Studies on the role of leucine and mTOR signaling in skeletal muscle physiology

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Abstract

Skeletal muscle is the largest metabolic organ, consuming about 20 % of the body daily expenditure of energy. Although the main function of skeletal muscle is “contraction” to move body and provide stability, it also works as a protein storage, serving as a source of amino acids to be utilized for energy production during food deprivation. Skeletal muscle tissue is composed by two basic types of fibers (slow and fast) with different metabolic, morphologic and contractile characteristics. Since slow twitch fiber (type I fiber) have a high oxidative capacity and prefer fatty acids as substrate, and are resistant to fatigue, it should be benefit to control muscle fiber type by exercise, drugs and nutrients.

Amino acids are taken in supplement form for a natural way to boost athletic performance or improve health since they play many critical roles in the body. Among these supplemental amino acids, branched chain amino acids (BCAAs), are utilize as an energy for skeletal muscle. Among the BCAAs, leucine is well known to regulate protein metabolism through the activation of mechanistic target of rapamycin (mTOR) signaling. Also, previous reports have demonstrated that mTOR signaling is involved in various cellular events, such as regulation of muscle fiber-type, fiber size, mitochondrial biogenesis and differentiation in skeletal muscle.

However, the role of leucine and mTOR signaling in skeletal muscle physiology has not been fully examined. Thus, in this doctoral thesis, to investigate the effects of leucine and mTOR signaling in skeletal muscle physiology including, fiber-type, myogenic differentiation and metabolic alteration, following studies were performed.

In chapter 2, it was investigated whether the acute oral administration of leucine affect muscle fiber-type and mitochondrial biogenesis in skeletal muscle of rats. Although the gene

expression of representative glycolytic enzymes (*Hk2* and *Eno3*) were not altered, leucine administration (135 mg/100 g B.W.) up-regulated the expression of slow-fiber related genes (*Myh7*, *Myl3* and *Tnni1*) and a mitochondrial biogenesis related gene (*Ppargc1a*) in the soleus and extensor digitorum longus (EDL) muscles compared with the control. In addition, leucine treatment also up-regulated the slow-fiber genes and mitochondrial gene expression in cultured C2C12 myotubes, while rapamycin inhibited the effects of leucine. The hypothesis was accepted that acute administration of leucine alone can up-regulate mitochondrial genes and slow-fiber related gene expression through mTOR signaling. The results suggested the possibility that leucine can alter fiber-type muscle cells and regulate metabolism in skeletal muscle.

In chapter 3, it was examined whether leucyl-tRNA synthetase (Lars), an intracellular sensor for leucine, is involved in the regulation of mTOR signaling and skeletal muscle physiology including, myogenic differentiation, hypertrophy and energy metabolism. By using small interfering (si)-RNA, it was shown that knockdown of Lars decreased phosphorylated p70 S6 kinase, a crucial downstream target of mTOR signaling, in C2C12 mouse myoblasts. Lars knockdown inhibited the differentiation of C2C12 myoblasts into myotubes, and this was accompanied with decreased level of Insulin-like growth factor 2 (*Igf2*) mRNA expression from the early stages of differentiation. The results suggested the possibility of an association between the mTOR–IGF2 axis and Lars in myogenic differentiation. However, Lars knockdown did not affect the hypertrophy of myotubes and energy metabolism (glycolysis and mitochondrial respiration) of myotubes. The results demonstrated for the first time that Lars is essential for the activation of mTOR signaling in skeletal muscle cells and myogenic differentiation through the induction of *Igf2* expression.

In chapter 4, the role of catabolism of BCAAs in C2C12 myoblasts were investigated. The catabolism of BCAAs is mediated by branched chain aminotransferase 2 (BCAT2) and branched chain alpha keto acid dehydrogenase (BCKDH) in mitochondria of skeletal muscle. Although BCAAs metabolism is basically assumed to be carried out in differentiated myofibers, it remains unclear whether BCAAs metabolic enzymes are expressed in undifferentiated myoblasts, and the physiological significance of BCAAs metabolism in myoblasts. Since the expression of BCAAs metabolic enzymes (*Bcat2*, *Bckdha* and *Bckdk*) were confirmed in both undifferentiated myoblasts and differentiated myotubes by qRT-PCR, the catabolism of BCAAs were promoted by the BCKDK inhibitor BT2 in C2C12 myoblasts. The activation of BCAAs catabolism by BT2 impaired C2C12 myoblasts proliferation and differentiation. The results suggested the possibility that increased BCAAs catabolism inhibits myoblasts proliferation and differentiation.

The findings should be important in the fields of nutrition, skeletal muscle physiology and metabolic disease, and contributes to the development of novel functional foods and supplements.

List of Abbreviation

18S : 18S ribosomal RNA

4E-BP1 : eukaryotic translation initiation factor 4E Binding Protein 1

AICAR : acadesine / AICA riboside

AMP : adenosine monophosphate

AMPK : AMP-activated protein kinase

ARS : aminoacyl-tRNA synthetases

ATP : adenosine triphosphate

B.W. : body weight

BCAAs : branched chain amino acids

BCAT2 : branched chain aminotransferase 2

BCKDC : branched chain α-keto acid dehydrogenase complex

BCKDH : branched chain alpha keto acid dehydrogenase

BCKDK : branched chain keto acid dehydrogenase kinase

BT2 : 3,6-dichlorobenzothiophene-2-carboxylic acid

DM : differentiation medium

DMEM : dulbecco's modified eagle's medium

ECAR : extracellular acidification rate

EDL : extensor digitorum longus

FBS : fatal bovine serum

GAPDH : glyceraldehyde-3-phosphate dehydrogenase

GCN2 : general control nonderepressible 2

H3 : histone h3

HS : horse serum

Igf2 : insulin-like growth factor 2

KMV : α-keto-β-methylvalerate

KIC : α-ketoisocaproate

KIV : α-ketoisovalerate

Lars : leucyl-tRNA synthetase

MEF2 : myocyte enhancer factor 2

MRFs : myogenic regulatory factors

mTOR : mechanistic target of rapamycin

MyHC : myosin heavy chain

Nprl2 : nitrogen permease regulator 2-like protein

OCR : oxygen consumption rate

PBS : phosphate-buffered saline

PGC1α : peroxisome proliferator-activated receptor γ coactivator 1 alpha

PI3K : phosphoinositide 3-kinase

PLD : phospholipase d

PPARδ : peroxisome proliferator-activated receptor delta

qPCR : quantitative real-time polymerase chain reaction

Rheb : ras homolog en- riched in brain

S6K1 : p70 ribosomal protein S6 kinase beta-1

(si)-RNA : small interfering-RNA

Sirt1 : sirtuin 1

T2D : type 2diabetes

TEAD1 : TEA domain family member 1

TSC1 : tuberous sclerosis complex1

YY1 : Ying-Yang 1

Chapter 1

General introduction

1. 1 The regulation of skeletal muscle fiber-types and metabolism

Skeletal muscle is the largest organ in the human body, that constitutes about 30-40% of body weights [1]. It works as a protein storage, serving as a source of amino acids to be utilized for energy production during food deprivation, and playing a central role in nitrogen flow during some disease states and starvation [2]. The main function of the skeletal muscle is “muscle contraction” to move body and provide stability. Skeletal muscle tissue is mainly composed of long multi-nucleated cells called myofibers that require large amount of ATP for muscle contraction. Skeletal muscle accounts for approximately 30% of the resting metabolic rate at rest, and this can be increased by up to 20-fold during exercise [3]. Muscle contraction depends on the contractile proteins, such as, myosin, actin, tropomyosin, and troponin. The present classification system for mammalian myofibers is based on myosin heavy chain (MyHC) isoforms, a major muscle contractile protein in skeletal muscle [4,5]. The major muscle fiber types are type I, IIa, IIx and IIb [6]. Rodent muscles express all four types of MyHC while IIb MyHC is not expressed in human muscle [7]. These four fibers are also classified into two types by their metabolic and functional differences. Type I fibers are also called slow twitch fiber. Slow twitch fibers have a high oxidative capacity and prefer fatty acids as substrate for ATP production, designed for low-intensity long-lasting contractions. Type II fibers are also called fast twitch fiber. Fast twitch fibers have a lower oxidative capacity and prefer glucose as substrate for ATP production, designed for high-intensity short-duration contractions. (Table 1)

Muscle tissue is composed by these different myofibers for their different roles. In order to supply the ATP demand for muscle contraction, skeletal muscle metabolizes large amounts of glucose, fatty acids, and amino acids to fuel. Since skeletal muscle is the largest insulin-responsive organ in the body, accounting for more than 80% of glucose disposal by insulin stimulation, it is the most important organ regarding to insulin resistance [8]. Furthermore, skeletal muscle is also the dominating organ for lipid metabolism [9]. Beta oxidation of fatty acid is the main energy source for skeletal muscle during fasting and exercise. Thus, fiber type composition of skeletal muscle impacts systemic energy metabolism. (Figure 1)

Table 1 Characteristics of skeletal muscle fibers

Fiber type	Slow twitch fibers (MyHC type I)	Fast twitch fibers (MyHC type II)
Mitochondria	High	Low
Myoglobin content	High (red fibers)	Low (white fibers)
Glycogen content	Low	High
Main ATP source	Fatty acids	Glycogen
Ratio to fatigue	Slow	Fast
Contractile speed	Slow	Fast

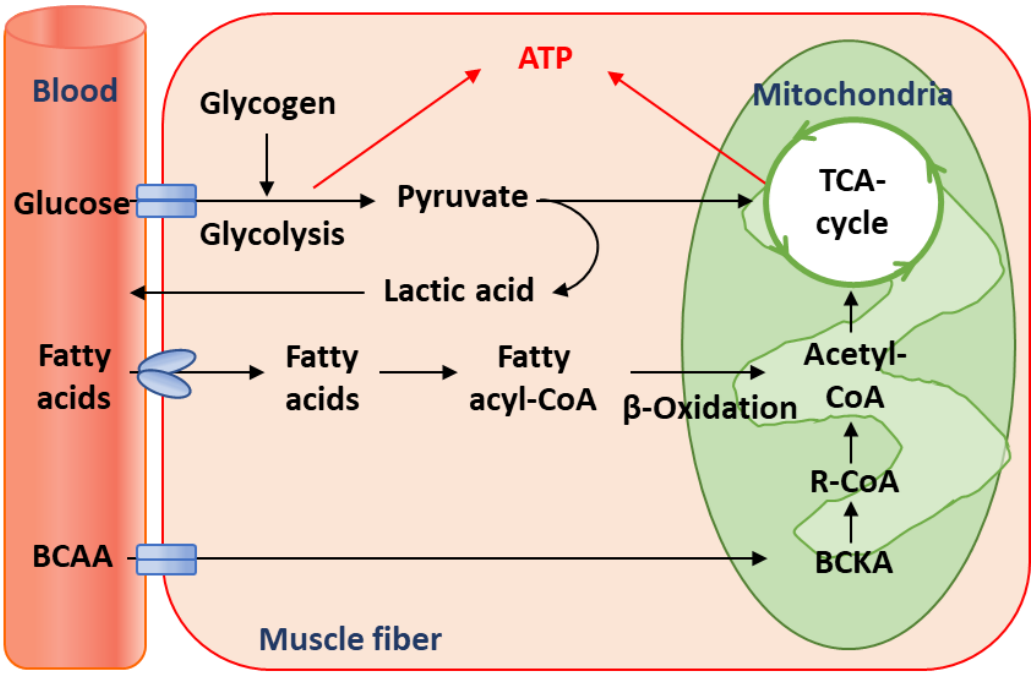

Figure 1 Energy metabolism in skeletal muscle

Skeletal muscle fiber type is transcriptionally regulated that begins with intra- and/or extracellular signals such as, hormones, mechanical stretch, calcium, and nutrients [10]. Myocyte enhancer factor 2 (MEF2), a transcriptional factor expressed highly in skeletal muscle, is a downstream target of these signals and regulates muscle metabolism by activating the transcriptional activity of two key metabolic mediator, Peroxisome Proliferator-Activated Receptor delta (PPARδ) and Peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC1α) [11-14]. Especially, PGC1α is known to regulate the expression of slow-twitch muscle fiber genes through coactivation with MEF2. Transgenic overexpression of PGC1α in the skeletal muscle leads to the characteristic of slow-twitch muscle fibers and oxidative metabolism [15,16]. Consistently, PGC1α knockout mice show exercise intolerant, lower mitochondrial content in skeletal muscle and myopathic features [17]. Thus, PGC1α is plausible to be one of master regulator of muscle fiber type and oxidative metabolism.

Muscle fiber types are firstly determined during embryonic stage and can be altered later by hormonal factors, exercise, drugs and nutrients [18,19]. Recent report demonstrated that Thyroid hormone negatively regulates the transcription of myosin heavy chain I indirectly through miR-133a, a microRNA enriched in fast type muscle, and transcriptional factor TEAD1 [20]. Both endurance and resistant exercise impacts systemic glucose and lipid homeostasis and alters muscle fiber type composition, which is regulated by the induction of PGC1a or PPARδ expression [21,22]. Endurance exercise increases the number of slow twitch fibers through the activation of AMP-activated protein kinase (AMPK), a sensor of intracellular energy status, and PGC1a leading to enhanced fatty acid utilization [23]. AICAR (Acadesine / AICA riboside), a pharmacologic activator of AMPK, is also reported to increase running endurance even in untrained mice through

the activation of AMPK and PGC1α [24,25]. Regarding to nutrients, so far, it has been reported that some natural nutrients alter the composition of muscle fiber type of experimental animals. A known most effective nutrient to change muscle fiber-type distribution is Resveratrol, a natural polyphenol enriched in the skin of grapes [23]. Resveratrol is well known for its antioxidant properties, also increases the resistance to muscle fatigue, consistent with increased mitochondrial activity and the transformation of muscle toward a slow type phenotype through PGC1α and Sirt1 [26]. In addition, supplementation of apple polyphenol which prepared from unripe apples containing 45% (w/w) of procyanidin dimers to pentadecamers, 25% phenolic acids (mainly chlorogenic acid), 10% phloretin glycosides (mainly phloridzin), 15% monomeric flavan-3-ols (mainly catechin monomer) and 5% other compounds (mainly, quercetin glycosides) increases endurance based on fiber-type composition in rat muscle [27]. Moreover, supplementation of beef extract which is rich in L-carnitine as well as carnosine, taurine and creatinine, increased muscle mass and slow type fiber in extensor digitorum longus (EDL) of Wistar rats [28].

As described above, since skeletal muscle is the largest metabolic organ, consuming about 20 % of the body daily expenditure of energy. Thus, it is of particular interest in the prevention of metabolic diseases such as obesity and type 2 diabetes (T2D) due to the central role that skeletal muscle plays key roles in metabolic homeostasis. Especially, since slow twitch fiber (type I fiber) have a high oxidative capacity and prefer fatty acids as substrate, and also lower proportion of slow fibers in metabolic syndrome muscle correlated with the severity of insulin resistance [29,30], it should be benefit to control muscle fiber type by exercise, drugs and nutrients. Although some natural nutrients or drugs have the ability to alter the muscle fiber types and metabolic characteristics, it is not plausible and realistic to take effective dose of certain drug

or nutrient for long-term due to its toxicity or cost.

