator-activated receptor α (PPAR α) (Harris et al., 2001). Little is known about the molecular level regulation of BDP.

3. System L amino acid transporter (LAT)

Amino acids are transported across the membrane by several amino acid transporters combined with the counter transport of ions such as Na^+, K^+, and Cl^- (Christensen, 1990; Palacín et al., 1998; Bröer, 2002). The uptake of a specific amino acid into a cell may occur by different transport systems. For example, glutamine can be transferred into hepatocytes by two secondary active transporters, system A and system N, and by system ASC exchanger (Bode, 2001). Amino acid exchangers such as system L, ASC, and y^+L may work as tertiary active transporters, employing differences in the transmembrane amino acid concentration caused by secondary active transport to facilitate the transport of other amino acids (Bröer, 2002; Meier et al., 2002).

System L amino acid transporter is an obligatory 1:1 exchanger of BCAAs, aromatic amino acids, and the non-metabolizable analogue 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), with a concomitant

efflux of cytoplasmic amino acids such as glutamine (Mastroberardino et al., 1998; Meier et al., 2002). System L amino acid transporter consists of two subunits: a catalytic light chain, and a heavy chain 4F2hC encoded by the SLc3A2 gene (Pineda et al., 1999; Ritchie and Taylor, 2001). The catalytic light subunit has four isoforms, LAT1-LAT4. Both LAT1 (encoded by the Slc7A5 gene) and LAT2 (encoded by the Slc7A8 gene) exhibit much higher extracellular (in the micromolar range) than intracellular (in the millimolar range) apparent substrate affinities. Furthermore, tissues such as skeletal muscle express both high-affinity (LAT1) and low-affinity (LAT2) system L amino acid transports for BCAAs and large neutral amino acids (Verrey et al., 2003), thus possibly helping to alleviate cellular starvation and aiding recuperation from starvation (Levy et al., 2011).

Amino acid transporters at the cell surface may have dual roles, acting as both sensors and carriers, to interact with intracellular nutrient-signaling pathways and thus regulate metabolism. For example, removal of LAT1 substrate leucine in the cell culture or loss of ASC2 or 4F2hc/LAT1 at the cell surface inhibits mTOR signaling (Fuchs and Bode, 2005). As expected, muscle-specific Slc7A5 knockout mice fed a high protein (30%) diet show mild insulin resistance and lower mTOR complex 1 (mTORC1) pathway activation in skeletal muscle (Poncet et al., 2014). Although sensing and signaling by amino acid transporters may help regulate the metabolic functions of cells and tissues, it remains unknown to what extent a specific amino acid transporter

such as LAT1 influences both the plasma and intracellular levels of amino acid and/or mTORC1 signaling.

4. mTOR

Evidence to date suggests that mTORC can integrate the flow of energy and nutrients to support cell metabolisms due to the function of mTOR protein kinase to simultaneously recognize energy-rich molecules, nutrients, and stress-related compounds (Zoncu et al., 2011). mTOR is an approximately 290 kDa protein and is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family. In mammalian cells, mTOR signals two different complexes, mTORC1 and mTORC2, which can be distinguished by their unique components and sensitivity to the immunosuppressant drug rapamycin (Loewith et al., 2002; Jacinto et al., 2004). mTORC1 is composed of regulatory-associated proteins of mTOR (RAPTOR), a 40 kDa proline-rich kinase B substrate (PRAS40), mammalian lethal with SEC13 protein 8 (mLST8), DEF domain-containing mTOR-interacting protein (DEPTOR), and mTOR (Sancak et al., 2007; Haar et al., 2007; Peterson et al., 2009; Yip et al., 2010). mTORC2 consists of rapamycin-insensitive companion of mTOR (RICTOR), PRAS40, mLST8, DEPTOR, mammalian stress-activated map kinase-interacting protein1 (mSIN1), protein observed with RICTOR (PROTOR), and mTOR (Sarbassov et al., 2004; Frias et al., 2006; Yang et al., 2006; Pearce et al., 2007; Peterson et al., 2009). Several studies suggest that

rapamycin binds to the small protein 12 kDa FK506-binding protein (FKBP12) and then rapamycin-FKBP12 inhibits RAPTOR-bound, but not RICTOR-bound, mTOR (Sarbassov et al., 2004). Rapamycin might impede mTORC1 by dissociating RAPTOR from mTOR, thus blocking the interaction of mTOR with certain substrates (Kim et al., 2002; Yip et al., 2010). However, longer-term treatment with rapamycin can obstruct mTORC2 in some tissues and cell lines, resulting from less mTOR for assembly into mTORC2 by formation of rapamycin-FKBP12 complex (Sarbassov et al., 2006).

In mammalian cells, mTORC1 signals to the phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein1 (4E-BP1) associated with mRNAs, and adjusts the initiation and progression of mRNA translation, thus regulating protein synthesis (Ma and Blenis, 2009). Furthermore, mTORC1 actively inhibits autophagy, and suppression of mTORC1 by rapamycin or by the removal of amino acids can prompt autophagy (Hosokawa et al., 2009; Thoreen et al., 2009). Recent studies suggest that mTORC2 does not phosphorylate S6K1 or 4E-BP1, but functions as an arbitrator of actin cytoskeletal arrangement and cell-cycle progression (Loewith et al., 2002; Jacinto et al., 2004; Sarbassov et al., 2004).

Upstream regulators of mTORC1 include four prime signals: nutrient levels, growth factors, energy status, and stress. Nutrients, and particularly amino acid levels, regulate mTORC1 through mediators such as lipid kinase vacuolar protein sorting 34 (Vps34), a sterile 20 family kinase mitogen-activated protein

kinase kinase kinase kinase 3 (MAP4K3), and Ras-related GTPases (Rags) (Findlay et al., 2007; Bar-Peled and Sabatini, 2014). Growth factors such as insulin can signal to mTORC1 by activating PI3K and Akt through receptor phosphorylation of insulin receptor substrate 1 (IRS-1) (Wullschleger et al., 2006). Furthermore, mTORC1 activity is decreased by chemical inhibitors of glycolysis and mitochondrial function, suggesting that mTORC1 recognizes the energy status of cells (Dennis et al., 2001) through a mechanism involving AMP-activated protein kinase (AMPK) (Hardie, 2007; Gwinn et al., 2008). The upstream regulators of mTORC2 remain unknown.

5. Aim of this thesis research

In the present study, it was addressed whether the effects of leucine administration on the plasma amino acid profile and the BCAA catabolism might be mediated through the activation of LAT and/or mTOR. The study consisted of two parts: in the first part, the plasma amino acid concentrations after leucine ingestion were examined using mice with treatment of the specific inhibitor BCH or rapamycin against LAT and mTORC1, respectively; the second part, the leucine-induced activation of cardiac BCKDH complex was evaluated in rapamycin-treated mice.

Chapter 2 Regulation of the plasma amino acid profile by leucine via the system L amino acid transporter[1]

1. Introduction

The BCAAs, leucine, isoleucine, and valine, cannot be synthesized endogenously in mammals (Hutson et al., 2005), and are therefore classified as nutritionally essential or indispensable amino acids. Mounting evidence indicates that BCAAs have various physiological and metabolic functions (Hutson et al., 2005; Lynch and Adams, 2014). It has been reported that intake of individual or combined BCAAs (Hargrove et al., 1988; Matsumoto et al., 2014) or ingestion of proteins (Am et al., 1992) affects the plasma amino acid profile. In addition of acute changes in the plasma amino acid profiles associated with ingestion of amino acids and proteins, the plasma amino acid profile has been the focus of increasing attention because of its prospective application in the diagnosis and assessment of disease and pathophysiological states (Noguchi et al., 2006; Hisamatsu et al., 2012). Moreover, repeated BCAA supplementation has been demonstrated to improve the abnormalities of the plasma amino acid profile as well as the nutritional status in patients with

[1] Zhen, H., Nakamura, K., Kitaura, Y., Kadota, Y., Ishikawa, T., Kondo, Y., Xu, M., and Shimomura, Y. (2015). Regulation of the plasma amino acid profile by leucine via the system L amino acid transporter. Biosci. Biotechnol. Biochem. *79*, 2057–2062. Biosci. Biotechnol. Biochem. grants the rights to the author(s) for this research article published in the Biosci. Biotechnol. Biochem. to be reprinted in the author(s) thesis.

liver cirrhosis (Kawaguchi et al., 2011). Although BCAA (especially leucine) administration has been shown to have a great impact on the plasma amino acid profile (Eriksson et al.,1981; Matsumoto et al., 2014), the physiological significance and the mechanism responsible for the regulation of plasma amino acid concentrations after leucine administration are largely unknown.