1. 2 Role of amino acid as signaling molecule in the regulation of skeletal muscle fiber type and myogenic differentiation

Besides being the building blocks of proteins, amino acids also function as substrates that are converted to different metabolites for required energy or physiological condition. The energy produced from amino acids accounts for 10-15% of the total energy produced in humans. The liver, kidneys, small intestines and skeletal muscle are organs that play an essential role in amino acid metabolism. Among them, skeletal muscle (primarily myofibers) store amino acids as protein and are known to be able to metabolize some kinds of amino acids and utilize as energy source [31]. In skeletal muscle, six amino acids, leucine, isoleucine, valine, asparagine, aspartate and glutamine, are metabolized by their own aminotransferases in the first catabolic step. Among these amino acids, BCAAs (Branched Chain Amino Acids; leucine, isoleucine, valine) are degraded by BCAT2 (Branched Chain AminoTransferase 2) and BCKDC (Branched-Chain α-Keto acid Dehydrogenase Complex) in mitochondria for energy supply, especially during exercise [32-34]. Skeletal muscle is also the main production organ of glutamine, an amino acid with many roles including intestinal microbiome, immune system and gut barrier function [35]. Furthermore, a half of amino acids in the skeletal muscle are released as alanine or glutamine (carriers of amine) to the blood circulation [36].

In addition, some amino acids are also known to function as signaling molecules to regulate cellular homeostasis [37]. For example, the mechanistic target of rapamycin (mTOR) signaling serves as a central regulator of cell metabolism and growth by regulating both protein

and mRNA synthesis [1,2]. Especially, it has been reported that leucine and arginine have the ability to activate mTOR signaling in various kinds of cell including skeletal muscle cells [38,39]. The mTOR signaling links intra- and extracellular cues, such as growth factors, stress, energy status, oxygen, and amino acids to control protein, lipid and energy metabolism.

Recent reports demonstrated that some amino acids are sensed by their different intracellular sensors to activate mTOR signaling [40-46]. Among these amino acids, leucine is known to strongly activate mTOR signaling to regulate protein metabolism in skeletal muscle both *in vitro* and *in vivo* [47-49]. In skeletal muscle cells, mTOR signaling also regulates mitochondrial DNA content and the expression of genes related to oxidative metabolism by mediating the nuclear association between PGC1α (Peroxisome proliferator-activated receptor-γ coactivator 1α) and the transcription factor YY1 (Ying-Yang 1) [50]. A previous report suggested that supplementation of chronic BCAAs increased the average lifespan of middle-aged mice, and this was accompanied by increased mitochondrial biogenesis in both cardiac and skeletal muscles through the activation of mTOR signaling [51]. Recent report also showed that skeletal muscle-specific deletion of Nprl2 gene, a negative regulator of mTORC1, induced aerobic glycolysis and fiber-type shift to fast-type muscle [52].

Although leucine may be a potent nutrient that alter muscle fiber type, metabolism and growth, there are some unclear points regarding its regulation and mechanisms. The following can be given as unsolved points,

1. Although supplementation of chronic BCAAs leads to the characteristic of slow-twitch muscle fibers and oxidative metabolism through the activation of mTOR signaling, it remains unclear whether leucine alone can directly alter the muscle fiber-type and metabolism.

2. Leucyl-tRNA synthetase (Lars) and Sestrin2 were identified as intracellular sensors of leucine for the activation of mTOR signaling. However, no evidence has been reported of the activation of mTOR signaling by leucine sensors in skeletal muscle cells, and the link between these sensors and muscle differentiation, metabolism.
3. BCAAs can be converted to branched chain keto acids, finally to acetyl-CoA or succinyl-CoA in mitochondria of skeletal muscle. However, it is not clear whether increased BCAAs catabolism affect muscle growth, myogenic differentiation.

In order to address these points, the present study aims to understand the mechanisms how amino acid signaling, especially leucine (and its metabolism), regulates skeletal muscle fiber-type, energy metabolism and myogenic differentiation though mTOR signaling by using both *in vitro* and *in vivo* experimental model. The major focus of this doctoral thesis is to elucidate the role of leucine and its metabolism on the skeletal muscle physiology, especially muscle fiber-type, metabolism and differentiation. The findings should be important in the fields of nutrition, skeletal muscle physiology and metabolic disease, and contributes to the development of novel functional foods and supplements.

1. 3 Structure of this thesis

This doctoral thesis is divided into several chapters based on specific research objectives. Chapter 1 is an introduction that provides research background information for a better understanding of the subject area of the research. Chapter 2 is an investigation on whether oral administration of Leucine acutely affects the fiber-type and metabolism of skeletal muscle though the activation of mTOR signaling. Chapter 3 is an investigation on whether leucyl-tRNA

synthetase, a known intracellular leucine sensor, is required for skeletal muscle cell differentiation, hypertrophy and energy metabolism. Chapter 4 is about investigating whether increased BCAAs catabolism by BCKDK inhibitor affect muscle growth, cell proliferation and myogenic differentiation. Finally, Chapter 5 summarizes the present findings and conclusion.

Except glycine, every amino acid can occur in two isomeric forms (called L- and D-forms), because of the possibility of forming two different enantiomers around the central carbon. In mammalian cells, only L-amino acids are manufactured and incorporated into proteins. Thus, only L-amino acids are dealt and L- is omitted in this doctoral study.

Chapter 2

Acute oral administration of leucine up-regulates slow-fiber and mitochondria related genes in skeletal muscle of rats

2. 1 Abstract

Branched-chain amino acids (BCAAs) promote both protein and mRNA synthesis through mechanistic target of rapamycin (mTOR) signaling. A previous report demonstrated that chronic BCAA supplementation increased mitochondrial biogenesis in the skeletal muscle of middle-aged mice through activation of mTOR signaling. In this chapter, it was investigated that the acute oral administration of leucine alone has the ability to alter the gene expression related to fiber-type and metabolism in skeletal muscle of young rats through the activation of mTOR signaling. Although the gene expression of representative glycolytic enzymes (*Hk2* and *Eno3*) were not altered, leucine administration (135 mg/100 g B.W.) up-regulated the expression of slow-fiber related genes (*Myh7, Myl3* and *Tnni1*) and a mitochondrial biogenesis related gene (*Ppargc1a*) in the soleus and extensor digitorum longus (EDL) muscles compared with the control. In addition, leucine treatment also up-regulated the slow-fiber genes and mitochondrial gene expression in cultured C2C12 myotubes, while rapamycin inhibited the effects of leucine. However, alanine, phenylalanine and valine treatment did not alter the expression of the fiber-type and metabolism related genes as observed in leucine. The results suggest that leucine may have the ability to alter skeletal muscle fiber-type toward slow fiber and oxidative metabolism by up-regulation of gene expression through mTOR signaling.

2. 2 Introduction

The mechanistic target of rapamycin (mTOR) signaling serves as a central regulator of cell metabolism and growth by regulating both protein and mRNA synthesis [53,54]. In skeletal muscle cells, mTOR signaling regulates mitochondrial DNA content and the expression of genes related to oxidative metabolism by mediating the nuclear association between Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) and the transcription factor Ying-Yang 1 (YY1) [50]. Muscle-specific deletion of mTOR or Raptor, one of the mTORC1 components, leads to impaired oxidative capacity that is more prominent in slow-type than fast-type muscles in mice [55,56]. In contrast, constitutively active mTOR generated by genetic deletion of TSC1 (Tuberous Sclerosis Complex1), a negative regulator of mTOR by targeting the small G protein (Rheb), leads to an increased mitochondrial capacity in the skeletal muscles [57]. The mTOR signaling is also activated/inactivated by sensing intracellular energy and nutrients. A previous report demonstrated that supplementation of chronic branched-chain amino acids (BCAAs) increased the average life span of middle-aged mice, and this was accompanied by increased mitochondrial biogenesis and Sirtuin1 expression in both cardiac and skeletal muscles through the activation of mTOR signaling [51]. Furthermore, among the BCAAs leucine is unique in its ability to strongly activate mTOR signaling both in skeletal muscle and cultured myotubes [48,58]. Oral administration of leucine alone (135 mg/100 g B. W.) has been reported to stimulate protein synthesis in the skeletal muscle of rats through the activation of mTOR signaling [48]. However, it remains unclear whether acute administration of leucine alone can alter the gene expression related to fiber-type and metabolism through mTOR signaling in skeletal muscle. Recently, unpublished microarray analyses from our lab revealed that administration of leucine (135 mg/100 g B. W.) altered global gene expression

after 3 hours (in approximately 2000 gene-sets) more actively than 1 hour (in approximately 500 gene-sets) in the gastrocnemius muscle of rats. Interestingly, the microarray analyses showed the trends of specific up-regulation of slow-fiber related genes (*Myh7, Myl3, Tnnt1, Tnni1* and *Tnnc1*) in gastrocnemius muscle with no alteration of fast-fiber related genes 3 hours after administration of leucine. Therefore, because leucine can activate mTOR signaling in skeletal muscles, it was hypothesized that one-shot oral administration of only leucine might alter the gene expression related to mitochondrial biogenesis and muscle function.

In this chapter, it was investigated whether a single oral administration of leucine activate mTOR signaling and affect the specific gene expression in skeletal muscle. It has been reported that maximal leucine-stimulated S6K1 phosphorylation occurred 1 hour after leucine administration and it returned to near the baseline level by 3 hours [59]. Thus, together with unpublished microarray data from our lab, it was hypothesized that the gene expression may be altered 3h after leucine administration.

To verify the hypothesis, leucine (135 mg/100 g B. W.) was administered to Wistar rats to activate mTOR signaling in skeletal muscle and the gene expression was examined by qPCR analysis 3 hours after the administration. Furthermore, to confirm whether the effect of leucine on the gene expression depends on the activation of mTOR signaling, C2C12 myotubes were treated with rapamycin, a well-known mTOR inhibitor, and the gene expression were examined by qPCR analysis. Since leucine can activate mTOR signaling which play a role in the regulation of both glycolytic and oxidative metabolism [50], it is important to understand the gene expression patterns in different fiber types (fast and slow) induced by the oral administration of leucine.

The aim of this study was to determine the effects of acute oral administration of leucine

on gene expression in skeletal muscle through mTOR signaling.

2. 3 Materials & Methods

2. 3. 1 Animal experiments

Male Wistar rats were used to investigate whether acute oral administration of leucine affect the gene expression in skeletal muscle through the activation of mTOR signaling. The Wistar rats (5 w old; Clea Japan, Tokyo, Japan) were housed with free access to water and a standard diet of AIN-93G (Nosan, Yokohama, Japan) for 10 days before the oral gavage experiment. Rats were food-deprived for 18 h and then administered saline (control) or leucine (135 mg/100 g B. W.) by oral gavage as described previously [48,60]. The concentration of leucine was equivalent to the amount of leucine consumed by the rats during 24 h of free access to an AIN-93 powdered diet [61]. All rats were sacrificed by decapitation and skeletal muscles were dissected from hindlimb. To examine the effect of leucine on the different fiber types, soleus (slow-type) and extensor digitorum longus (EDL) (fast-type) muscles were dissected for protein and gene expression analyses from 6 individual rats 1 h or 3 h after leucine administration, respectively. All animal experiments were performed in accordance with the guidelines for the care and use of animals required by the Animal Experimentation Committee of Utsunomiya University.

2. 3. 2 Cell culture

To investigate whether the activation/inactivation of mTOR signaling affect the specific gene expression, cultured C2C12 myotubes were used as a model of skeletal muscle. C2C12

myoblasts were purchased from the ATCC and maintained in growth media (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin mixture) at 37 °C and 5% CO_2. For experimentation, cells were seeded in 6-well plates and differentiated in 2% HS-DMEM until myotubes was formed (4 days) after the cells reached 80–90% confluency. Cells were cultured with fresh DMEM for 3 h before amino acid treatment. The culture medium was then replaced with DMEM containing leucine, alanine, phenylalanine or valine (10 mM). Proteins and total RNAs were extracted from the myotubes 10 min and 1 h after the amino acid treatment, respectively. To inactivate mTOR signaling, the myotubes were treated with 20 nM rapamycin (Cell Signaling Technology, MA, USA, #9904) 30 min before leucine treatment.

2. 3. 3 RNA extraction and quantitative real-time PCR

Expression of target and reference genes were monitored using a qRT-PCR according to the previous study [62]. *Actb* used as the reference gene. Total RNAs were isolated from 4 individual wells of cultured C2C12 myotubes of each group, and soleus and EDL muscles of 6 individual rats of each group according to the regular Trizol-chloroform protocol. cDNA was synthesized from 1 μg of total RNA by a reverse-transcriptase iScript (Bio-rad, CA, USA) and qRT-PCR was performed using the MyiQ2 real-time PCR system (Bio-rad). The primer sets were designed by Primer3. Primer validation was performed by analyzing the standard curve and the presence of a single peak in the melting curve after qPCR analysis. Primer sequences for rats were as follows: *Actb* forward, AGCCATGTACGTAGCCATCC; *Actb* reverse, CTCTCAGCTGTGGTGGTGAA; *Tnni1*forward, GACTGGAGGAAGAACGTGGA; *Tnni1*reverse, GCTTGAACCCAAGAGAGCTG; *Tnnc1* forward, GGCTGCCTTCGATATCTTTG;

Tnnc1 reverse, CCTCAGACTTCCCTTTGCTG; *Tnnt1* forward,

CGAGGAACAGGCAGAAGATG; *Tnnt1* reverse, TAGCGCAATGAGTTCCTCCT;

Myh7 forward, CCTCGCAATATCAAGGGAAA; *Myh7* reverse,

TACAGGTGCATCAGCTCCAG; *Myl3* forward, AATCCTACCCAGGCAGAGGT;

Myl3 reverse, CCCTCCACGAAGTCCTCATA; *Myod* forward, TCCCTTCCCATGTTCAGGAC;

Myod reverse, CCAACACCTGAGCGAATGAG; *Ppargc1a* forward,

TGATGTGAATGACTTGGATACAGACA; *Ppargc1a* reverse,

GCTCATTGTTGTACTGGTTGGATATG; *Tfam* forward, AGTTGGGCGAAGTGATCTCA;

Tfam reverse, GCATTCAGTGGGCAGAAGTC; *Atp5o* forward,

AAAAGGAGTTGCTGCGAGTG; *Atp5o* reverse, CCTAGGCGACCATTTTCAGC;

Cycs forward, GGCAAGCATAAGACTGGACC; *Cycs* reverse, GTCTGCCCTTTCTCCCTTCT;

Ndufa5 forward, ACAGGAGCAGCTTCTACGTT; *Ndufa5* reverse,

TTGTTAGCCTCTCGTGTGGA; *Hk2* forward, AATGCCCTGTTCTCTGCTCT;

Hk2 reverse, TGTTCGGATGTAACCAGGCT; *Eno3* forward, AGAACAATGAGGCCCTGGAA;

Eno3 reverse, AAGGACTTGTACAGCTCCCC.

Primer sequences for mouse (C2C12) were as follows:

Actb forward, AGCCATGTACGTAGCCATCC; *Actb* reverse, CTCTCAGCTGTGGTGGTGAA;

Tnni1 forward, TCATGCTGAAGAGCCTGATG; *Tnni1* reverse,

GGAGGCATTTGGCTTCAATA; *Tnnc1* forward, CTGCAGGAGATGATTGACGA;

Tnnc1 reverse, TGTAGCCATCAGCGTTTTTG; *Tnnt1* forward,

TGCACTAAAAGACCGCATTG; *Tnnt1* reverse, GCTTCTGTTCTGCCTTGACC;

Myh7 forward, ACTCAAGCGGGAGAACAAGA; *Myh7* reverse,

ACCTGGGACAACTCCAACTG; *Myl3* forward, GATGCTGACACCATGTCTGG; *Myl3* reverse, TAAGGCCACAGGGTGGATAC; *Myod* forward, TTGAGAGATCGACTGCAGCA; *Myod* reverse, ACTTCTGCTCTTCCCTTCCC; *Ppargc1a* forward, TGATGTGAATGACTTGGATACAGACA; *Ppargc1a* reverse, GCTCATTGTTGTACTGGTTGGATATG; *Tfam* forward, TGCAACTCTCCCCAGAAGAG; *Tfam* reverse, ACGTCTCTCCTGGATTTGCA; *Atp5o* forward, GCTGAAAATGGTCGCCTAGG; *Atp5o* reverse, TGGTTTGGACTCAGGAAGCT; *Cycs* forward, GTTCAGAAGTGTGCCCAGTG; *Cycs* reverse, GTCTGCCCTTTCTCCCTTCT; *Ndufa5* forward, AGGGTGGTGAAGTGGAAGAG; *Ndufa5* reverse, CCACCACTGACATGAGGT; *Hk2* forward, CATGTTCCCTCCCCTGTTCT; *Hk2* reverse, AGGTTGAGCTGAAGAGGGAC; *Eno3* forward, GTCCCAGCTGCTACCTAGAG; *Eno3* reverse, CGTGCTTTGTCTCCATCTCG.

2. 3. 4 Protein extraction and immunoblot analyses

Proteins were extracted from 3 individual wells of cultured C2C12 myotubes of each group, and soleus and EDL muscles of 6 individual rats of each group. The samples were then homogenized in SDS sample buffer containing 125 mm Tris–HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS and 10% glycerol. Extracted proteins were separated on acrylamide gels, and then transferred onto PVDF membranes (GE Healthcare). A blocking solution of 5% BSA was used. The chemidoc XRS Imager (Bio-rad) was used for evaluating the bands. For the evaluation of phosphorylation levels of p70 S6 kinase (S6K1) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) in muscles from rats, multiple electrophoretic forms (α, β , γ) resolved

by SDS-PAGE were calculated according to the previous study [8,15]. The most highly phosphorylated γ form exhibits the slowest electrophoretic mobility in gel. The phosphorylation level of S6K1 was expressed as the percentage of the β and γ forms to the total S6K1 forms by immunoblot with separation of each form. The phosphorylation level of 4E-BP1 was also expressed as the percentage of γ forms to the total 4E-BP1 forms by immunoblot with separation of each form. The phosphorylation level of S6K1 in C2C12 myotubes was determined by the ratio of phosphorylated-S6K1 (T389) to total S6K1. The antibodies used for the immunoblot analysis were: rabbit p70 S6 Kinase (total S6K1) (Santa Cruz Biotechnology, TX, USA, sc-230, dilution 1:500), rabbit phospho-p70 S6 Kinase (T389) (Cell Signaling Technology, #9234, dilution 1:1000), and rabbit 4E-BP1 (Santa Cruz Biotechnology, sc-6936, dilution 1:800).

2. 3. 5 Statistical analyses

All data are presented as the mean ± standard error of the mean (SEM). The P values less than 0.05 were considered significant and all assessment of significance was performed with unpaired two-tailed student's t-test or one-way ANOVA with Tukey's post hoc test using Prism6 (GraphPad Software, La Jolla, CA). Post hoc power analyses to estimate the power of the test were performed using Power software at a type-1 error probability of 5%.

2. 4 Results

Leucine is known to activate S6K1 and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), the critical downstream targets of mTOR signaling for protein synthesis, in skeletal muscle 1 hour after oral administration [48]. To confirm the activation of

mTOR signaling by oral administration of leucine in the present study, immunoblots were performed to analyze the phosphorylation levels of S6K1 and 4E-BP1 in the skeletal muscles. The soleus and extensor digitorum longus (EDL) muscles were used for the analysis of S6K1 and 4E-BP1 to investigate the responses of leucine on the different fiber types. Soleus and EDL muscle are representative of slow-type and fast-type muscle in the hindlimb of rats, respectively. Soleus muscle mainly contains slow fibers (type 1) with some fast fibers (type 2a) whereas EDL muscle contains mainly fast fibers (2b and 2x). Morphologically, soleus muscle has predominantly oxidative slow fiber distribution and have greater capillary structure for oxidative metabolism than EDL muscles to transport nutrients and oxygen. Differentiated C2C12 myotubes are known to express dominantly MyHC2a and MyHC1 [63].

To evaluate the phosphorylation levels of S6K1 and 4E-BP1, the ratio of multiple electrophoretic forms (α, β, γ) resolved by SDS-PAGE according to a previous study was employed [48]. The phosphorylation levels of S6K1 and 4E-BP1 were increased 1 hour after leucine administration in both soleus and EDL muscles compared with the control (Fig. 2.1). Unexpectedly, the phosphorylation levels of S6K1 and 4E-BP1 were higher in soleus muscle than EDL muscle, both before (basal) and after leucine administration. To determine whether differential phosphorylation levels of S6K1 and 4E-BP1 in soleus and EDL muscles depend on the differential expression of amino acid transporter among the different fiber types of muscle, the gene expression of transporters for leucine (Slc7a5/LAT1, Slc3a2/4F2hc) and others (Slc36a1/PAT1, Slc38a2/SNAT2) were measured by qRT-PCR. However, the expression levels of these amino acid transporters were not altered between soleus and EDL muscle (Fig. 2.2), therefore, the differential phosphorylation levels of S6K1 and 4E-BP1 in soleus and EDL muscles

might depend on other factors.

Moreover, a previous report demonstrated that chronic BCAA supplementation increased mitochondrial biogenesis in the skeletal and cardiac muscles of middle-aged mice through the activation of mTOR signaling [51]. Because leucine can activate mTOR signaling in both the soleus and EDL muscles of rats, it was considered that administration of leucine alone acutely could up-regulated the gene expression related to mitochondrial biogenesis and muscle function. Also, since our unpublished microarray analyses showed that administration of leucine altered global gene expression after 3 hours (in approximately 2000 gene-sets) more actively than 1 hour (in approximately 500 gene-sets) in the gastrocnemius muscle of rats, therefore it was measured the gene expression in both soleus and EDL muscles of rats 3 hours after the oral administration of leucine by qRT-PCR in the present study.

In order to determine whether administration of leucine altered the expression of genes related to fiber-type and energy metabolism (glycolysis and mitochondrial respiration) in skeletal muscle, mRNA of fiber-type related genes (*Myh7, Myl3, Tnnt1, Tnni1, Tnnc1, Myod*), mitochondrial biogenesis (*Ppargc1a, Tfam*), mitochondrial respiratory chain (*Atp5o, Cycs, Ndufa5*) and glycolytic enzymes (*Hk2, Eno3*) were measured by qRT-PCR. Although, the expression levels of *Hk2* and *Eno3*, which are representative glycolytic enzymes were more highly expressed in fast-type muscle than slow-type muscle [64], but were not altered 3 hours after oral administration of leucine. Besides, the expression of slow-fiber related genes (*Tnni1, Myh7, Myl3*) and *Ppargc1a* were up-regulated in both the soleus and EDL muscles 3 hours after oral administration of leucine compared with the control (Fig. 2.3 and 4). Also, the mitochondrial respiratory chain genes (*Tfam, Atp5o, Cycs*) expression were also significantly up-regulated in the

soleus muscle 3 h after oral administration of leucine (Fig. 2.4). In contrast, the expression of *Myod* was significantly down-regulated after leucine administration in EDL muscle (Fig. 2. 3).

Additionally, to confirm whether the effects of leucine on gene expression was regulated through the activation of mTOR signaling, C2C12 myotubes were treated with leucine and rapamycin, a specific, similar to the results observed in the muscles of rat (Fig. 2.1), Leucine treatment up-regulated the slow-fiber and mitochondria-related gene expression in the C2C12 myotubes, while rapamycin completely inhibited the effects of leucine (Fig. 2.5). Nevertheless, treatment with other essential or non-essential amino acids (alanine, phenylalanine and valine) did not increase the phosphorylation level of S6K1, and there were no alterations in the gene expression (Fig. 2.6). These results suggest that leucine exerts specific effects on gene expression in skeletal muscle through the activation of mTOR signaling.

2. 5 Discussion

A lot of studies have confirmed that leucine is the most effective activator of mTOR signaling among BCAAs both in skeletal muscle and cultured myotubes [48,58]. Activated mTOR signaling has been shown to promote not only protein synthesis but also gene expression related to oxidative metabolism by regulating PGC1α [50]. A previous report suggests that chronic supplementation of a BCAA mixture has the ability to promote mitochondrial biogenesis through activation of mTOR signaling in skeletal muscles of middle-aged animals [51]. However, the acute effect of administration of leucine alone on gene expression remains unclear.

In this chapter, the results showed that acute administration of leucine alone could up-regulate mitochondrial and slow-fiber related genes, even in the skeletal muscles of young

animals. The acute administration of leucine activated mTOR signaling and up-regulated gene expression both in the soleus (slow-type) and EDL (fast-type) muscles. Unexpectedly, the phosphorylation levels of S6K1 and 4E-BP1 were higher in soleus muscle than EDL muscle both before (basal) and after leucine administration. It is known that there are differences in the rate of protein synthesis among the different fiber types, usually higher in slow-type muscle than fast-type muscle [65]. Nevertheless, since the gene expression of the transporters for leucine and other amino acids (*Slc36a1, Slc38a2, Slc7a5, Slc3a2*) were not altered between soleus and EDL muscles, and that the different phosphorylation levels might depend on the capillary density of the different fiber-type muscles. Because soleus muscles have predominantly oxidative myofiber distribution and greater capillary structure for oxidative metabolism than EDL muscles to transport nutrients and oxygen [66,67], it was considered that the greater basal blood flow used to transport nutrients and oxygen in soleus muscle might contribute to higher phosphorylation levels of S6K1 and 4E-BP1 than those found in EDL muscle.

Leucine administration also up-regulated slow-fiber genes related to muscle contraction (*Tnni1, Myh7, Myl3*) in both the soleus and EDL muscles of young rats. Interestingly, *Myod* expression was down-regulated after leucine administration in fast-type EDL muscle. This may be because *Myod* is more highly expressed in fast-type than slow-type muscle, and is also known to be a transcriptional regulator of the fast-type fiber phenotype [68-70], therefore leucine administration might have induced a muscle fiber-type shift from fast to slow by down-regulation of *Myod* in adult skeletal muscle.

In addition, mTOR regulates mitochondrial biogenesis and oxidative metabolism in skeletal muscle cells by mediating transcription of PGC1α-YY1 [50]. Deletion of mTOR or

Raptor in skeletal muscle leads to defective oxidative metabolism, while sustained activation of mTORC1 by genetic deletion of TSC1 (Tuberous Sclerosis Complex1) causes increased oxidative capacity in all muscles [57]. Previous reports demonstrated the restricted effect of chronic BCAA supplementation on mitochondrial biogenesis through activation of mTOR signaling in the skeletal and cardiac muscles of middle-aged mice [51]. The results showed that leucine administration acutely up-regulated *Ppargc1a* (2.9 fold) and expression of mitochondrial respiratory chain genes (*Tfam, Atp5o, Cycs*), even in the soleus muscle of young rats. The effects of leucine on gene expression were stronger and more predominant in soleus muscle than EDL muscle. These results were consistent with muscle-specific deletion of mTOR and Raptor in mice skeletal muscle which resulted in an impaired oxidative capacity in their muscles [56,57].

Also, in cultured myotubes, leucine is important for both protein synthesis and myogenic differentiation [58,71]. Long-term treatment of BCAA or leucine increased mitochondrial gene expression in cultured C2C12 myotubes or HL-1 mouse cardiomyocytes through the activation of mTOR signaling [51,72]. The results showed that leucine treatment acutely, up-regulated gene expression in cultured C2C12 myotubes by activating mTOR signaling but rapamycin cancelled the effects. These results were similar to those observed in young rats. Consistent with our results, elevated mitochondrial gene expression was observed in $TSC^{-/-}$ cells where mTOR was constitutively activated [50]. It was also confirmed by gene expression analysis that treatment with valine, alanine and phenylalanine did not activate mTOR signaling. Recent studies have identified Leucyl-tRNA synthetase (Lars) and Setrin2 as the intracellular sensors required for leucine to activate mTORC1 signaling [40-44,73], suggesting the possibility of a specific effect of leucine on gene expression in skeletal muscle cells.

In conclusion, the hypothesis is accepted that acute administration of leucine alone can up-regulate mitochondrial genes and slow-fiber related gene expression through mTOR signaling. The results suggested the possibility that leucine can alter fiber-type muscle cells and regulate metabolism in skeletal muscle, however, there may be limitations. This is because the hypothesis in this study is based on the data of 1.35 g/ kg B.W. of leucine administration to rats [[59,74] and unpublished microarray], it did not comment on the dose-dependent effect of leucine on the gene expression. Actually, oral administration of 0.675 g/ kg B.W. of leucine (equivalent to half dosage of this study) is sufficient for leucine maximally to activate mTOR signaling in gastrocnemius muscle [74]. Whether lower dose of leucine could up-regulate the specific genes in the skeletal muscle needs to be further investigated.

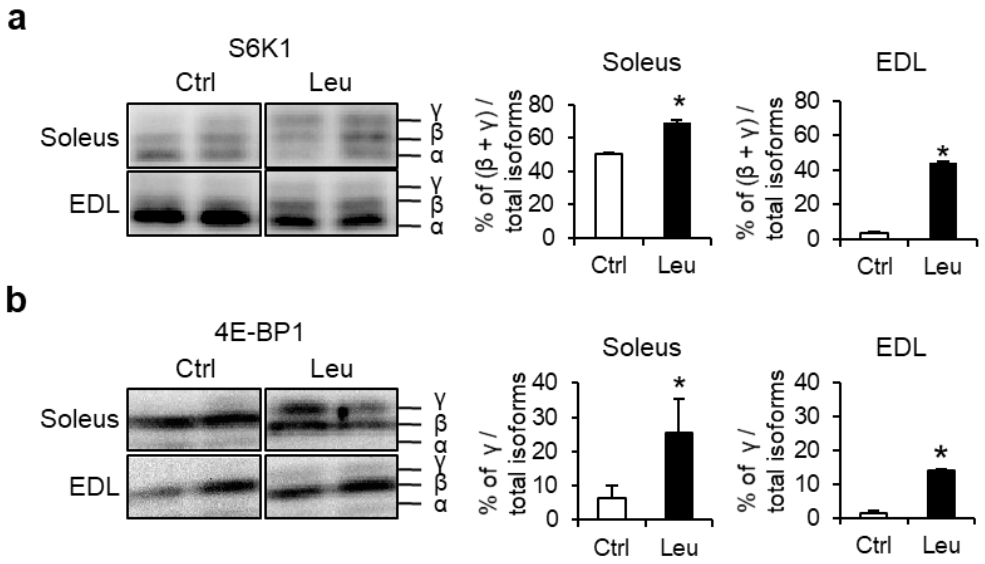

Figure 2.1 Phosphorylation of S6K1 and 4E-BP1 in the soleus and EDL muscles of food-deprived rats after oral administration of leucine.