The BCAAs leucine, isoleucine, and valine share the same transport system across the plasma membrane the LAT. The transport of BCAAs in and out of cells and membrane-bound intracellular compartments involves the movement of ions, including Na^+, H^+, K^+, and/or Cl^-, as well as the movement of other amino acids by antiporters (Taylor, 2014). LAT, a Na^+-independent amino acid transporter, functions in the movement of neutral amino acids, including several essential amino acids (Nicklin et al., 2009). Therefore, it is of interest to consider the role of LAT in the regulation of leucine-induced alterations in the plasma amino acid profile.

BCAAs (particularly leucine) function both as activators of protein synthesis and inhibitors of proteolysis to increase muscle mass, primarily through the upregulation of mammalian target of rapamycin complex 1 (mTORC1) (Dodd and Tee, 2012). While leucine-induced mTORC1 activity plays an import role in the regulation of protein turnover, its involvement in the regulation of plasma amino acid concentrations after protein ingestion or leucine administration remains unknown.

In this study, the functions of LAT and mTORC1, respectively, in the

regulation of plasma amino acid concentrations were examined by using the specific inhibitors BCH and rapamycin, and demonstrated that LAT is, but mTORC1 may not be, involved in leucine-induced alterations in plasma amino acid concentrations.

2. Materials and methods

2-1 Reagents.

Heparin was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan), rapamycin was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), BCH was from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA), and leucine was provided by Ajinomoto Co., Inc. (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2-2 Treatment solutions.

BCH solutions (final concentrations of 0.5-4% (w/v)) for intraperitoneal (i.p.) injection were prepared by dissolving BCH in 5% (v/v) ammonia neutralized with HCl. The neutralized ammonia solution without BCH was used as the corresponding vehicle (Veh). A rapamycin stock solution was prepared by dissolving 11.25 mg/mL rapamycin in 99.5% ethanol. To prepare the rapamycin solution (0.45 mg rapamycin/mL (4% ethanol)) used for injection, the stock solution was diluted with 5% (w/v) polyethylene glycol-400 (PEG) and 5% (w/v) Tween 80 (Hartman et al., 2012) immediately prior to use. The solution without rapamycin was used as the corresponding Veh. Leucine was dissolved in saline at a concentration of 2% (w/v).

2-3 Animals.

The Animal Care Committee of Nagoya University Graduate School of Bioagricultural Sciences approved all the procedures for the care and use of animals in this study. Male ddY mice aged 7-8 weeks were obtained from Japan SLC Inc. (Hamamatsu, Japan) and were maintained in a conventional animal room under controlled temperature (22 ± 1 °C) and 12-h light-dark cycle (lights on at 08:00) conditions. All the animals were ad libitum fed a commercial diet (CE2; CLEA Japan, Tokyo, Japan) and tap water for 1 week during acclimatization and were then used in experiments.

2-4 Experimental design for BCH treatment.

In the first experiment, the temporal effect of BCH treatment on leucine-induced alterations in plasma BCAA concentration was examined. Mice were fasted for 12 h before the experiment, which was initiated at 23:00 one day before the experiment. At ~11:00 on the experiment day, animals were arbitrarily divided into 2 groups: Veh and BCH groups. To assess the temporal effect of BCH administration in relation to leucine administration, 4 mg BCH (using the 4% BCH solution) or vehicle were i.p. injected into mice 3 h before oral administration of leucine (0.45 g leucine/kg body weight (BW)) (Kadota et al., 2012) or saline (Sal), resulting in 4 groups of mice: Veh/Sal, Vel/Leu, BCH/Sal, and BCH/Leu (n = 3 per group). Blood (~50 μ L) was collected into a heparinized tube from tail immediately before administration of

BCH or Veh (at -180 min), and 0 (before), 30, 60, 90, 120, and 180 min after administration of leucine or saline. In the second experiment, the dose-dependent effect of BCH treatment on plasma BCAA concentrations after leucine administration was examined. The same procedure as described above was followed, except that (1) BCH at 0, 2, 4, or 6 mg/mouse was injected, (2) all groups of mice (n = 3 per group) were orally administered leucine, and (3) blood was collected from tail at 0, 30, and 60 min after leucine administration.

In the third experiment, four groups of mice (n = 4 in each group) were prepared as described in the first experiment. The mice were fasted for 12 h as outlined above and were arbitrarily divided into BCH and Veh groups. Animals were then injected with 6 mg BCH or Veh, respectively, 3 h before administration of leucine or saline as above. Blood was collected from tail at 0, 30, and 60 min after administration of leucine or saline.

2-5 Experimental design for rapamycin treatment.

In order to examine the function of mTORC1 in the regulation of plasma amino acid profiles after leucine administration, mice were treated with rapamycin (Rap) following the same procedure as for the third BCH experiment. Mice were arbitrarily divided into two groups: Veh and Rap groups. Mice were i.p. injected with 4.5 mg/kg BW rapamycin or vehicle. The dose of rapamycin chosen was able to completely inhibit mTORC1 activity (Hartman et

al., 2012). Three hours after injection of Rap or Veh, each group was subdivided into two groups: saline (Veh/Sal and Rap/Sal) and leucine (Veh/Leu and Rap/Leu) groups (n = 6-8 per group). These subgroups were orally administered saline or leucine (0.45 g/kg BW) (Kadota et al., 2012) respectively. The temporal and dose-dependent effects of rapamycin administration used in this experiment have been reported previously (Hartman et al., 2012). Blood samples were collected from tail at 0, 30, and 60 min after administration of saline or leucine. Skeletal muscles (gastrocnemius and plantaris muscles) were also collected from mice at 60 min after administration of saline or leucine under anesthesia, and the animals were killed by exsanguination.

2-6 Analyses of plasma amino acids.

In the first BCH experiment, the sum of the three BCAAs in the plasma obtained after centrifugation of blood was measured spectrophotometrically using leucine dehydrogenase (Beckett et al., 1996). In the other experiments, plasma samples were deproteinized as reported previously (Parvy et al., 1995), and the free amino acid concentrations in the preparations were analyzed using an automatic amino acid analyzer (JLC-500/V; JEOL, Tokyo, Japan).

2-7 Analysis of phosphorylation of p70S6 kinase 1 (S6K1) in skeletal muscle.

Western blotting analyses to quantify the phosphorylation of S6K1 in skeletal muscle were conducted according to the method reported previously

(Bajotto et al., 2011).

2-8 Statistical analyses.

Values are expressed as means ± SEM. In the BCH experiment, the number of mice in each group was the same, and the data were analyzed using a two-way ANOVA and Fisher' s PLSD test. In the rapamycin experiment, the number of mice in the groups differed, and the data were analyzed using a two-way ANOVA and the Turkey-Kramer method. Values of $P < 0.05$ were considered significantly different.

3. Results

3-1 Temporal effects of BCH treatment on plasma BCAA concentration after leucine administration

In the first experiment, BCH (4 mg/mouse) was injected into the BCH/Sal and BCH/Leu groups 3 h before administration of leucine or saline. The plasma BCAA concentration in the Veh/Sal or BCH/Sal group was largely unchanged during the 6-h experimental period (from -180 to 180 min) (Fig. 1). The plasma BCAA concentration in the Veh/Leu group increased quickly, peaking 30 min after leucine administration and then decreased to the same level as in the Veh/Sal group 60 min after leucine administration (Fig. 1). Although the plasma BCAA concentration in the BCH/Leu group exhibited a similar peak after leucine administration as the Veh/Leu group, it decreased comparatively slowly, resulting in a BCAA concentration at 60 and 90 min after leucine administration that was significantly higher than in the Veh/Leu group. These results suggest that 4 mg BCH treatment 3 h before leucine administration significantly suppressed BCAA transport from the blood to the cells.

In preliminary experiments, the effects of BCH treatment at 1, 3, or 5 h before leucine administration were examined; however, the BCH effect of reducing the rate of decrease in plasma BCAA levels after peaking was not observed (data not shown). Therefore, the 3 h time point before leucine

administration was chosen as the appropriate timing for BCH treatment in the following experiments.