(a) Phosphorylation level of S6K1 was expressed as the amount of S6K1 in the b and g forms as percentages of total S6K1 by immunoblot with separation of each form (left). Two representative bands from 6 individual muscles are shown. Ctrl indicates data from untreated rats; Leu indicates data from rats 1 h after administration of leucine. Right graphs show relative intensity of phosphorylated S6K1 (% of β + γ / total isoforms). (b) Phosphorylation level of 4E-BP1 was expressed as the amount of 4E-BP1 in the g forms as percentages of total 4E-BP1 by immunoblot with separation of each form (left). Two representative bands from 6 individual muscles are shown. Ctrl indicates data from untreated rats; Leu indicates data from rats 1 h after administration of leucine. Right graphs show relative intensity of phosphorylated 4E-BP1 (% of γ / total isoforms). All assessments of significance were performed with unpaired two-tailed Student's t-test (*$p < 0.05$). Values are means ± SEM ($n = 6$).

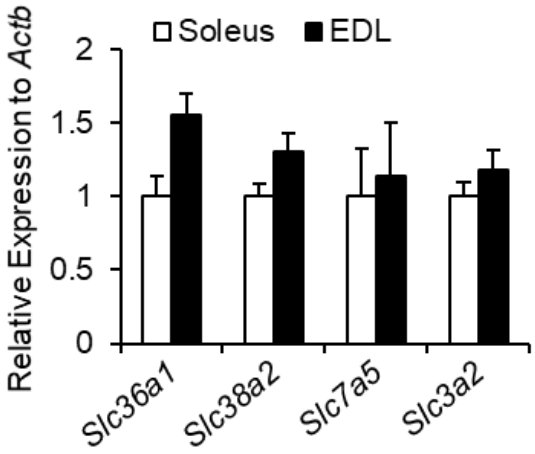

Figure 2.2 Comparison of the gene expression of amino acid transporters in the soleus and EDL muscles measured by qRT-PCR.

Expression levels were calculated relative to *Actb* and the data are expressed as the fold increase. All assessments of significance were performed with unpaired two-tailed Student's t-test (*p <0.05). Values are means ± SEM (n = 6).

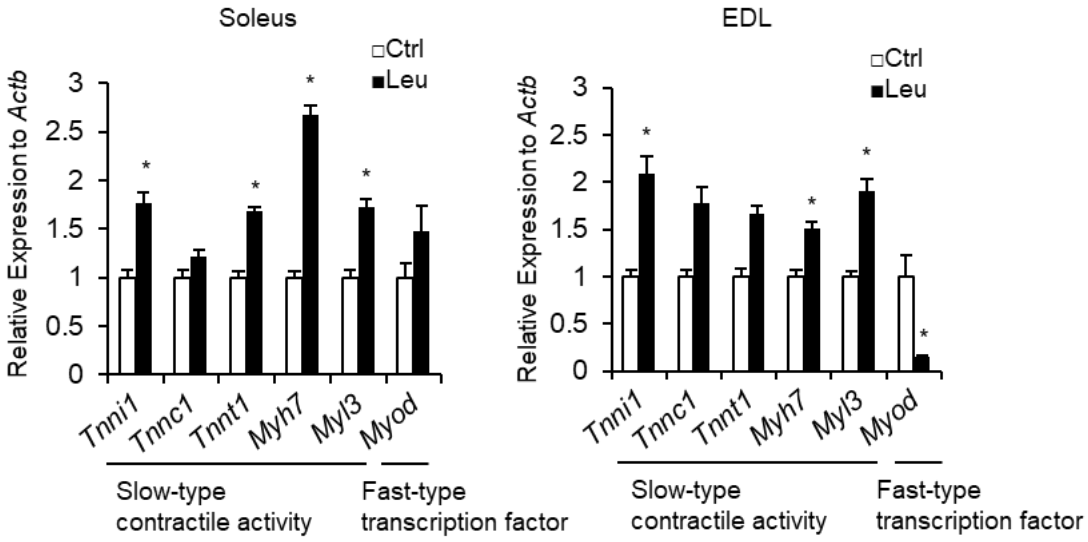

Figure 2.3 Increased expression of slow-fiber and mitochondria related genes after oral administration of leucine.

qRT-PCR analyses of muscle enriched genes in soleus and EDL muscles 3 h after oral administration of leucine. Expression levels were calculated relative to *Actb* and the data are expressed as the fold increase. All assessments of significance were performed with unpaired two-tailed Student's t-test (*p <0.05). Values are means ± SEM (n = 6).

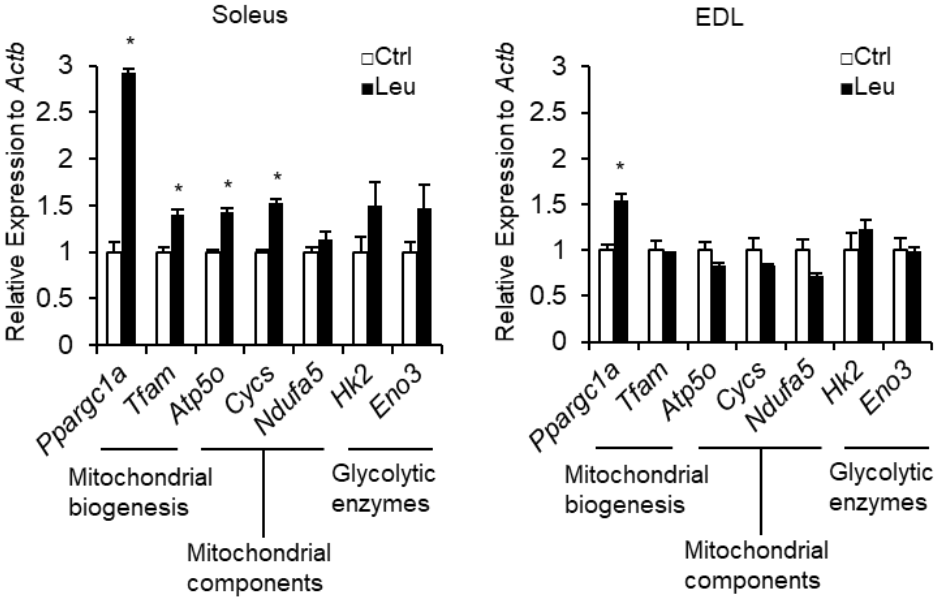

Figure 2.4 Increased expression of slow-fiber and mitochondria related genes after oral administration of leucine.

qRT-PCR analyses of mitochondria related and glycolytic enzyme genes in soleus and EDL muscles 3 h after oral administration of leucine. Expression levels were calculated relative to *Actb* and the data are expressed as the fold increase. All assessments of significance were performed with unpaired two-tailed Student's t-test (*$p < 0.05$). Values are means ± SEM ($n = 6$).

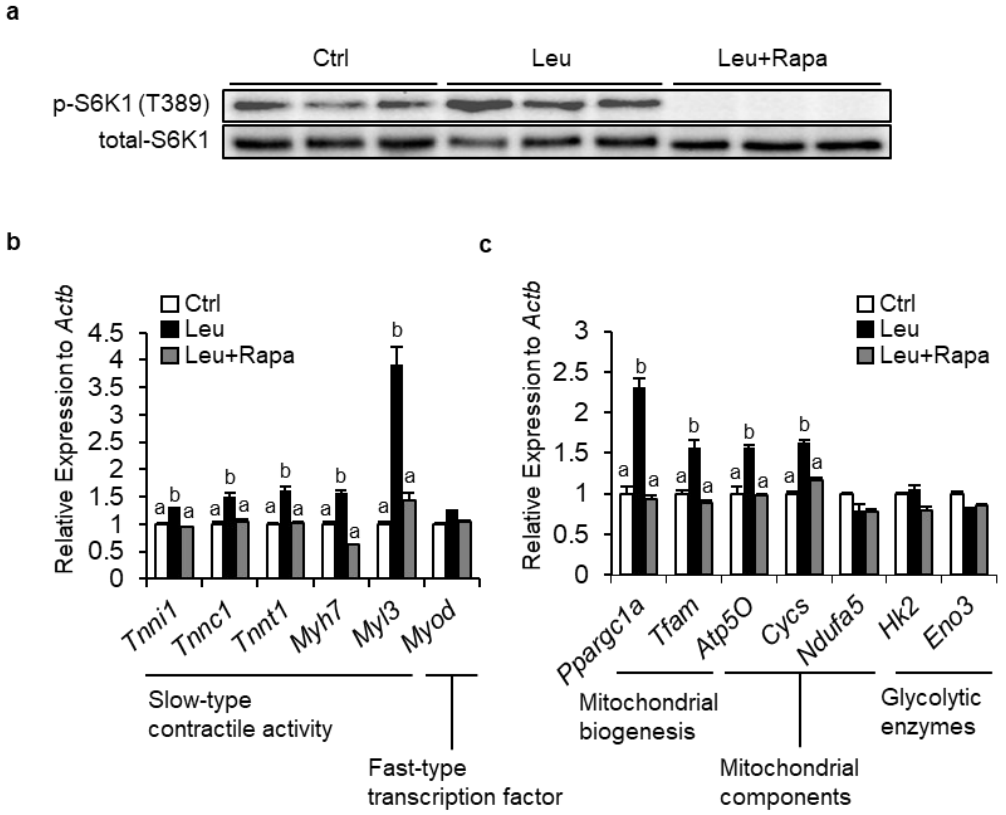

Figure 2.5 The effects of leucine on gene expression in C2C12 myotubes in the presence and absence of rapamycin.

(a) Phosphorylation level of S6K1 was measured by immunoblot of p-S6K1 (T389)/total-S6K1 in C2C12 myotubes with/without leucine and 20 nM rapamycin (10 min after treatment). qRT-PCR analyses of muscle enriched genes (b) and mitochondria related and glycolytic enzyme genes (c) in C2C12 myotubes with/without L-leucine and 20 nM rapamycin (1h after treatment). Expression levels were calculated relative to *Actb* and the data are expressed as the fold increase. Different superscripts indicate a significant difference between two groups. All assessments of significance were performed with one-way ANOVA with Tukey's post hoc test (*$p < 0.05$). Values are means ± SEM ($n = 4$).

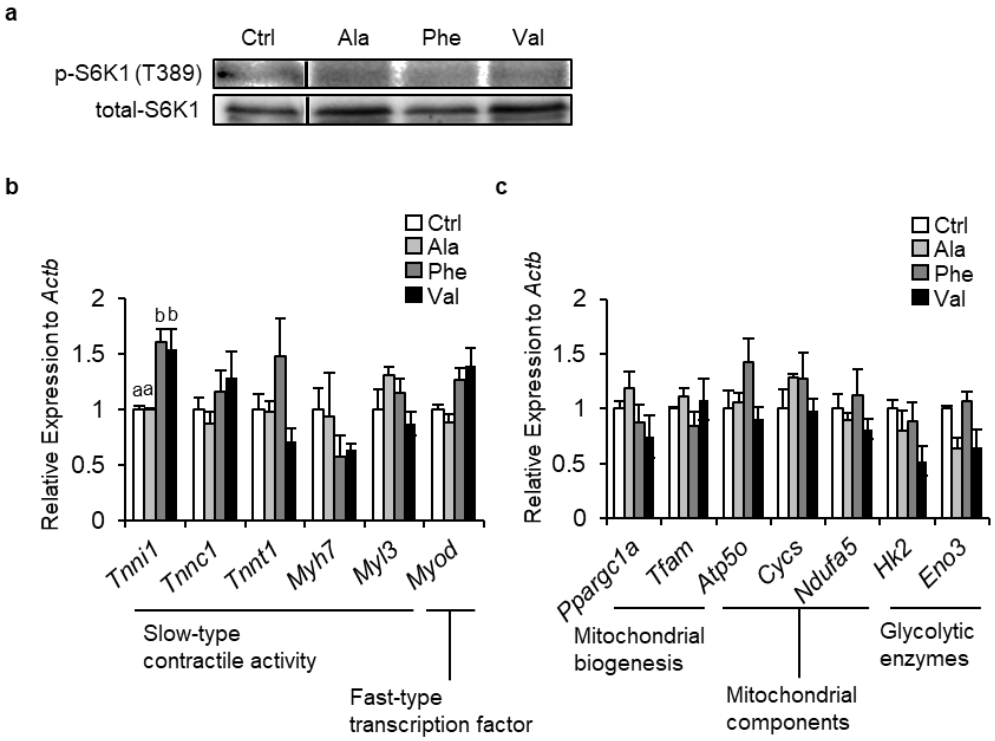

Figure 2.6 The effects of alanine, phenylalanine, and valine on gene expression in C2C12 myotubes.

(a) Phosphorylation level of S6K1 was measured by immunoblot of p-S6K1 (T389)/total-S6K1 in C2C12 myotubes treated with alanine, phenylalanine, and valine (10 min after treatment). qRT-PCR analyses of muscle enriched genes (b) and mitochondria related and glycolytic enzyme genes (c) in C2C12 myotubes treated with alanine, phenylalanine, and valine (1h after treatment). Expression levels were calculated relative to *Actb* and the data was expressed as the fold increase. Different superscripts indicate a significant difference between the groups. All assessments of significance were performed with one-way ANOVA with Tukey's post hoc test (*$p < 0.05$). Values are means ± SEM (n = 4).

Chapter 3

Leucyl-tRNA synthetase is required for the myogenic differentiation of C2C12 myoblasts, but not for hypertrophy or metabolic alteration of myotubes

3. 1 Abstract

Mechanistic target of rapamycin (mTOR) signaling controls skeletal muscle cell differentiation, growth, and metabolism by sensing the intracellular energy status and nutrients. Recently, leucyl-tRNA synthetase (Lars) was identified as an intracellular sensor of leucine involved in the activation of mTOR signaling. However, there is still no evidence for the activation of mTOR signaling by Lars and its physiological roles in skeletal muscle cells. In this chapter, it was determined the potential roles of Lars for the activation of mTOR signaling, skeletal muscle cell differentiation, hypertrophy, and metabolism using small interfering (si)-RNA knockdown. siRNA-mediated knockdown of Lars decreased phosphorylated p70 S6 kinase and inhibited the differentiation of C2C12 mouse myoblasts into myotubes, as evidenced by a decreased fusion index and decreased mRNA and protein expression levels of myogenic markers. Importantly, si-Lars decreased the level of Insulin-like growth factor 2 (*Igf2*) mRNA expression from the early stages of differentiation, indicating the possibility of an association between the mTOR–IGF2 axis and Lars. However, Lars knockdown did not decrease phosphorylated mTOR in differentiated myotubes, nor did it affect the hypertrophy of myotubes as evidenced by measuring their diameters and detecting the mRNA and protein expression of hypertrophy markers. Similarly, an extracellular flux analyzer showed that Lars knockdown did not affect the metabolism (glycolysis and mitochondrial respiration) of myotubes. These results demonstrate that Lars is required for skeletal muscle

differentiation through the activation of mTOR signaling, but not for hypertrophy or metabolic alteration of myotubes.

3. 2 Introduction

Mechanistic target of rapamycin (mTOR) signaling regulates cell differentiation, growth, and metabolism by sensing intracellular energy conditions and nutrients [53,54]. In skeletal muscle cells, mTOR signaling plays crucial roles during various cellular processes such as myogenesis, hypertrophy, and metabolic alterations. During the differentiation of cultured myoblasts, mTOR signaling was found to be essential in the response to growth factor withdrawal for normal myotube formation by autocrine actions [75-77]. Growth factors induced by the activation of mTOR signaling initiate myoblast differentiation as a molecular link between nutrients and skeletal muscle development. In differentiated myocytes, mTOR signaling integrates cell growth by regulating both protein synthesis and degradation, while the genetic activation of mTOR signaling was sufficient to cause hypertrophy and prevent atrophy [78,79].

The oral administration of leucine, a known activator of mTOR signaling, stimulates protein synthesis in skeletal muscle [47,48]. mTOR signaling also regulates mitochondrial biogenesis and the expression of genes associated with oxidative metabolism by mediating the nuclear association between peroxisome proliferator-activated receptor-coactivator 1α (PGC1α) and the transcription factor Yin-Yang 1 (YY1) [50]. The muscle-specific ablation of mTOR or Raptor, a component of mTORC1, causes impaired oxidative capacity in mice [55,56]. In contrast, constitutive activation of mTOR by the genetic ablation of tuberous sclerosis complex 1, a negative regulator of mTOR, leads to increased mitochondrial capacity in skeletal muscle [57]. Also, as

described in the chapter 2, the oral administration of leucine alone up-regulated the expression of slow fiber and mitochondria-related genes in skeletal muscle tissue of rats through the activation of mTOR signaling.

Recent discoveries have identified intracellular sensors for several amino acids and the mechanisms to activate mTOR signaling. For example, leucyl-tRNA synthetase (Lars) and Sestrin2 were identified as intracellular sensors for leucine [40-45,73,80]. Lars functions not only as an enzyme that attaches leucine onto leucyl-tRNA but also as a sensor for leucine. Lars directly binds to Rag GTPase, a known mediator of amino acid signaling to mTORC1, in a leucine-dependent manner and acts as a GTPase-activating protein for Rag GTPase to activate mTOR signaling. However, no evidence has been reported of the activation of mTOR signaling by Lars in skeletal muscle cells.

In this chapter, it was examined whether the activation of mTOR signaling by Lars in cultured C2C12 cells, and the physiological roles of Lars in myoblast differentiation, and myotube hypertrophy and metabolism by determining the effects of Lars knockdown. Our results suggest for the first time a link between Lars and mTOR activation in muscle cells and the physiological role of myoblast differentiation.

3. 3 Materials and methods

3. 3. 1 Cell culture and small interfering (si)RNA knockdown

C2C12 myoblasts were purchased from ATCC (Manassas, VA) and maintained in growth medium (DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin mixture) at 37°C with 5% CO_2. For experimentation, cells were seeded in 6- or 12-well plates and

differentiated in 2% HS-DMEM until myotubes formed (5 days) after the cells reached approximately 90% confluency. For siRNA knockdown experiments, cells in each well were transfected with 10 nM Lars or scramble siRNAs (MSS272497; Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (Invitrogen) for 48 h (during myogenic differentiation or after the formation of myotubes).

3. 3. 2 Animal experiments

Male Wistar rats (5-weeks-old; Clea Japan, Tokyo, Japan) were housed with free access to water and a standard diet. All animal experiments were performed in accordance with the Guidelines for the care and use of animals required by the Animal Experimentation Committee of Utsunomiya University. Soleus and extensor digitorum longus (EDL) muscles were dissected from 6 individual rats for protein and gene expression analyses, respectively.

3. 3. 3 Immunostaining and myotube analysis

Cells were stained with anti-total MyHC (MF20) antibody to evaluate differentiation and hypertrophy. Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and permeated with 0.2% Triton X-100 for 10 min at room temperature. They were then blocked with 3% bovine serum albumin (BSA) in PBS + 0.05% Tween-20 (PBST), and incubated with MF20 antibody at 1:100 dilution at 4°C overnight. After rinsing in PBST, cells were incubated with secondary Alexa Fluor 594-labeled chicken anti-mouse antibodies (A21201, Thermo Fisher Scientific) (1:500) and 4',6-diamidino-2-phenylindole at room temperature for 1 h. Fluorescent images were obtained with a Leica microscope (DM5000B). Myotube nuclei were

counted in approximately 100 randomly chosen MF20-positive cells containing three or more nuclei. The fusion index was calculated as the ratio of nuclei in MF20-positive myotubes to the total number of nuclei in the field for five random fields. To determine the myotube diameter, five fields were chosen randomly, and three myotubes were measured per field using ImageJ software. The average diameter per myotube was calculated as the mean of three measurements taken along the long axis of the myotubes.

3. 3. 4 RNA extraction and quantitative real-time PCR (qRT-PCR)

The expression of target and reference genes was monitored using qRT-PCR, with *Actb* and *18s* as reference genes. Total RNA was isolated from cultured C2C12 myoblasts, myotubes, and soleus and EDL muscle of rats according to the regular TRIzol–chloroform protocol. cDNA was synthesized from 1 μg of total RNA using iScript reverse transcriptase (Bio-Rad), and qRT-PCR was performed using the MyiQ2 real-time PCR system (Bio-Rad). The primer sets were designed by Primer3 software. Primer sequences for C2C12 were as follows:
Actb forward, AGCCATGTACGTAGCCATCC; *Actb* reverse, CTCTCAGCTGTGGTGGTGAA; *Tnni1* forward, TCATGCTGAAGAGCCTGATG; *Tnni1* reverse, GGAGGCATTTGGCTTCAATA; *Myh7* forward, ACTCAAGCGGGAGAACAAGA; *Myh7* reverse, ACCTGGGACAACTCCAACTG; *Myh3* forward, CAATAAACTGCGGGCAAAGAC; *Myh3* reverse, CTTGCTCACTCCTCGCTTTCA; *Myh8* forward, GAACTTGAAGGAGAGGTCGA; *Myh8* reverse, GAGCACATTCTTGCGGTCTT; *Tnnc2* forward, GGGAAGAGCGAAGAGGAACT; *Tnnc2* reverse, TGGATGGACACGAACAAAGA; *Igf2* forward, ATCCACATCTGCTGGAAGGT;

Igf2 reverse, CGCTTCAGTTTGTCTGTTCG; *18s* forward, GTAACCCGTTGAACCCCATT; *18s* reverse, CCATCCAATCGGTAGTAGCG; *Lars* forward, AATCCCTTGGGCTCTGAT; *Lars* reverse, CCACCTGACAAAGGAATCGT.

Rat primer sequences were as follows:

Actb forward, AGCCATGTACGTAGCCATCC; *Actb* reverse, CTCTCAGCTGTGGTGGTGAA; *Lars* forward, TTCAAGGATGCTCCATTTCC; *Lars* reverse, ACGGATACAGGCCACTTGTC.

3. 3. 5 Protein extraction and immunoblot analyses

Proteins were extracted from cultured C2C12 myotubes, and soleus and EDL muscles, and homogenized in SDS sample buffer containing 125 mm Tris–HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS, and 10% glycerol. Extracted proteins were separated on acrylamide gels, transferred onto PVDF membranes (GE Healthcare), and blocked using 5% BSA. A ChemiDoc XRS Imager (Bio-Rad) was used to evaluate bands. The following antibodies were used for immunoblot analysis: Lars (Cell Signaling Technology, 13868, dilution 1:1000), MF20 (eBioscience, 14-6503-82, dilution 1:1000), GAPDH (Santa Cruz Biotechnology, sc-32233, dilution 1:2000), α-tubulin (Cell Signaling Technology, 2144S, dilution 1:1000), histone H3 (Sigma-Aldrich, H0164, dilution 1:1000), p-p70 S6 kinase (T389)(Cell Signaling Technology, 9234S, dilution 1:1000), p70 S6 Kinase (Cell Signaling Technology, 2708, dilution 1:1000), p-Akt (S473)(Cell Signaling Technology, 4060S, dilution 1:1000), Akt (Cell Signaling Technology, 9272S, dilution 1:1000), p-mTOR (S2448)(Cell Signaling Technology, 2971, dilution 1:1000), and mTOR (Cell Signaling Technology, 2983P, dilution 1:1000).

3. 3. 6 Metabolism measurement

The oxygen consumption rate was measured using a Seahorse XFp Extracellular Flux Analyzer and Cell Mito Stress Test kit (Agilent Biosciences) without supplements (basal), after the addition of 1 μM oligomycin, after the addition of 1 μM carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone, and after the addition of 2 μM rotenone according to the manufacturer's protocol. Data were calculated by WAVE software.

3. 3. 7 Statistical analysis

All data are presented as means ± SEM. *P* values less than 0.05 were considered significant and all assessment of significance was performed with a Student's *t*-test.

3. 4 Results

To determine the expression of Lars in cultured myoblasts and skeletal muscle tissue, qRT-PCR and immunoblotting were performed. In rat skeletal muscle tissue, *Lars* mRNA expression was significantly higher in fast-type EDL muscle than slow-type soleus muscle, but Lars protein expression did not differ between the different fiber types (Fig. 3.1). Both Lars mRNA and protein were broadly expressed during myoblast differentiation (Fig. 3.1).

To investigate the potential role of Lars in the activation of mTOR signaling in skeletal muscle cells, siRNA was used for *Lars* mRNA knockdown in C2C12 myoblasts. si-Lars-transfected cells showed a 70% decrease in *Lars* mRNA expression compared with scramble siRNA (si-Ctrl)-transfected cells (Fig. 3.2). Lars protein expression (Lars/H3) was also significantly decreased following si-Lars transfection (Fig. 3.2). The phosphorylation level of p70 S6K1 (T389), one of the

downstream targets of mTOR signaling, was decreased in si-Lars-transfected myoblasts (both p-S6K1/S6K1 and p-S6K1/H3) (Fig. 3.2). Total Akt protein (Akt/H3) was increased and the phosphorylation level Akt (S473) (p-Akt/Akt) was decreased in myoblasts treated with si-Lars. These data suggest that the effect of Lars knockdown is associated with p70 S6K1 phosphorylation in C2C12 myoblasts.

Next, to determine the potential role of Lars in myoblast differentiation, C2C12 myoblasts transfected with si-Lars or si-Ctrl were induced to differentiate, and their differentiation status was assessed by measuring marker gene expression and the fusion index. Myoblasts transfected with si-Lars showed reduced expression of Lars mRNA and protein during myogenic differentiation (Fig. 3.3). Phosphorylated mTOR (S2448) and MyHC (MF20) protein expression was also decreased in si-Lars-transfected myoblasts compared with si-Ctrl-transfected myoblasts during myogenic differentiation (Fig. 3.3). Measurement of the fusion index showed that myotubes formed from si-Lars-transfected myoblasts contained fewer nuclei on average than those from si-Ctrl-transfected myoblasts (Fig. 3.4). These results suggested that Lars knockdown inhibited the differentiation of C2C12 myoblasts into myotubes. Furthermore, the expression of myogenic marker genes (*Myh3*, *Myh8*, *Myh3*, *Tnnc2*, and *Myog*) was dramatically decreased in si-Lars-transfected myoblasts compared with those transfected with si-Ctrl during myogenic differentiation (Fig. 3.4). The expression of *Igf2*, a critical myogenic regulator whose expression was regulated by mTOR signaling, was also decreased in si-Lars-transfected myoblasts compared with controls from an early stage of differentiation (Fig. 3.4). These results further indicated that Lars knockdown inhibited the differentiation of C2C12 myoblasts into myotubes through the mTOR–IGF2 axis.

mTOR signaling regulates protein turnover in skeletal muscle cells. To determine the

potential role of Lars in myotube hypertrophy, myotubes transfected with si-Lars or si-Ctrl for 48 h were assessed by measuring marker gene expression and myotube diameters. Differentiated myotubes transfected with si-Lars showed a decreased level of Lars mRNA and protein expression compared with those transfected with si-Ctrl (Fig. 3.5). However, the expression levels of genes associated with muscle maturation (*Myh7* and *Tnni1*) and the phosphorylation level of mTOR (S2448) were not altered between si-Lars- and si-Ctrl-transfected myotubes (Fig. 3.5). There was also no difference in myotube diameters or their distribution between si-Lars- and si-Ctrl-transfected myotubes (Fig. 3.6). These results indicated that Lars knockdown did not affect the hypertrophy of C2C12 myotubes.

In skeletal muscle cells, mTOR regulates mitochondrial biogenesis and oxidative metabolism by mediating the transcription of PGC1α–YY1 [9]. To determine the potential role of Lars in myotube metabolism, mitochondrial respiration and glycolysis of myotubes transfected with si-Lars or si-Ctrl were measured using an extracellular flux analyzer. Similar to the results of hypertrophy experiments, the oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and other parameters (basal OCR, spare respiratory chain, proton leak, and ATP production) were not altered between si-Ctrl- and si-Lars-transfected myotubes (Fig. 3.7). These data indicated that Lars knockdown did not affect the metabolism of C2C12 myotubes.

3. 5 Discussion

mTOR signaling regulates various cellular processes such as proliferation, differentiation, growth, and metabolic alterations by sensing extracellular hormones (growth factors) or the intracellular energy status and nutrients. Recent studies have uncovered detailed mechanisms of the

activation of mTOR signaling through identifying sensors for amino acids, especially Lars and Sestrin2 as intracellular sensors of leucine [41-45,73,80]. Lars is an aminoacyl tRNA-synthetase that attaches leucine onto leucyl-tRNA. Aminoacyl-tRNA synthetases (ARS) are essential and general components of the genetic code that are sustained in all types of cell. Because of their essential role in protein synthesis, no ARS-knockout mice have yet been generated. Thus, no evidence has been obtained for the activation of mTOR signaling by Lars in skeletal muscle cells.

In this chapter, the role of Lars in the activation of mTOR signaling during myogenesis, muscle hypertrophy, and metabolic alteration using siRNA knockdown in cultured C2C12 cells was investigated. Expression analyses confirmed the broad expression pattern of Lars protein in both differentiating myoblasts and muscles of different fiber types. Unexpectedly, *Lars* mRNA expression was higher in EDL than soleus muscle whereas Lars protein was same among different muscles. This might be caused by the higher rate of protein synthesis in slow-type soleus muscle than fast-type EDL muscle [65].

siRNA targeting Lars successfully knocked down Lars mRNA and protein expression in myoblasts, leading to decreased levels of phosphorylated p70 S6K1 (T389). Total Akt protein (Akt/H3) was increased and the phosphorylation level Akt (S473) (p-Akt/Akt) was decreased in myoblasts treated with si-Lars. However, although p-S6K1/H3 and p-S6K1/S6K1 were both decreased by si-Lars, p-Akt/H3 was not altered in myoblasts treated with si-Lars. Because H3 bands reflect total cell number applied for immunoblot as a reference protein, it was considered that increased Akt protein by Lars knockdown lead to decreased level of p-Akt. Increased Akt protein by Lars knockdown might be defensive response against decreased mTOR activation. These results

suggest, for the first time, a possible role for Lars as a specific signaling molecule in the activation of mTOR in muscle cells.