3-2 Dose-dependent effects of BCH on plasma BCAA concentrations after leucine administration

In the second experiment, the dose-dependent effects of BCH were examined by injection of 0, 2, 4, and 6 mg BCH/mouse. The plasma leucine concentration peaked 30 min after leucine administration regardless dose of the BCH treatment (Fig. 2), as described above. The BCH treatment dose-dependently reduced the decrease in leucine concentration after peaking and appeared to suppress the leucine-induced reduction in the concentrations of other BCAAs (Fig. 2). Therefore, BCH treatment at 6 mg/mouse was used in the following experiment.

3-3 Effects of BCH treatment on plasma amino acid profile after leucine administration.

In the third experiment, four groups of mice (Veh/Sal, Veh/Leu, BCH/Sal, and BCH/Leu) were prepared and temporal changes in the plasma amino acid concentrations for 60 min after leucine administration were examined. In order to clarify the changes in the amino acid profile, the plasma concentrations of amino acids at 60 min after leucine administration were calculated relative to the concentration before leucine administration (the relative 60-min concentration). Leucine administration significantly increased the plasma leucine concentration, peaking at 30 min after administration, as described

above (Fig. 3 A). The relative 60-min concentration of plasma leucine was significantly greater in the BCH/Leu group than in the Veh/ Leu group (Fig. 3 B). Leucine administration gradually decreased plasma concentrations of isoleucine, valine, methionine, phenylalanine, and tyrosine, resulting in significantly lower relative concentrations at 60 min of these amino acids in the Veh/Leu group than in the Veh/Sal group (Fig. 3 C-L). However, leucine administration did not affect the plasma concentrations of the other amino acids measured (data not shown). The leucine-induced decreases in plasma concentrations of the amino acids were significantly suppressed by BCH treatment, and the relative 60-min concentrations of isoleucine (Fig. 3 D), methionine (Fig. 3 H), phenylalanine (Fig. 3 J), and tyrosine (Fig. 3 L) were almost the same in the BCH/Leu and BCH/Sal groups. The relative 60-min valine concentration (Fig. 3 F) was also significantly higher in the BCH/Leu group than in the Veh/Leu group; however, the leucine-induced decrease in valine concentration was not completely blocked by BCH treatment.

3-4 Effects of rapamycin treatment on plasma BCAA concentrations after leucine administration

The effects of rapamycin treatment on plasma amino acid profiles after leucine administration were examined by the same method as that for the third BCH experiment, except for that rapamycin was used in place of BCH. Leucine administration showed a similar increase in the plasma leucine concentration in mice with or without rapamycin treatment, whereas the peak level at 30 min

after leucine administration was slightly, but significantly, higher in the Rap/Leu group than in the Veh/Leu group (Fig. 4 A). Thereafter, the plasma leucine concentration declined, and the relative 60-min concentration in the Veh/Leu and Rap/Leu groups did not differ. Leucine administration decreased plasma concentrations of isoleucine and valine, and these decreases were largely unaffected by rapamycin treatment; the relative 60-min concentrations of plasma isoleucine and valine were not different between the Veh/Leu and the Rap/Leu groups, although the decrease in plasma isoleucine concentration at 30 min after leucine administration was somewhat suppressed in the rapamycin-treated group (Fig. 4 C-F). Rapamycin treatment did not affect the leucine-induced alterations in plasma concentrations of the other amino acids measured (data not shown). These results indicate that the rapamycin treatment had much less effects on the leucine-induced alterations in the plasma amino acid profile than the BCH treatment.

In order to confirm the efficacy of rapamycin, the phosphorylation state of S6K1 of skeletal muscle was measured in this experiment. The rapamycin treatment completely inhibited leucine-induced phosphorylation of muscle S6K1 (Fig. 5).

4. Discussion

In this study, the results indicated that the elevated plasma leucine concentration after leucine administration was associated with significant decreases in plasma concentrations of isoleucine, valine, methionine, phenylalanine, and tyrosine in mice. These results are comparable with previous findings in humans administered single BCAAs (Hagenfeldt et al., 1980; Eriksson et al., 1981; Matsumoto et al., 2014) or three BCAAs combined (Shimomura et al., 2009). Notably, it was previously demonstrated that this change in plasma amino acid profiles was not observed with isoleucine or valine administration in humans (Matsumoto et al., 2014). Since leucine and the other amino acids whose plasma concentrations were affected by leucine administration are substrates for LAT, BCH was employed as a selective inhibitor of the amino acid transporter and clearly showed that BCH treatment slowed the rate of decrease in plasma leucine concentrations after peaking and almost completely blocked the leucine-induced decreases in the other amino acids described above. These findings suggest that leucine plays an important role in the regulation of the plasma amino acid profile and that LAT is involved in the mechanism of leucine action.

It is interesting to consider that the peak concentration of plasma leucine at 30 min after leucine administration was not affected by BCH treatment, suggesting that absorption of leucine in the gut was not affected by BCH treatment because LAT is not a main BCAA transporter in the gut (Castagna et

al., 1997). The other interesting point is that the leucine-induced decreases in the plasma amino acid concentrations were greater at 60 min than at 30 min after leucine administration, suggesting that there is a lag time for the leucine action to decrease the plasma concentrations of other amino acids. It might be explained by the hypothesis that the elevated leucine concentration in the cells (such as muscle cells), but not in the circulation, may be responsible for activation of the LAT, resulting in promotion of the influx of other amino acids into cells from the circulation. It has been reported that there are four isoforms of the system L amino acid transporter, LAT1-LAT4 (Kanai et al., 1998; Segawa et al., 1999; Babu et al., 2003; Bodoy et al., 2005). Although all of the isoforms are strongly inhibited by BCH, substrate specificity differs slightly among isoforms; all LATs have a high affinity for BCAAs, methionine, and phenylalanine compared to other amino acids, but only LAT1 and LAT2 show high affinity for tyrosine (Kanai et al., 1998; Segawa et al., 1999). The Km values for these amino acids are lower in LAT1 than in LAT2, but the tissue distribution of expression is wider in LAT2 than in LAT1. In this study, leucine administration affected the plasma concentrations of BCAAs, methionine, phenylalanine, and tyrosine, suggesting that LAT1 and LAT2 may be the main modulators responsible for the effects of leucine. However, the regulation of LAT gene expression might not be involved in the leucine effects, as reported previously (Gran and Cameron-Smith, 2011). Therefore, it was hypothesized

that LAT1 and LAT2 in the plasma membrane may be activated and/or increased by the elevation of cytoplasmic concentration of leucine.

On the other hand, rapamycin administration had much less effects on leucine activity than BCH treatment, although the plasma concentrations of leucine and isoleucine 30 min after leucine administration were slightly affected by the rapamycin treatment. The rapamycin treatment was confirmed to effectively inhibit mTORC1 activity as measured by the phosphorylation of muscle S6K1. These results suggest that mTORC1 may be involved little in the effects of leucine to stimulate amino acid transport from the circulation into cells. Further studies are required to clarify the role of mTORC1 in the regulation of plasma amino acid concentrations.

The leucine-induced decreases of plasma amino acid concentration observed in this study do not occur in feeding of the regular diets, because proteins in the diet contain all of the amino acids required for protein synthesis. Rather, the leucine action, which may stimulate the amino acid influx into cells from the circulation, may contribute to increase the availability of amino acids for protein synthesis in cells. From a clinical point of view, it is important to clarify the regulation of plasma amino acid profiles, especially plasma BCAA concentrations, because these changes are associated with the onset and development of diseases such as hepatocarcinogenesis associated with liver cirrhosis (Kawaguchi et al., 2014), pancreatic adenocarcinoma (Mayers et al., 2014), and diabetes associated with insulin resistance (Lynch and Adams,

2014). Further detailed studies on the regulation of amino acid profiles will contribute to the diagnosis and treatment of these diseases.