Under amino acid deprivation, uncharged tRNAs activate the serine/threonine protein kinase, general control nonderepressible 2 (GCN2), which senses amino acid deficiency. This phosphorylates eIF2α to repress protein translation and inactivate mTOR signaling [81]. In the present study, si-Lars decreased the level of phosphorylated p70 S6K1 in myoblasts, even during culture in DMEM which contains sufficient amino acids for the activation of mTOR. This further suggests a link between mTOR signaling and leucine sensing by Lars.

mTOR signaling is essential for the differentiation of cultured myoblasts in response to growth factor withdrawal by autocrine actions, such as IGF2 (known as the mTOR–IGF2 axis) [77]. IGF2 initiates myoblast differentiation as a molecular link between nutrients and skeletal muscle development through PI3K–Akt signaling [76,77]. Phospholipase D (PLD), hydrolase for phosphatidylcholine to yield choline and phosphatidic acid, is also known to regulate the expression of IGF2 by activation of mTOR during myogenic differentiation [82]. Although recent reports suggest that Lars directly activates the class III PI-3-kinase Vps34 and mediates activation of a Vps34-PLD1-mTORC1 pathway in HEK293 and MEF cells [73], it still remains unclear whether Lars functions as a link between mTOR and IGF2 in skeletal muscle cell. In this chapter, it was found that si-Lars dramatically inhibited myogenic differentiation from an early stage of its induction with decreased levels of phosphorylated mTOR. This inhibition of differentiation by si-Lars correlated with a drastic down-regulation of *Igf2* mRNA expression. These results demonstrate that Lars acts as a direct link between intracellular leucine sensing and the mTOR–IGF2 axis during myogenic differentiation, as part of the Lars–mTOR–IGF2 axis. The down-regulation of *Igf2*

expression by si-Lars coincided with the decreased expression of other factors such as *Myh3*, *Myh8*, *Myh7*, *Tnnc2* and *Myog* which are involved in the differentiation of skeletal muscle cells.

mTOR signaling acts as an integral cell growth regulator controlling protein turnover, so is up-regulated during muscle hypertrophy and down-regulated during muscle atrophy in differentiated myocytes. Indeed, the genetic activation of mTOR signaling was sufficient to cause hypertrophy and prevent atrophy [78]. However, it showed that si-Lars did not affect myotube hypertrophy, although si-Lars successfully knocked down Lars mRNA and protein expression in differentiated myotubes. Similarly, si-Lars had no effect on phosphorylated mTOR levels. This might reflect a compensatory system for sensing leucine through another intracellular sensor, Sestrin2. Although the affinity of Lars for leucine is reported to be similar to that of Sestrin2, it remains unclear how they orchestrate leucine sensing to activate mTOR signaling. Moreover, the expression of Sestrin2 was reduced with aging in skeletal and cardiac muscle [83,84], suggesting that the sensors might function at different developmental stages.

Similar to the results of myotube hypertrophy, the knockdown of Lars in myotubes had no effect on OCR (mitochondrial respiration) or ECAR (glycolysis). mTOR controls both oxidative metabolism and glycolysis in skeletal muscle using the mTOR–PGC1α–YY1 and mTOR–HIF1α (Hypoxia Inducible Factor 1α) axes, respectively [50,85]. Muscle-specific deletion of YY1, a transcriptional regulator for mitochondrial biogenesis, showed mitochondrial dysfunction with decreased OCR in mice [86]. Moreover, chronic supplementation of branched-chain amino acid mixture promoted mitochondrial biogenesis through the activation of mTOR signaling in the skeletal muscle of middle-aged mice [51]. These studies suggest that mTOR signaling regulates metabolic alteration by regulating mitochondrial biogenesis in skeletal muscle. Although Lars was

successfully knocked down by siRNA in differentiated myotubes in the present study, other leucine or other amino acid sensing systems might compensate by activating mTOR signaling, leading to the normal expression of genes associated with mitochondrial biogenesis and oxidative metabolism. Further studies need to be done for understanding the mechanism of mTOR activation by amino acids in skeletal muscle.

In conclusion, the results demonstrated for the first time that Lars is essential for the activation of mTOR signaling in skeletal muscle cells and myogenic differentiation thought the induction of *Igf2* expression. The fact that Lars knockdown did not affect the activation of mTOR signaling, myotube hypertrophy, or metabolic alteration suggests a compensatory effect of leucine sensing by another sensor.

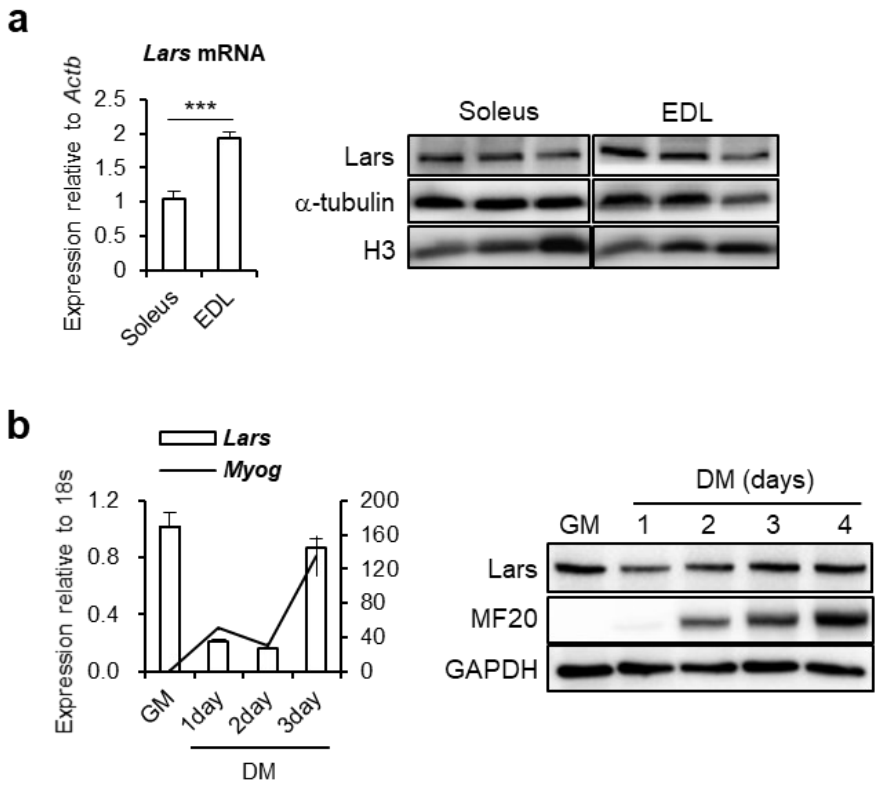

Figure 3.1 Expression analysis of Lars in skeletal muscle.

a) Comparison of Lars mRNA (left) and protein (right) expression between slow-type soleus muscle and fast-type extensor digitorum longus (EDL) muscle of rats (n = 6). Three representative bands were shown. b) Relative expression levels of Lars mRNA (left) and protein (right) expression during myoblast differentiation. Significance was determined with the two-tailed Student's t-test (***$p < 0.001$).

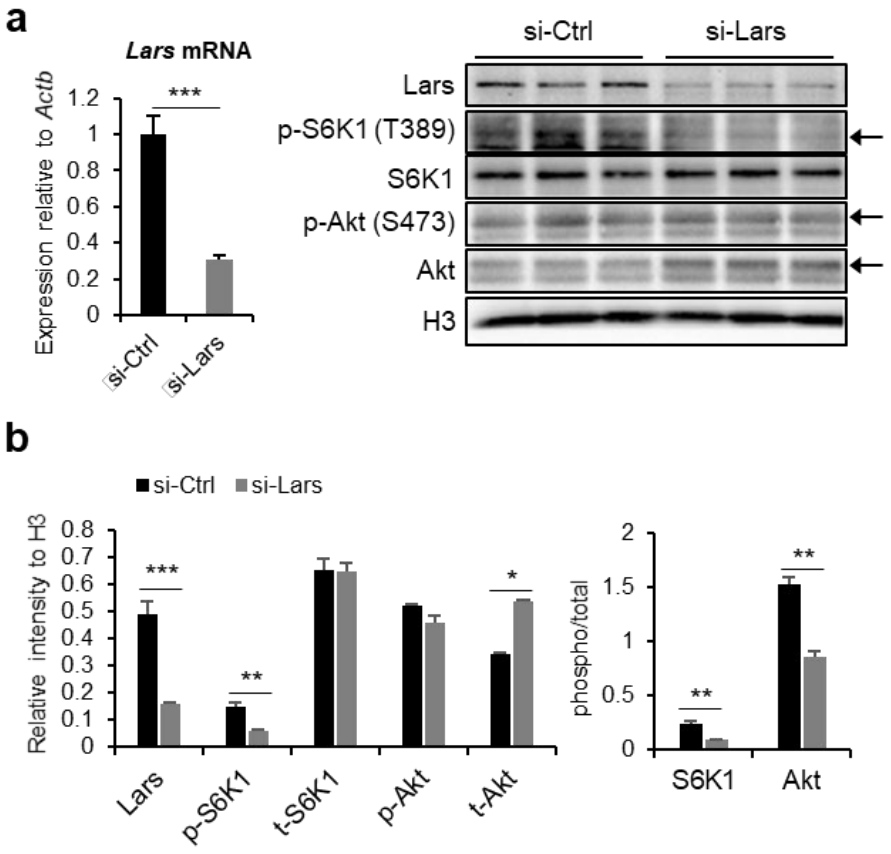

Figure 3.2 Knockdown efficiency of siRNA targeting Lars, and its effect on the activation of mTOR signaling in myoblasts.

a) The efficiency of Lars knockdown was determined by quantitative qPCR in myoblasts. mRNA expression levels were calculated relative to *18s* or *Actb* and the data are expressed as a fold-increase (n=5). b) Lars knockdown decreased the phosphorylation of S6K1 (T389), a downstream target of mTOR. Protein and phosphorylated levels were calculated relative to Histon H3 or total protein (n = 3) (lower). Significance was determined with the two-tailed Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

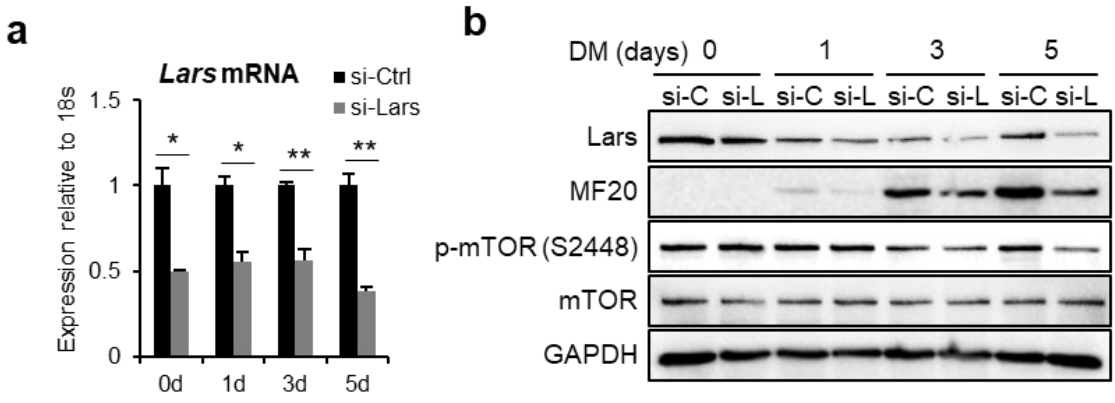

Figure 3.3 The effect of Lars knockdown on myoblast differentiation.

a) The efficiency of Lars knockdown was determined by qPCR during myoblast differentiation. b) The effect of Lars knockdown on MyHC expression (anti-MF20) and the phosphorylation level of mTOR (S2448) during myoblast differentiation. Significance was determined with the two-tailed Student's t-test (*$p < 0.05$, **$p < 0.01$) (n = 4).

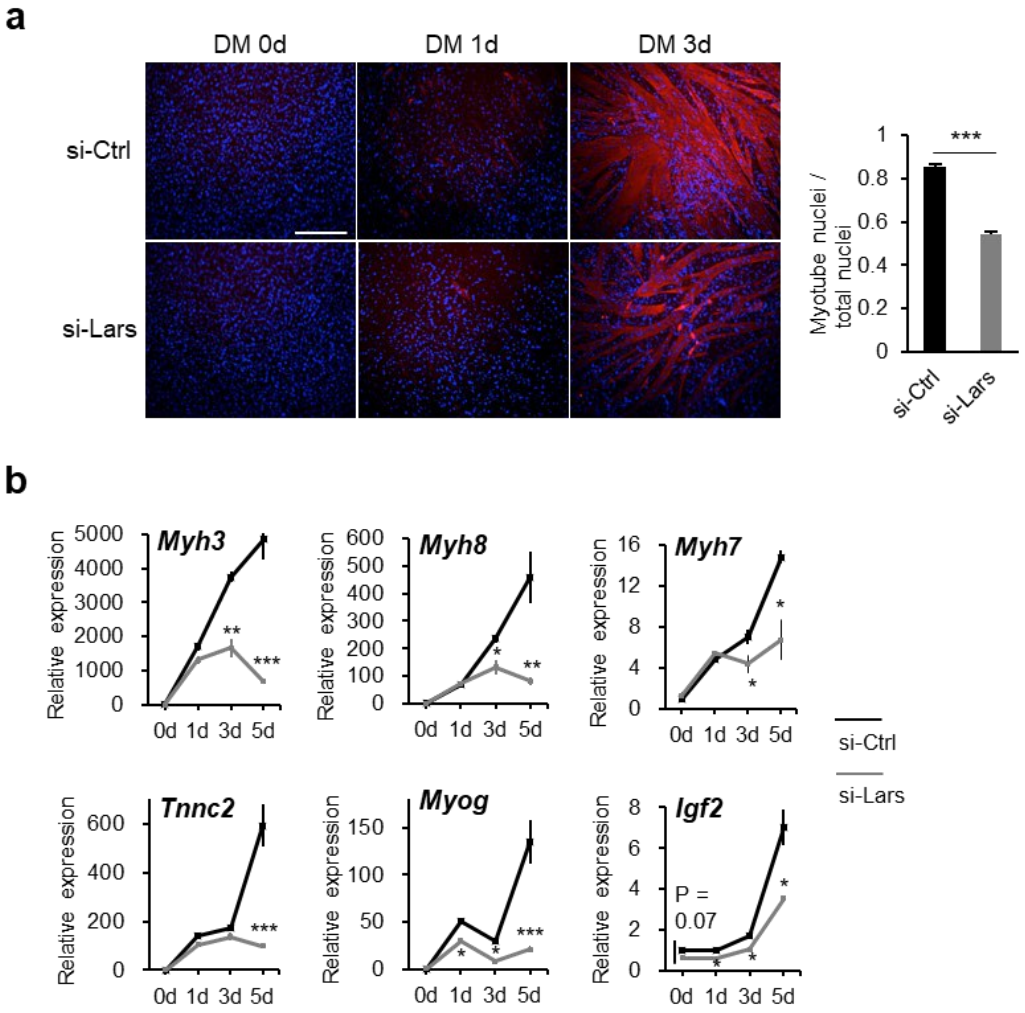

Figure 3.4 The effect of Lars knockdown on myoblast differentiation.

a) Representative images of immunostaining with the MF20 antibody during myoblast differentiation (left). The fusion index was calculated from the ratio of the number of nuclei in MF20-positive myotubes to the total number of nuclei in one field for five random microscopic fields. Bar = 100 μm. b) Time-course qPCR analysis of the effect of Lars knockdown on the expression of myogenic differentiation markers (*Myh3*, *Myh8*, *Myh3*, *Tnnc2*, *Myog* and *Igf2*). mRNA expression levels were calculated relative to *18s* and the data are expressed as a fold-increase. Significance was determined with the two-tailed Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) (n = 4).