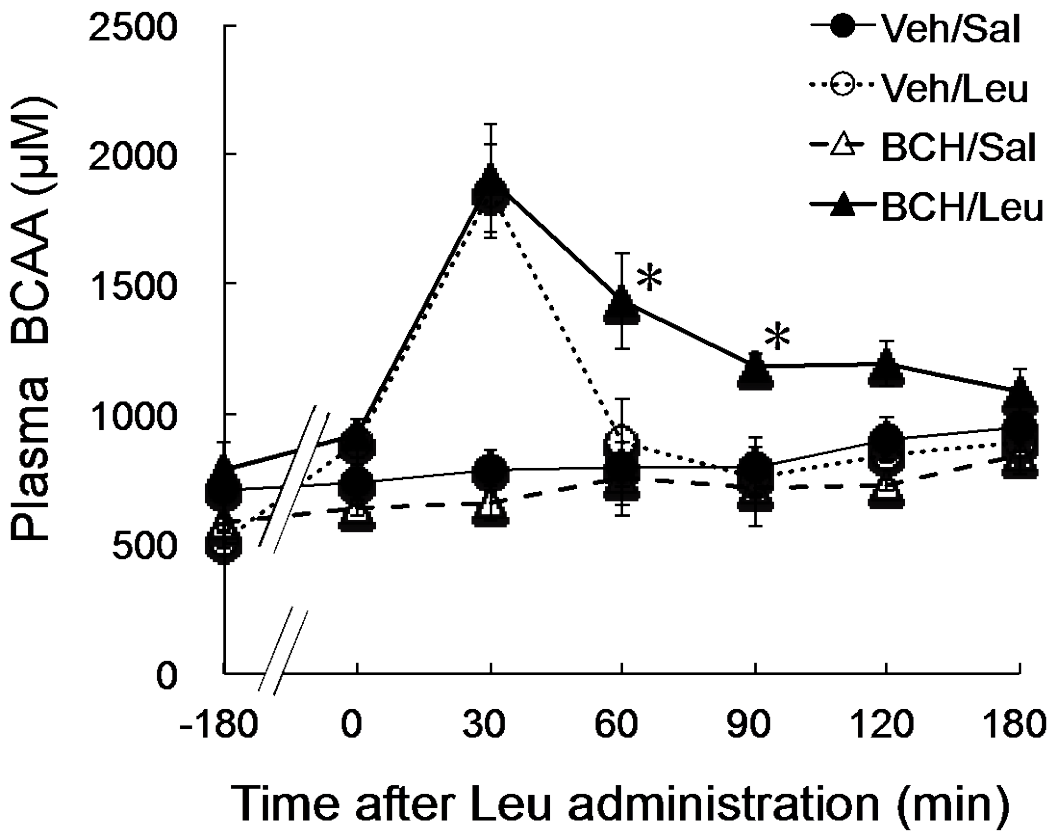

Fig. 1. Temporal effects of BCH treatment on plasma BCAA concentrations after leucine administration.

Notes: BCAA levels were measured spectrophotometrically with leucine dehydrogenase in mice with or without BCH treatment. Values are presented as means ± SE (n = 3). *$P < 0.05$ vs. other groups in the same time point.

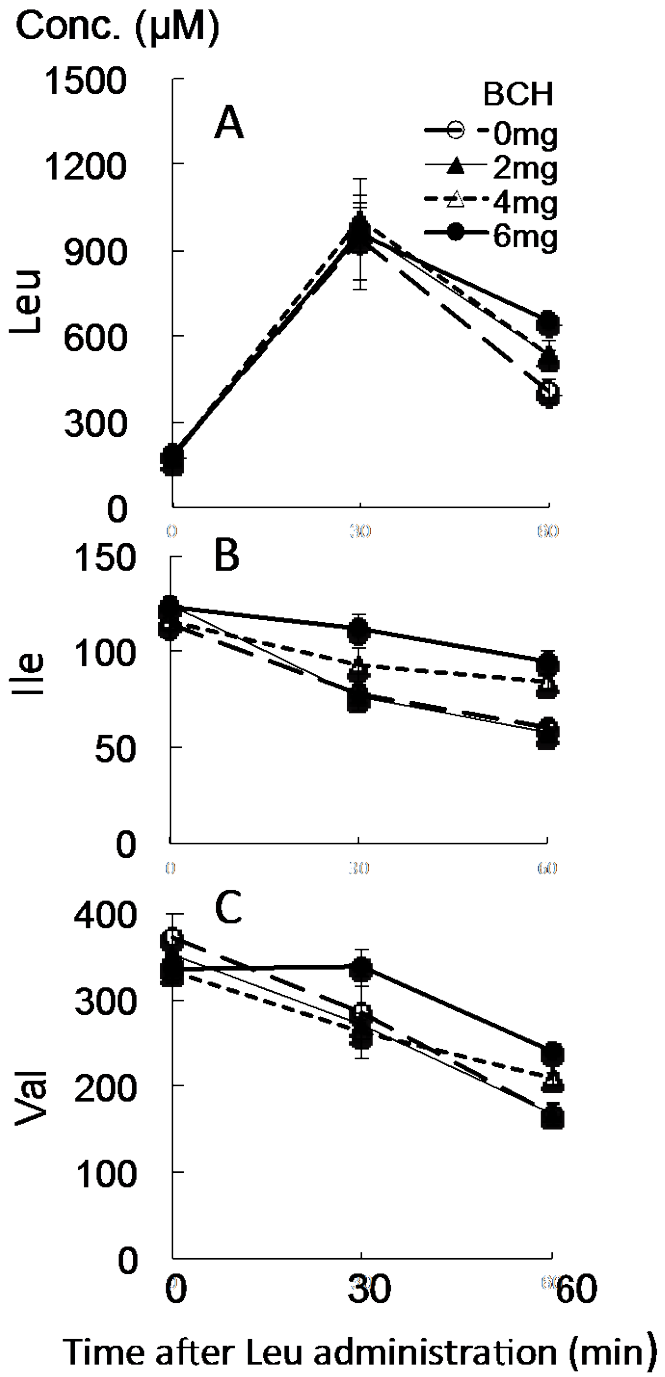

Fig. 2. Dose-dependent effects of BCH treatment on plasma BCAA concentrations after leucine administration.

Notes: Plasma concentrations of leucine (A), isoleucine (B), and valine (C) in mice with different doses (0–6 mg/mouse) of i.p. injected BCH after leucine administration. Values are presented as means ± SE (n = 3).

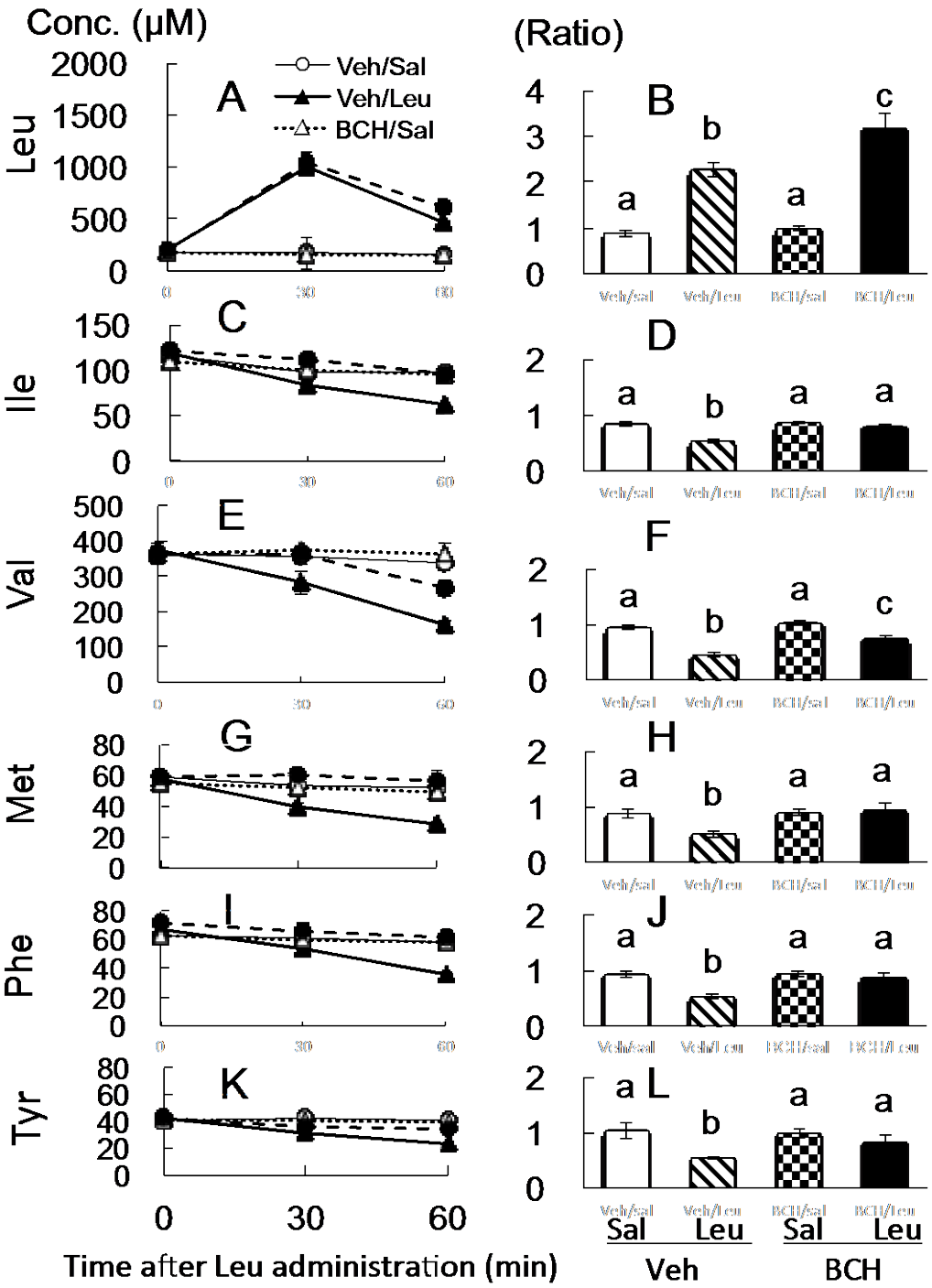

Fig. 3. Effects of BCH treatment on plasma amino acid profiles after leucine administration.

Notes: Plasma concentrations of leucine (A), isoleucine (C), valine (E), methionine (G), phenylalanine (I), and tyrosine (K) after 0.45 g/ kg BW leucine

administration in mice with or without 6 mg BCH injection. The ratio of plasma concentration of leucine (B), isoleucine (D), valine (F), methionine (H), phenylalanine (J), and tyrosine (L) at 60 min after leucine administration to those immediately before leucine administration in mice with or without BCH treatment. Values are presented as means ± SE (n = 4). Means without a common letter are significantly different, $P < 0.05$.

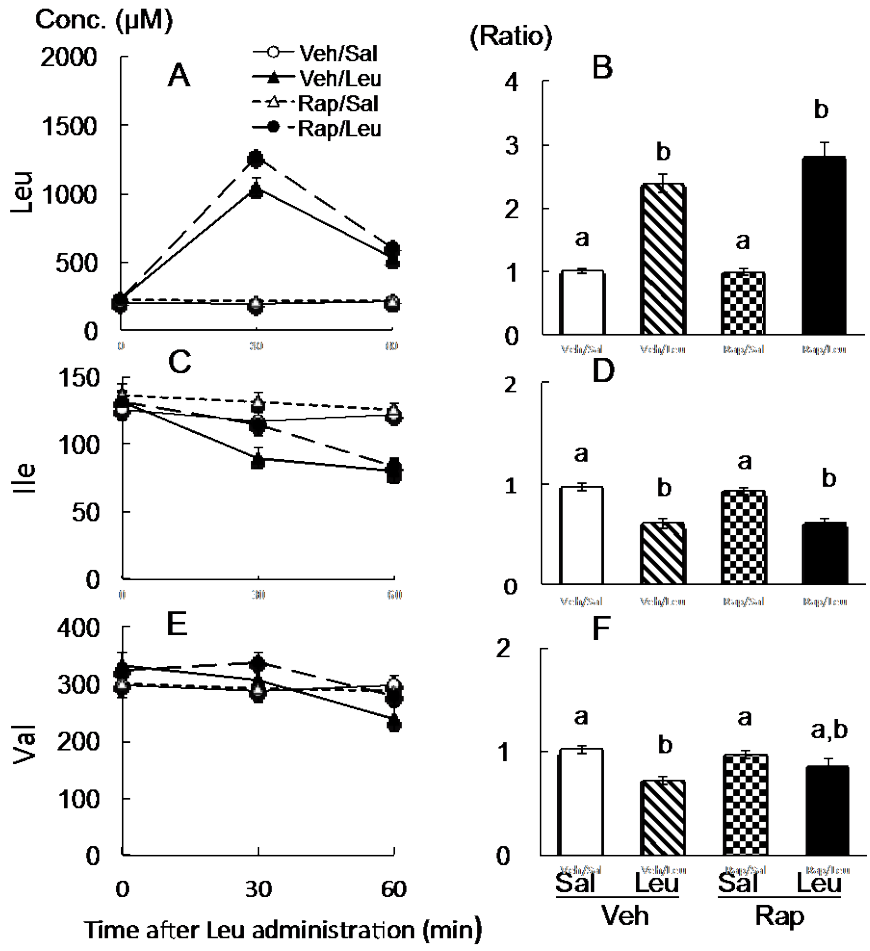

Fig. 4. Effects of rapamycin treatment on plasma BCAA concentrations after leucine administration.

Notes: Plasma concentrations of leucine (A), isoleucine (C), and valine (E) after 0.45 g/kg BW leucine administration in mice with or without 4.5 mg/kg rapamycin injection. The ratio of plasma concentration of leucine (B), isoleucine (D), and valine (F) at 60 min after leucine administration to those immediately before leucine administration in mice with or without rapamycin treatment. Values are presented as means ± SE (n = 6–8). Means without a common letter are significantly different, $P < 0.05$.

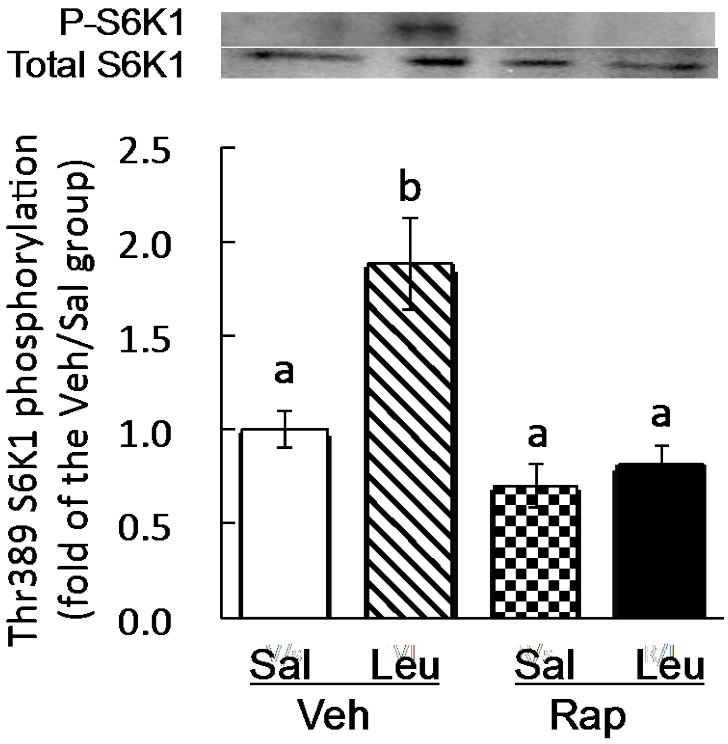

Fig. 5. Effect of rapamycin treatment on leucine-induced phosphorylation of S6K1 in skeletal muscle.

Notes: Skeletal muscles (gastrocnemius and plantaris muscles) were obtained in the experiment of rapamycin treatment. Total and phosphorylated S6K1 were measured by Western blotting analysis using anti-total-S6K1 and anti-phospho-S6K1 (Thr389) antibodies. Representative blots for both total and phosphorylated S6K1 (P-S6K1) are shown in the upper panel. Bar graph gives quantification of phosphorylated S6K1 relative to the Veh/Sal group of mice. Values are presented as means ± SE (n = 6). Means without a common letter are significantly different, $P < 0.05$.

Chapter 3　mTORC1 is involved in the regulation of BCAA catabolism in mouse heart[2]

1. Introduction

BCAAs, including leucine, isoleucine, and valine, are essential amino acids in mammals and must be continuously acquired from the diet. Since BCAAs regulate protein synthesis (Hutson et al., 2005; Anthony et al., 2000b), glucose metabolism (Lynch and Adams, 2014), and neurotransmitter synthesis (Cole et al., 2012), the catabolism of BCAAs must be tightly regulated. The first two steps of the catabolic pathway are common to the three BCAAs. BCAT catalyzes the first reversible transamination of BCAAs to produce BCKAs, and the BCKDH complex catalyzes the second irreversible oxidative decarboxylation of BCKAs to form corresponding coenzyme A (CoA) esters (Shimomura et al., 2004). The BCKDH complex catalyzes the rate-limiting step of BCAA catabolism and therefore is likely to be tightly controlled.