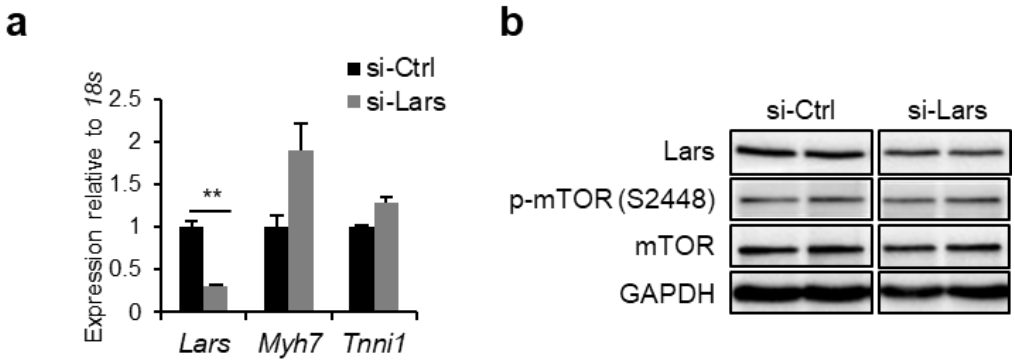

Figure 3.5 The effect of Lars knockdown on myotube hypertrophy.

a) The efficiency of Lars knockdown and marker expression were determined by qPCR during myotube hypertrophy. Expression levels were calculated relative to *18s* and the data are expressed as a fold-increase. b) The effect of Lars knockdown on the phosphorylation level of mTOR (S2448) during myotube hypertrophy. Significance was determined with the two-tailed Student's t-test (**$p < 0.01$) (n = 4).

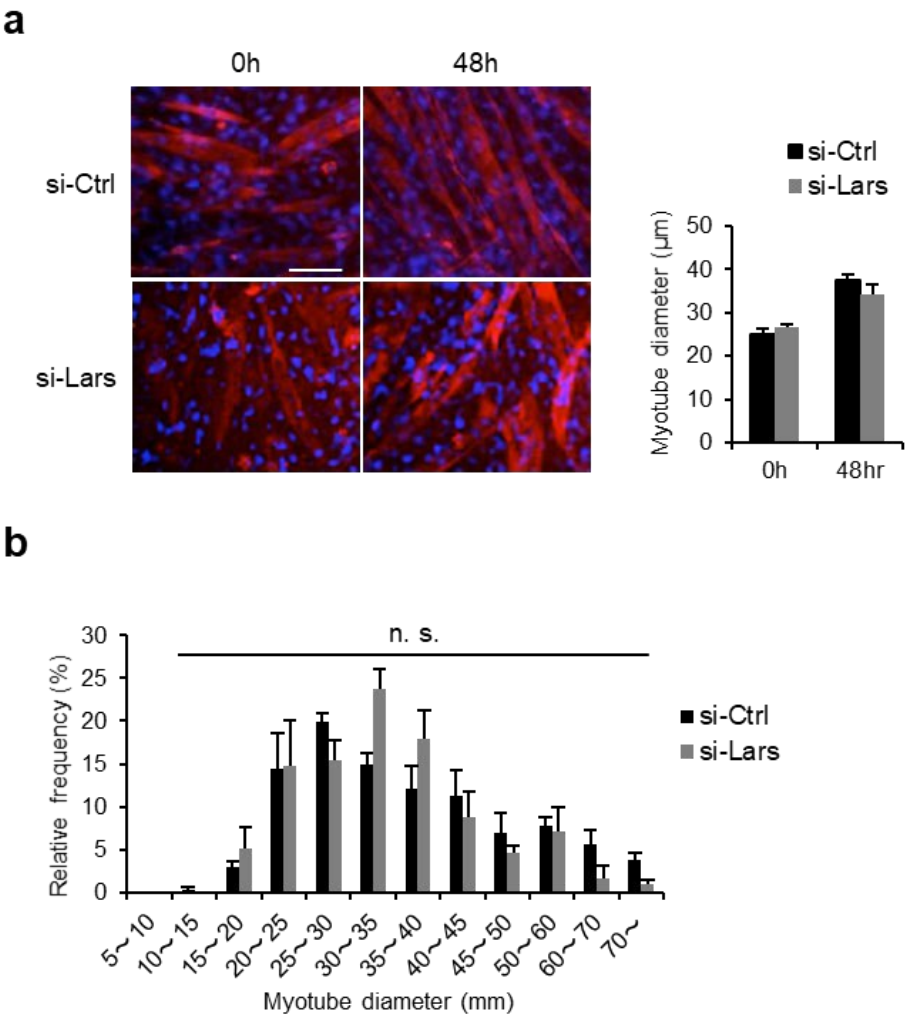

Figure 3.6 The effect of Lars knockdown on myotube hypertrophy.

a) Representative images of immunostaining with the MF20 antibody before and after siRNA transfection (left). Myotube diameters were measured to evaluate hypertrophy. Bar = 100 μm. b) The distribution frequency of myotube diameters. Significance was determined with the two-tailed Student's t-test (n = 4).

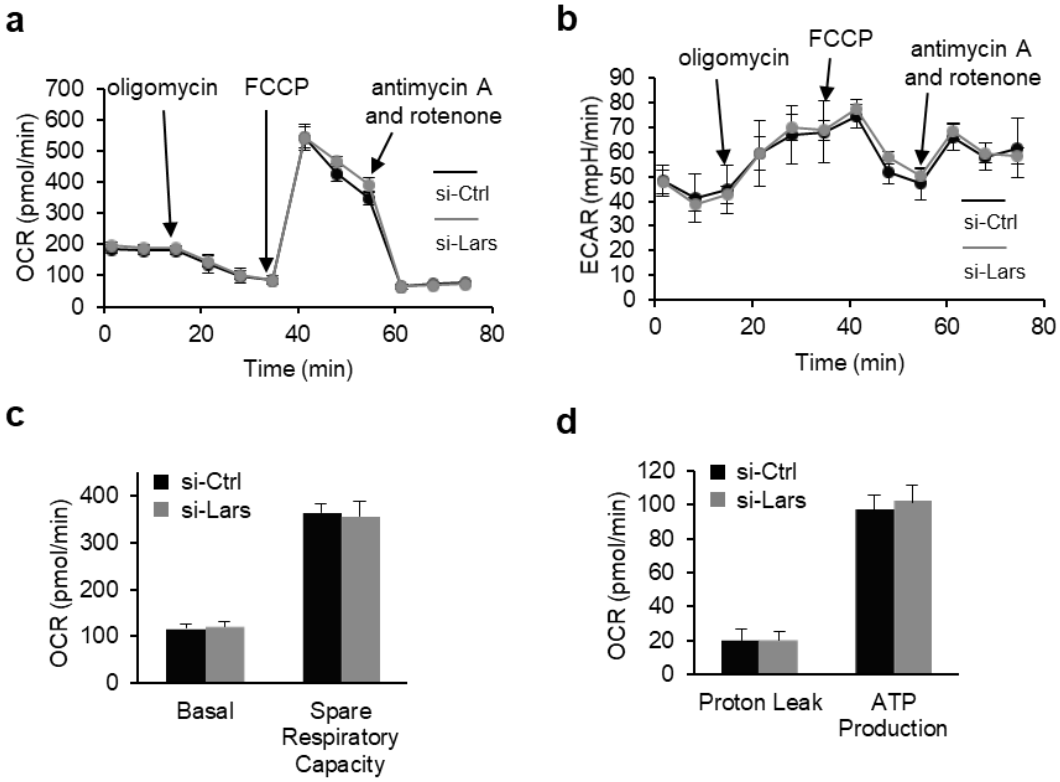

Figure 3.7 The effects of Lars knockdown on myotube metabolism.

The effect of Lars knockdown on mitochondrial respiration (a) and the glycolysis of myotubes (b) were measured by an extracellular flux analyzer. (c and d) Basal OCR, spare respiratory chain, proton leak, and ATP production were calculated by the Cell Mito Stress Test Kit using WAVE software (n = 3).

Chapter 4

Inhibition of branched-chain keto acid dehydrogenase kinase ameliorates the proliferation and differentiation of C2C12 myoblast

4. 1 Abstract

The catabolism of branched chain amino acids (BCAAs) is mainly carried out in skeletal muscle myofibers. It is mediated by branched chain aminotransferase 2 and branched chain alpha keto acid dehydrogenase (BCKDH) in mitochondria for energy supply, especially during exercise. BCKDH kinase (BCKDK) is a negative regulator of BCAAs catabolism by its inhibitory phosphorylation of the BCKDH E1a subunit. Suzuki et al. (from our lab) have demonstrated that 1hour treatment of BT2, an inhibitor of BCKDK, decreased the glycolysis of C2C12 differentiated myotubes compared to the control [87]. Although BCAAs metabolism is basically assumed to be carried out in differentiated myofibers, it remains unclear whether BCKDK is expressed in undifferentiated myoblasts, and the physiological significance of BCAAs metabolism in myoblasts. In this chapter, an in vitro assessment of BT2 on C2C12 myoblasts proliferation and differentiation was performed. The results suggest that activation of BCAAs catabolism by the BCKDK inhibitor BT2 impairs C2C12 myoblasts proliferation and differentiation.

4. 2 Introduction

As mentioned above in the chapter 1, leucine, isoleucine, valine, asparagine, aspartate and glutamine, are metabolized by their own aminotransferases in the first catabolic step in skeletal muscle. Among them, BCAAs (Branched Chain Amino Acids; leucine, isoleucine, valine) are

degraded by BCAT2 (Branched Chain AminoTransferase 2) and BCKDC (Branched-Chain α-Keto acid Dehydrogenase Complex) in mitochondria for energy supply, especially during exercise. Suzuki et al. (from our lab) have reported that activation of branched chain amino acids catabolism by BT2, a BCKDK (branched chain keto acid dehydrogenase kinase) inhibitor, impaired the glycolysis of C2C12 myotubes [87,88]. Although amino acids catabolism, especially BCAAs catabolism, is assumed to be carried out in differentiated myofibers, it is unclear whether BCKDK is also expressed in undifferentiated myoblasts. Thus, in this chapter, the expression levels of BCAAs metabolic enzymes (*Bcat2*, *Bckdha* and *Bckdk*) were measured by qRT-PCR. Also, since the biological and physiological significance of BCAAs metabolism in myoblasts is still unclear, in this chapter, the effect of enhanced BCAAs catabolism by BT2 on C2C12 myoblast proliferation and myogenic differentiation was investigated.

4. 3 Materials, and Methods

4. 3. 1 Cell culture and reagents

C2C12 myoblasts were purchased from ATCC (Manassas, VA, USA). C2C12 myoblasts at early passage (3-10) were used for experiment. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin mixture at 37 °C with 5% CO_2. For myogenic differentiation, myoblasts were cultured in 2% HS-DMEM until myotubes formed (5 days) after the cells reached 80-90% confluency. For gene and protein expression analyses, cells were seeded on 12-well miniplates (n = 6, each group) or 6-well miniplates (n = 3, each group), respectively. BT2 (3,6-dichlorobenzo[b]thiophene-2-carboxylic acid) (Axon Medchem, Groningen, Netherland) was used to inhibit BCKDC kinase for the activation of BCAAs catabolism [88].

4. 3. 2 Cell proliferation assay

Cell proliferation assay was assessed with a Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacture's protocol with slight modifications [89]. C2C12 myoblasts were seeded in 96-well miniplates at a density of 3000 cells/well in DMEM containing 10% FBS for 24 hours. The culture medium was removed and replaced with DMEM containing 1% FBS and BT2 (10-100 μM). After 24 hours of culture, cell proliferation was assessed using Cell Counting Kit-8.

4. 3. 3 RNA extraction and quantitative real-time polymerase chain reaction

Expression of target and reference genes was measured using a quantitative real-time polymerase chain reaction (qRT-PCR) according to the previous report [90]. *Gapdh* was used as the reference gene. The significance of differences in mRNA was calculated by 2-ΔΔCt method. Total RNAs were isolated from 6 individual wells of cultured C2C12 myoblasts according to the regular Trizol-chloroform protocol. cDNA was synthesized from 1 μg of total RNA by a reverse-transcriptase iScript (Bio-Rad, Hercules, CA, USA), and qRT-PCR was performed using LightCycler 96 (Roche Diagnostics, Mannheim, Germany). The primer sets were designed by Primer3. The primer sequences are as follows:

Gapdh forward, TTGCCATCAACGACCCCTTC; *Gapdh* reverse, TTGTCATGGATGACCTTGGC; *Myog* forward, ACCTTCCTGTCCACCTTCAG; *Myog* reverse, CACCGACACAGACTTCCTCT; *Myh3* forward, CAATAAACTGCGGGCAAAGAC; *Myh3* reverse, CTTGCTCACTCCTCGCTTTCA; *Bcat2* forward, CGGACCCTTCATTCGTCAGA; *Bcat2* reverse, CCATAGTTCCCCCCCAACTT;

Bckdha forward, CCAGGGTTGGTGGGATGAG; *Bckdha* reverse, GGCTTCCATGACCTTCTTTCG; *Bckdk* forward, GATCCGAATGCTGGCTACTCA; *Bckdk* reverse, GCCAACAAAATCAGGCTTGTC; *Mat1a* forward, GTGCTGGATGCTCACCTCAAG; *Mat1a* reverse, CCACCCGCTGGTAATCAACC; *Sccpdh* forward, AGTAATCCAGCCTCACTTGATGA; *Sccpdh* reverse, CCCCACAGATGTCAATACAACTT; *Pah* forward, TTGTCCTGGAGAACGGAGTC; *Pah* reverse, CTGGATTCAATGTGTGTCAGGTT.

4. 3. 4 Protein extraction and immunoblot analyses

Proteins were extracted from 3 individual wells of cultured C2C12 myoblasts of each group. The samples were homogenized in SDS sample buffer containing 125 mm Tris–HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS and 10% glycerol. Extracted proteins were separated on acrylamide gels, and then transferred onto PVDF membranes (GE Healthcare). A blocking solution of 5% BSA was used. The chemidoc XRS Imager (Bio-rad) was used for evaluating the detected bands. Total myosin heavy chain was measured by MF20 antibody (eBioscience, 14–6503–82, dilution 1:1000) to determine the differentiation level of C2C12 myoblasts. β-actin was used as internal standard (Cell Signaling Technology, #4967, dilution 1:1000).

4. 3. 5 Statistical analysis

All data are presented as means ± SEM. P values less than 0.05 were considered significant and all assessment of significance was performed with unpaired 2-tailed Student's t-test or 1-way analysis of variance (ANOVA) with Tukey post hoc test using Prism6 (GraphPad

Software, La Jolla, CA, USA).