The BCKDH complex is regulated by a phosphorylation-dephosphorylation cycle. BDK is responsible for inactivation of the complex by phosphorylation of the E1α sub unit of the complex (Harris et al., 2001; Shimomura et al., 2001),

[2] Hongmin Zhen, Yasuyuki Kitaura, Yoshihiro Kadota, Takuya Ishikawa, Yusuke Kondo, Minjun Xu, Yukako Morishita, Miki Ota, Tomokazu Ito, and Yoshiharu Shimomura. mTORC1 is involved in the regulation of BCAA catabolism in mouse heart. FEBS Open Bio. *In Press.* *FEBS Open Bio grants* grants the rights to the author(s) for this research article published in the FEBS Open Bio to be reprinted in the author(s) thesis.

and BDP is responsible for reactivation of the complex by dephosphorylation (Damuni and Reed, 1987). BDK is considered to be a primary regulator of the BCKDH complex, because BDK activity is negatively correlated with the activity state of BCKDH complex (Shimomura et al., 2006). It was reported that protein starvation (Kobayashi et al., 1999), diabetes (Lombardo et al., 1999; Doisaki et al., 2010) and hormones (Doering et al., 1998; Shimomura et al., 2001) influence BDK expression to regulate the BCKDH activity in the long term. In acute regulatory mechanisms, BDK is downregulated by the transamination products of BCAAs (especially KIC) in an allosteric manner, which is accompanied by dissociation of BDK from the complex (Harris et al., 2004; Shimomura et al., 2004; Shimomura et al., 2006).

Among BCAAs, leucine is the most physiologically active amino acid, because it is not only an essential amino acid but also signals to mTORC1, which in turn activates S6K1 to promote mRNA translation (Proud, 2002). However, the exact cellular mechanisms regulating leucine activation of the mTORC1 signaling system remain undefined. Some reports have proposed that leucine-induced mTORC1 activation might be connected with the structural characteristics of leucine molecules (Lynch et al., 2000) or the second regulatory step of leucine catabolism (Xu et al., 2001). However, Beugnet et al. (2003) suggested that intracellular amino acid levels might be involved in mTORC1 activation, a finding supported by a study using cultured cells with enzymatic defects in leucine catabolism that showed that

leucine-induced activation of mTORC1 was related to leucine pool size and repletion capacity, but not leucine oxidation capacity (Schriever et al., 2013). From these findings, the correlation between mTORC1 signaling and the leucine catabolic pathway does not seem to be physiologically important. However, it has been clearly demonstrated that an increase in the cellular concentration of leucine activates both mTORC1 signaling and the BCAA catabolic pathway. Therefore, further studies are required to clarify the relationship between these systems. In the present study, we used rapamycin-treated mice to investigate the involvement of mTORC1 in the regulation of BCKDH complex activity.

2. Materials and methods

2-1 Materials

1,2-Diamino-4,5-methylenedioxybenzene (DMB) was obtained from Dojindo Laboratories (Kumamoto, Japan). Lambda protein phosphatase was acquired from New England BioLabs (Beverly, MA, USA). Rabbit polyclonal antibodies against total-S6K1, phospho-S6K1 (Thr389) and goat anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Cell Signaling Technology (Danvers, MA, USA) or Bio-Rad Laboratories (Hercules, CA, USA) for use in Western blot analyses. Antibody against the BCKDH complex and BDK were prepared as described previously (Doisaki et al., 2010). Enhanced chemiluminescence (ECL) reagents and immobilon polyvinylidene difluoride (PVDF) membranes were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). All other reagents were of analytical grade and were purchased from Wako (Osaka, Japan) or Sigma Aldrich Japan (Tokyo, Japan).

2-2 Animals and experimental design

The Animal Care Committee of Nagoya University Graduate School of Bioagricultural Sciences approved all procedures for the animal experiments in this study. Male ddY mice (aged 7 weeks, approximately 34 g body weight) were obtained from Japan SLC Inc. (Hamamatsu, Japan) and were maintained in a conventional animal room with controlled temperature (22 $\pm$ 2°C) and a 12

h light-dark cycle (lighting on at 08: 00). They were fed a commercial diet (CE2; CLEA Japan, Tokyo, Japan) and tap water for 1 week ad libitum during acclimatization in the animal room and were then used in experiments.

Mice were fasted for 12 h before experiment initiation at 2300. Animals were arbitrarily divided into 2 groups: vehicle (Veh) and rapamycin (Rap) groups. Mice in each respective group were injected intraperitoneally with rapamycin 4.5 mg/kg BW or vehicle. The rapamycin was prepared by diluting a stock solution (11.25 mg/ml in 99.5% (v/v) ethanol, stored at -30°C) to a final concentration of 4% (v/v) ethanol in the vehicle immediately prior to use. The vehicle consisted of 5% polyethylene glycol (PEG)-400 and 5% Tween 80 (Hartman et al., 2012).

Three hours after injection of vehicle or rapamycin, each group was subdivided into 2 subgroups: the saline (Veh/Sal or Rap/Sal) and leucine (Veh/Leu or Rap/Leu) subgroups. These subgroups of mice were orally administered saline (22.5 ml/kg BW) or 2% leucine solution in saline (0.45 g leucine/kg BW) (Ishiguro et al., 2006), respectively. Thirty minutes after administration of saline or leucine, the mice were anesthetized with isoflurane and then sacrificed by collecting blood from the inferior vena cave to prepare plasma for measuring amino acid concentrations. The heart was then removed and immediately freeze-clamped at liquid nitrogen temperature and stored at -80°C until analyses.

2-3 Amino acid analysis

Plasma samples were mixed with 3% sulfosalicylic acid to give a final concentration of 1.5% and then centrifuged at 3000 rpm at 4°C for 15 min to remove precipitated proteins. To prepare deproteinized tissue extracts, ~50 mg pulverized frozen heart was homogenized in 300 µl of 3% sulfosalicylic acid and centrifuged as described previously (Armstrong and Stave, 1973). The amino acid concentrations in the supernatants obtained were analyzed using an automatic amino acid analyzer (JLC-500/V; JEOL, Tokyo, Japan).

2-4 Cardiac BCKA concentrations

Cardiac concentrations of BCKAs were analyzed using high-performance liquid chromatography (HPLC) (Kato et al., 2011). Briefly, ~30 mg pulverized frozen heart was homogenized in 180 µl of 0.8 M perchloric acid and then centrifuged at 15,000 rpm at 4°C for 15 min to remove precipitated proteins. The BCKAs in the supernatant were reacted with DMB to produce fluorescent compounds that could be separated and quantified by the HPLC system (Shimadzu Co., Kyoto, Japan).

2-5 Cardiac BCKDH complex activity

Cardiac BCKDH complex activity was measured as described previously (Nakai et al., 2000). The enzyme activity was defined as the rate of formation of 1 µmol of NADH/min at 30°C. Both actual and total activities were measured; the former corresponded to in vivo dephosphorylated enzyme levels and the

latter was obtained by treating the tissue extract with lambda protein phosphatase to fully dephosphorylate enzymes (Obayashi et al., 2001). The activity state of the enzyme is defined as the percentage of actual activity relative to total activity (Nakai et al., 2000).

2-6 Abundance of the BDK bound to the BCKDH complex

The BDK bound to the BCKDH complex was quantified by the method of immunoprecipitation of BCKDH complex in the heart extract and Western blotting of BDK and the complex as described previously (Obayashi et al., 2001).

2-7 Quantification of phospho-S6K1 at Thr 389

Approximately 20 mg of pulverized frozen heart was homogenized in ice-cold buffer (50 mM HEPES (pH 7.4 with NaOH), 1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 100 mM NaF, 50 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 5 μg/ml aprotinin, 0.1 mg/ml trypsin inhibitor, 0.1 mM TPCK) using a motor-driven Teflon homogenizer, and the homogenates were centrifuged at 10,000 rpm at 4°C for 15 min to remove debris. The extracts containing 20 μg protein were loaded for SDS-PAGE and transferred to PVDF membranes. The membranes were then probed with antibodies against S6K1 and phospho-S6K1 (Thr 389) (Ishiguro et al., 2006).

2-8 Statistics

Values are expressed as means ± SEM. The data were analyzed by a two-way ANOVA and the Tukey-Kramer method. $P < 0.05$ was considered significantly different.

3. Results

3-1 Plasma and cardiac BCAA concentrations

It was reported that plasma leucine concentrations showed a peak 30 min after oral administration of leucine in mice (Zhen et al., 2015). Therefore, in the present study, we chose this time point to sacrifice the mice for collection of blood and heart. We confirmed that the plasma leucine concentration in Veh/Leu mice was markedly higher than that in Veh/Sal mice (197 ± 23 μM for Veh/Sal vs. 1,479 μM for Veh/Leu). The same levels of plasma leucine were observed in Rap/Leu and Rap/Sal mice, respectively.

Cardiac leucine concentrations in Veh/Leu and Rap/Leu mice were also significantly higher than those of Veh/Sal and Rap/Sal mice, respectively (Table 1). The leucine concentrations were not significantly different between the Veh and Rap groups, but tended to be higher in the latter compared to the former (Table 1).

Cardiac concentrations of isoleucine and valine in Veh/Leu mice and Rap/Leu mice were significantly lower than those in Veh/Sal mice and Rap/Sal mice, respectively (Table 1). The concentrations of these amino acids tended to be higher in the Rap group than in the Veh group.

3-2 Cardiac BCKA concentrations

Since KIC is an important physiological factor to activate the BCKDH complex through inhibition of BDK (Shimomura et al., 2004), we measured

cardiac BCKA concentrations. Alterations in concentrations of the three BCKAs showed the same trends as those of the BCAA concentrations, although the BCKA concentrations were less than 1% of the BCAA concentrations (Table 2).

3-3 Cardiac BCKDH complex activities

Cardiac BCKDH complex activity was measured in mice under the same conditions as described above. The total activities of the cardiac BCKDH complex were not different among the 4 groups of mice (Fig. 6 A). The actual activity of the complex in Veh/Sal mice was as low as ~7% of the total activity, and that in Veh/Leu mice was significantly higher (~40% of the total activity) than that in Veh/Sal mice (Fig. 6 B). The actual activity in Rap/Sal mice was the same as that in Veh/Sal mice, while activity in Rap/Leu mice tended to be higher, but was not significantly different from that in Rap/Sal, and thus was significantly lower than that in Veh/Leu mice (Fig. 6 B).

3-4 Abundance of the BDK bound to BCKDH complex

It has been reported that the BDK exists as two forms, a form bound to the BCKDH complex and a free form, and the former only is suggested to be active (Obayashi et al., 2001). Therefore, the abundance of bound BDK was measured in the immunoprecipitates with the antibody against the complex. The abundance of the bound BDK expressed by the ratio of BDK/E2 subunit (n=3 in each group) was 1.00 ± 0.16 for Veh/Sal, 0.52 ± 0.02 for Veh/Leu, 0.67

± 0.28 for Rap/Sal, and 0.67 ± 0.23 for Rap/Leu. Although there was no significant difference among 4 groups, the abundance of the bound BDK tended to be lower in Veh/Leu mice than in other groups of mice.

3-5 Phosphorylation of S6K1

Phosphorylation of the Thr 389 site of S6K1 is catalyzed by mTORC1; thus, phosphorylation was quantified to determine the activity of mTORC1. The phosphorylation state of S6K1 Thr 389 was significantly higher in Veh/Leu mice than in Veh/Sal mice (Fig. 7). On the other hand, the phosphorylation states in Rap/Sal and Rap/Leu mice were not different and were markedly lower than those in Veh/Sal and Veh/Leu mice (Fig. 7).

4. Discussion

In the present study, we showed that leucine administration significantly increased cardiac leucine concentrations and activated the cardiac BCKDH complex in control mice (Veh group). The leucine-induced activation of the BCKDH complex was previously reported in rat skeletal muscle (Fujii et al., 1994) and was explained through inhibition of the BDK by KIC formed from leucine (Shimomura et al., 2004). This explanation also accounts for the activation of the BCKDH complex observed in the present study, because the cardiac concentration of KIC was significantly increased following leucine administration. On the other hand, the leucine-induced activation of the cardiac BCKDH complex in mice was significantly suppressed by treatment with rapamycin (Rap group), although cardiac concentrations of leucine and KIC were significantly increased by leucine administration. The rapamycin treatment almost completely abolished phosphorylation of S6K1 Thr 389, indicating that rapamycin fully inhibited cardiac mTORC1 in vivo. These findings suggest that leucine-induced activation of the cardiac BCKDH complex may be, in part, mediated by mTORC1, although its mechanism remains to be elucidated. This is the first research showing that mTORC1 may be involved in the regulation of BCAA catabolism because the BCKDH complex is the rate-limiting enzyme in BCAA catabolism.

Lynch et al. (2003) examined the responses of mTORC1 and BCKDH complex in rat adipose tissue to plasma leucine concentrations and found that

mTORC1 activity, measured by phosphorylation of S6K1 Thr 389, was more sensitive to an increase in plasma leucine concentration than activation of the BCKDH complex, analyzed by the phosphorylation state of BCKDH E1α Ser 293. It is concluded in this study that activation of the BCKDH complex is not required for activation of mTORC1 signaling. This conclusion is supported by the study of Schriever et al. (2013) using cultured cells. However, that study did not examine effects of mTORC1 activity on the activation of the BCKDH complex. In the present study, we demonstrated that mTORC1 may have a role in the activation of BCKDH complex and have therefore provided a new viewpoint for the relationship between mTORC1 signaling and BCAA catabolism. Furthermore, we observed that cardiac KIC concentrations tended to be higher in the rapamycin treated mice than in control mice, which is consistent with the suppression of the leucine-induced activation of BCKDH complex by the rapamycin treatment. Even this high KIC concentration in rapamycin treated mice, the BCKDH complex was not significantly activated.

It is interesting to consider how mTORC1 mediates leucine-induced activation of the BCKDH complex. The activity state of the complex *in vivo* should be determined by the balance of the activities between BDK and BDP. It has been reported that the bound BDK is an active form of the kinase and that its abundance is inversely correlated with the activity state of the BCKDH complex (Shimomura et al., 2006). In the present study, there was a tendency that the abundance of bound BDK was decreased by leucine administration

and that this phenomenon was suppressed by the rapamycin treatment, suggesting that mTORC1 may act on BDK for activation of the complex. However, BDP is not ruled out of the mechanisms, because this phosphatase is an important enzyme for activation of the complex. Unfortunately, it is technically difficult to measure the BDP activity in tissue extracts.

It has been reported that leucine administration decreases plasma concentrations of isoleucine and valine in rats (Ishiguro et al., 2006) and humans (Matsumoto et al., 2014). In the present study, cardiac concentrations of isoleucine and valine were significantly decreased by leucine administration, suggesting that leucine regulates the three BCAA concentrations not only in the circulation but also in muscle tissues. The leucine-induced activation of the cardiac BCKDH complex may contribute to the decreases in isoleucine and valine concentrations.

As reported previously, BCAAs, especially leucine, are physiologically active amino acids. Although the regulation of BCAA catabolism has been intensively studied (Shimomura et al., 2006; Lynch and Adams, 2014), the present study presented a novel viewpoint in the concept of the regulation of BCAA catabolism. Further studies are warranted to clarify the mechanisms responsible for the regulation of BCAA catabolism as well as the physiological and clinical functions of BCAAs.

Table 1. Cardiac BCAA concentrations 30 min after leucine administration

BCAA	Veh		Rap	
	Sal	Leu	Sal	Leu
	(nmol/g wet weight tissue)			
Leucine	123 ± 21[a]	563 ± 70[b]	155 ± 8[a]	485 ± 25[b]
Isoleucine	97 ± 9[a]	60 ± 4[b]	122 ± 2[c]	68 ± 6[b]
Valine	131 ± 12[a,c]	67 ± 13[b]	145 ± 8[a]	80 ± 13[b,c]

Values are means ± SE, n=5. Means not sharing the same superscript letters in the same row are significantly different, $P < 0.05$.

Table 2. Cardiac BCKA concentrations 30 min after leucine administration

BCKA	Veh		Rap	
	Sal	Leu	Sal	Leu
	(nmol/g wet weight tissue)			
KIC	0.23 ± 0.04[a]	0.78 ± 0.07[b]	0.34 ± 0.04[a]	1.16 ± 0.17[b]
KIV	0.24 ± 0.05[a]	0.10 ± 0.02[b]	0.24 ± 0.03[a]	0.18 ± 0.02[a,b]
KMV	0.14 ± 0.03	0.06 ± 0.01	0.17 ± 0.03	0.10 ± 0.03

Values are means ± SE, n=5-6. Means not sharing the same superscript letters in the same row are significantly different, $P < 0.05$.

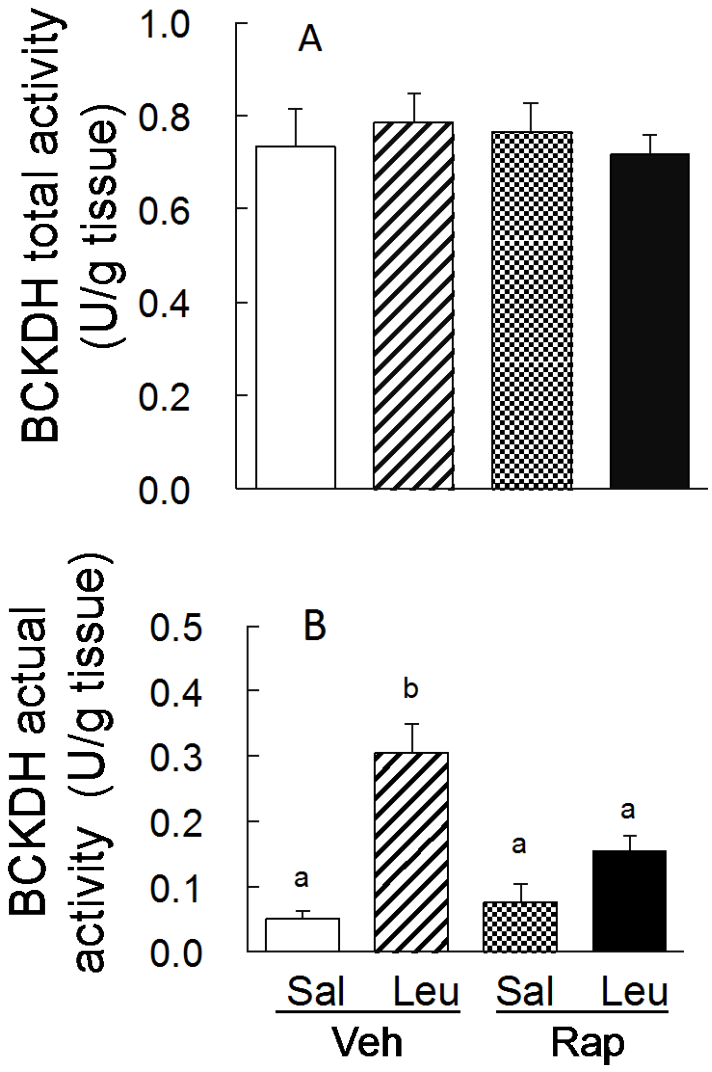

Fig. 6. Cardiac BCKDH complex activity in mice. (A) Total and (B) actual activities of the cardiac BCKDH complex.

Notes: Fasted mice were injected intraperitoneally with rapamycin (4.5 mg/kg body weight) or vehicle. Three hours later, one-half of each groups of mice were orally administrated saline or leucine. Hearts were collected from mice 30 min after oral administration of saline or leucine. Values are mean ± SE, n=5-7. Means not sharing the same letters are significantly different, $P < 0.05$.

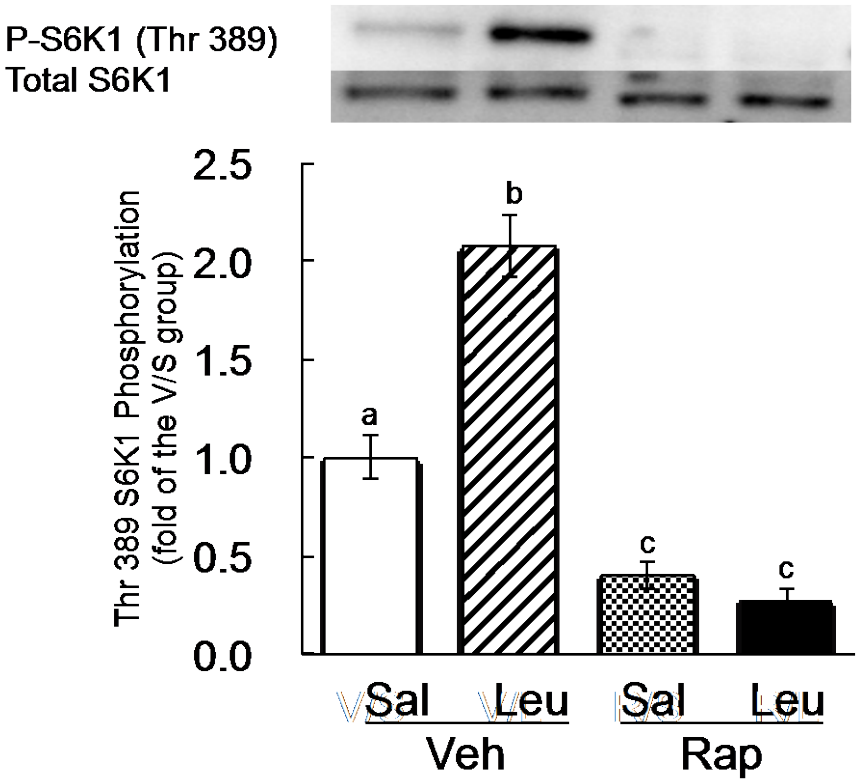

Fig. 7. Phosphorylation of S6K1 Thr 389 in mouse heart.

Notes: Total and phosphorylated S6K1 were measured by western blot analysis using anti-total-S6K1 and anti-phospho-S6K1 (Thr 389) antibodies, respectively. Representative data for total and phosphorylated S6K1 (PS6K1) are shown in the upper panel. The bar graph gives quantification of the relative amounts of phosphorylated S6K1 for each group (mean ± SE, n=5-7). Means not sharing the same letters are significantly different, $P < 0.05$.

Concluding remarks

In the first study, it was found that the elevated plasma leucine concentration after leucine administration was associated with significant decreases in plasma concentrations of isoleucine, valine, methionine, phenylalanine, and tyrosine in mice. These results are comparable with previous findings in humans administered single BCAA (Hagenfeldt et al., 1980; Eriksson et al., 1981; Matsumoto et al., 2014) or three BCAAs combined (Shimomura et al., 2009). Notably, it was previously demonstrated that this change in plasma amino acid profiles was not observed with isoleucine or valine administration in humans (Matsumoto et al., 2014). Since leucine and the other amino acids whose plasma concentrations were affected by leucine administration are substrates for LAT, BCH was used as a selective inhibitor of the amino acid transporter and it was clearly showed that BCH treatment slowed the rate of decrease in plasma leucine concentrations after peaking and almost completely blocked the leucine-induced decreases in the other amino acids described above. These findings suggest that leucine plays an important role in the regulation of the plasma amino acid profile and that LAT is involved in the mechanism of leucine action. Since it has been demonstrated that the plasma amino acid profile is usable for diagnosis of some types of diseases, the plasma leucine concentration might be important in diseases-associated changes in plasma amino acid profiles.

In the second study, it was shown that leucine administration significantly increased cardiac leucine concentrations and activated the cardiac BCKDH complex in control mice (Veh group). The leucine-induced activation of the BCKDH complex was previously reported in rat skeletal muscle (Fujii et al., 1994) and was explained through inhibition of the BDK by KIC formed from leucine (Shimomura et al., 2004). This explanation also accounts for the activation of the BCKDH complex observed in the present study, because the cardiac concentration of KIC was significantly increased following leucine administration. On the other hand, the leucine-induced activation of the cardiac BCKDH complex in mice was significantly suppressed by treatment with rapamycin (Rap group), although cardiac concentrations of leucine and KIC were significantly increased by leucine administration. The rapamycin treatment almost completely abolished phosphorylation of S6K1 Thr 389, indicating that rapamycin fully inhibited cardiac mTORC1 in vivo. These findings suggest that leucine-induced activation of the cardiac BCKDH complex may be, in part, mediated by mTORC1. This is the first research showing that mTORC1 may be involved in the regulation of BCAA catabolism because the BCKDH complex is the rate-limiting enzyme in BCAA catabolism. Further studies are required to clarify the role of mTORC1 in the regulation of BCAA catabolism.

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