4. 4 Results

To have the insight of the metabolic differences of BCAAs between myoblasts and myotubes, the gene expression of *Bcat2* (Branched chain aminotransferase 2), *Bckdha* (Branched chain α−keto acid dehydrogenase E1 component alpha chain), *Bckdk* (Branched chain keto acid dehydrogenase kinase) were measured by qRT-PCR. AML12 hepatocytes were used as positive control for some enzymes that is assumed to be exclusively expressed in the liver. Although the expression level of *Bckdha* was not altered between myoblasts and myotubes, the expression levels of *Bcat2* and *Bckdk* in myotubes were about half of those of myoblasts, suggesting the different metabolism of BCAAs in different stages (Fig. 4.1). Since *Bcat2* and *Bckdk* were highly expressed in myotubes compared to myoblasts, it is hypothesized that catabolism of BCAAs have role in myoblasts. To investigate the effect of BCAAs catabolism on myoblasts proliferation and myogenic differentiation, C2C12 myoblasts were treated with BT2, an inhibitor for BCKDK. For the evaluation of myoblasts proliferation, myoblasts were cultured for 24 hours and then relative cell proliferation rate was measured by Cell Counting Kit-8. The results show that 24 hours of BT2 (50 and 100 μM) treatment significantly impairs the proliferation of C2C12 myoblasts compared to control (Fig. 4.2). Next, to examine the effect of BT2 on myogenic differentiation, myoblasts were collected at day 0, 2 and 5 after induction of differentiation with or without BT2 and then myogenic marker genes and protein expression were measured by qRT-PCR and immunoblot. The results show that 100 μM of BT2 treatment significantly decreased the expression of *Myog* and *Myh3,* early and late myogenic differentiation marker genes, respectively (Fig. 4.3). BT2 treatment also

decreased the expression of total MyHC, a major myogenic differentiation marker (Fig. 4.3). Microscopic observation also shows that BT2 treated myoblasts have less myotubes, suggesting the inhibitory effect of BCAAs catabolism on myogenic differentiation (Fig. 4.4).

4. 5 Discussion

Although it is well known about the positive effects of branched-chain amino acids (BCAA) in regulating skeletal muscle protein metabolism, much less is known about the effects of catabolism of BCAAs in regulating myoblasts proliferation and myotube formation. In this chapter, it was investigated whether increased catabolism of BCAAs affect myoblasts proliferation and myogenic differentiation by using BT2, a BCKDK (branched chain keto acid dehydrogenase kinase) inhibitor.

Muscle cell differentiation is an essential process in converting mono-nucleated myoblasts to terminally differentiated, multi-nucleated myotubes. It accompanies a drastic change in proteome such that there is an abundance of contractile myofibrillar proteins, including myosin heavy/light chain, troponin, and tropomyosin during myogenic differentiation. Since these contractile myofibrillar proteins need to be synthesized de novo, and given the significance of BCAAs in regulating protein metabolism, it is plausible to expect a role of these amino acids during differentiation. BCAAs activate mTOR signaling pathway that is required for myogenic differentiation [82]. In addition, skeletal muscle cell can use leucine as a source of cholesterol [91], which can be utilized in making myotube cell membranes during differentiation. BCAAs are also used as a fuel for oxidative metabolism in mitochondria of skeletal muscle cell during differentiation [92,93]. Leucine deprivation results in differentiation failure accompanied with abnormal expression of myogenic regulatory factors (MRFs) *Myf5* and *Myod* [94,95]. These studies

provide pivotal roles for BCAAs and/or their metabolites (or catabolism itself) in regulating skeletal muscle cell differentiation.

In this chapter, the effects of increased BCAAs catabolism by BT2 on myoblast proliferation and differentiation were examined. Although the data revealed that BT2 suppressed the cell proliferation and differentiation of C2C12, limitations of the research should be acknowledged. Although mRNA expression levels of BCAAs metabolic enzymes (*Bcat2*, *Bckdha* and *Bckdk*) were quantified by qRT-PCR, actual protein expression levels of them were not measured. Recent report has demonstrated that expression of E1α subunit of BCKD protein increased during myogenic differentiation, while its mRNA *Bckdha* was not altered [96]. Also, since the levels of α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV), and α-ketoisovalerate (KIV), metabolites of BCAAs, or other metabolites of BCAAs were not quantified in this study, it is obscure whether these or other metabolites directly inhibits the myoblasts proliferation and differentiation.

In conclusion, the results suggested the possibility that increased BCAAs catabolism inhibits myoblasts proliferation and differentiation.

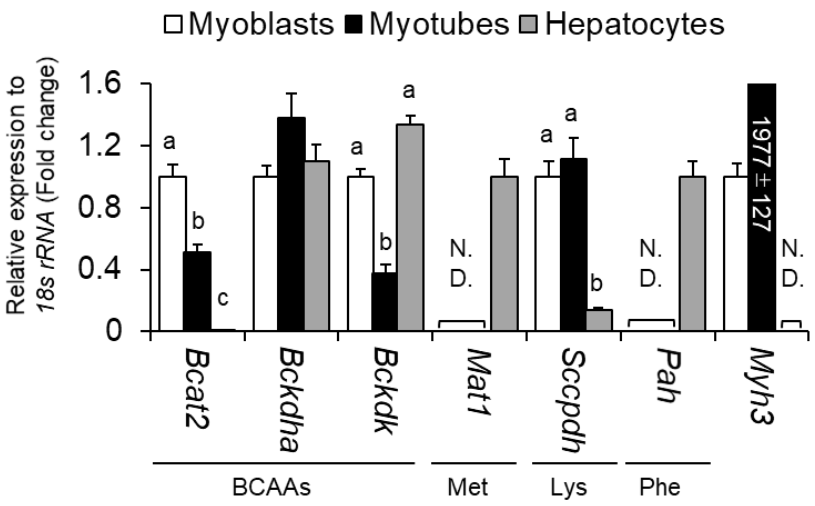

Figure 4.1 Gene expression analysis related to amino acid metabolism.

The expression of genes related to amino acid metabolic enzymes (*Bcat2, Bckdha, Bckdk, Mat1 a, Sccpdh,* and *Pah*) were measured by qRT-PCR. *Myh3* was used as a myogenic differentiation marker. AML12 hepatocytes were used as positive control for some amino acid metabolic enzymes. *Mat1* a and *Pah* were not detected in either myoblasts or myotubes (N.D.). mRNA expression levels were calculated relative to 18s rRNA and the data are expressed as a fold-increase. Different superscripts indicate a significant difference between the two groups. All assessments of significance were performed with one-way analysis of variance with Tukey post hoc test. Values are means ± SEM (n = 4).

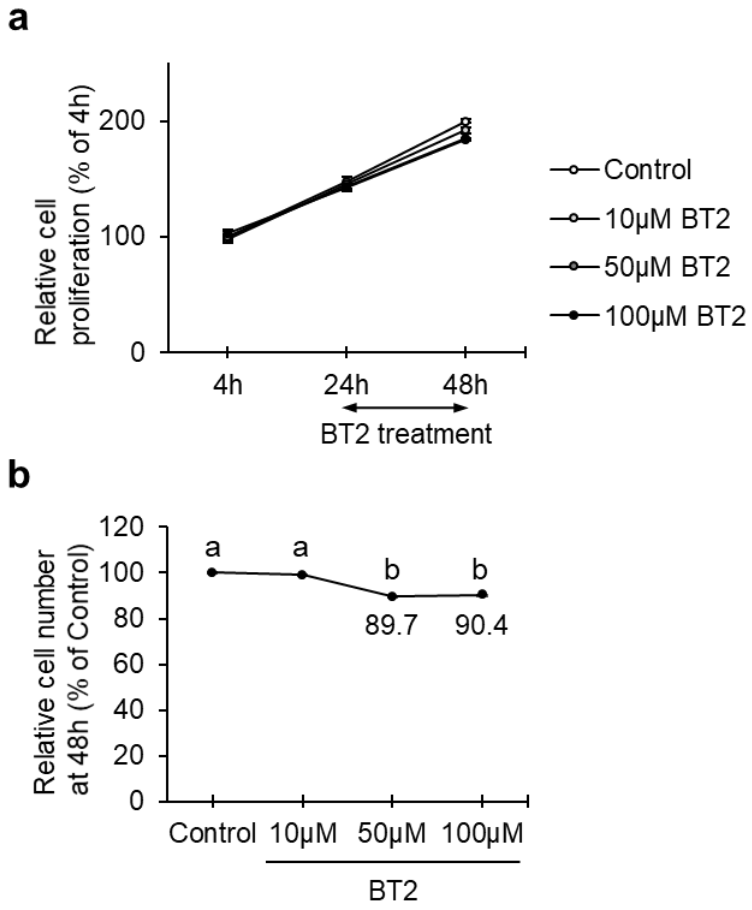

Figure 4.2 The effect of BT2 on C2C12 myoblast proliferation.

C2C12 myoblasts were cultured in DMEM containing 1% FBS and BT2 (10, 50, 100 μM). Cell Counting Kit-8 was used to measure the relative number of myoblasts. a) Relative cell proliferation rates were presented as percentage of 4h. b) Relative cell number 24 h after the treatment of BT2 was presented. Different superscripts indicate a significant difference between 2 groups. All assessments of significance were performed with 1-way ANOVA with Tukey post hoc test ($p < 0.05$). Values are means ± SEM (n = 12–14).

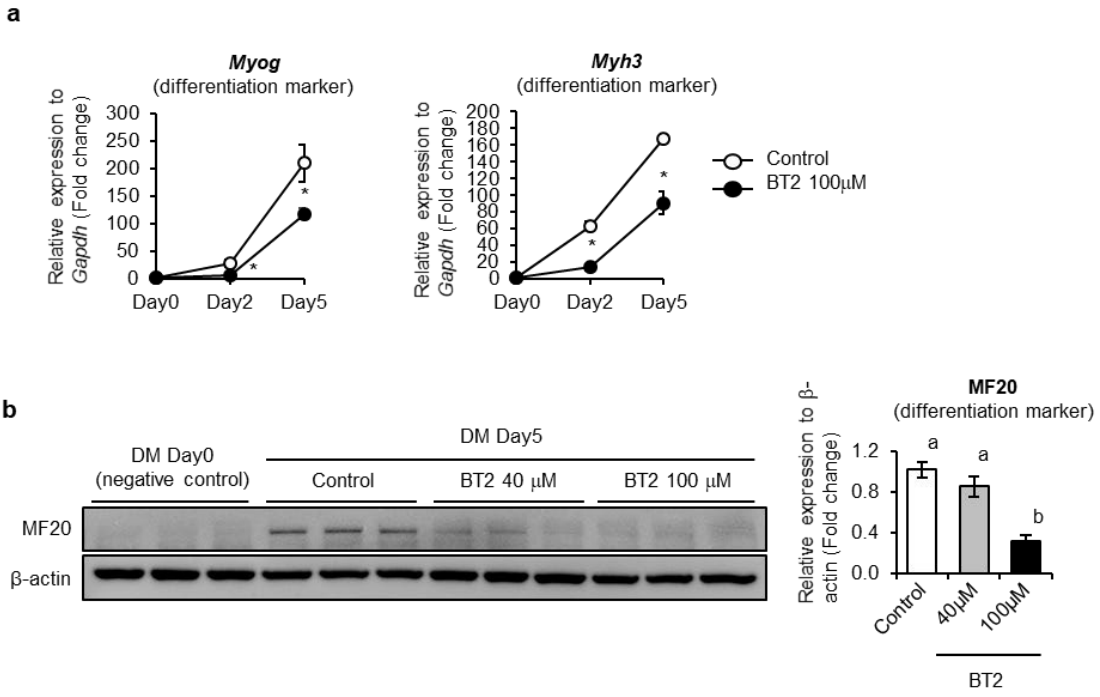

Figure 4.3 The effect of BT2 on C2C12 myoblast differentiation.

a) Time-course qRT-PCR analysis of the effect of BT2 treatment (100 mM) on the expression of myogenic differentiation markers (*Myh3* and *Myog*) of C2C12 myoblasts. mRNA expression levels were calculated relative to *Gapdh* and the data are expressed as a fold-increase. Significance was determined with the two-tailed Student's t-test (vs. control, *p < 0.05) (n = 6). Values are expressed as means ± SEM. b) The effect of BT2 treatment (40 μM and 100 μM) on total MyHC expression (anti-MF20) of C2C12 myoblasts for 5 days after induction of differentiation. Myoblasts at DM 0day (cultured in growth media) is used as negative control. Graph shows the relative intensity of each band after normalization to b-actin. Different superscripts indicate a significant difference between 2 groups. All assessments of significance were performed with 1- way ANOVA with Tukey post hoc test ($p < 0.05$) (n = 3). Values are expressed asmeans ± SEM

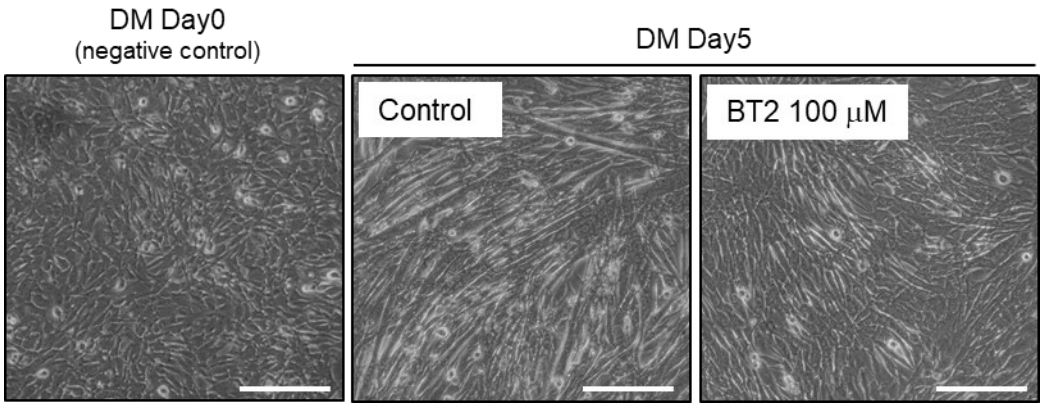

Figure 4.4 The effect of BT2 on C2C12 myoblast differentiation.

Representative images of Control and BT2-treated (100 μM) C2C12 myoblasts for 5 days after induction of differentiation. Myoblasts at DM day0 was shown as negative control. Bar = 100 μm.

Chapter 5

General discussion and conclusion General discussion and conclusion

An understanding of the mechanism of skeletal muscle energy metabolism, hypertrophy and differentiation is important for the prevention and treatment of metabolic diseases and muscle dysfunction. Recently, some amino acids and nutrients which are known to be biologically safe had been studied as regulator of skeletal muscle metabolism. BCAAs, especially leucine is well known as a trigger for skeletal muscle anabolism through the activation of mTOR signalling. Although the mTOR signalling is well known to regulate various cellular events, such as protein metabolism, energy metabolism, cell proliferation and differentiation, its role has not been fully examined.

In our laboratory, unpublished microarray analyses revealed that administration of leucine (135 mg/100 g B. W.) up-regulated slow-fiber related genes in gastrocnemius muscle of rat with no alteration of fast-fiber related genes 3 hours after administration of leucine. Thus, it was anticipated that one-shot oral administration of only leucine might alter the gene expression related to mitochondrial biogenesis and muscle function. To further understanding of the unidentified physiological roles of leucine, in this doctoral thesis, it was aimed to investigate the effects of leucine on fiber-type, metabolism, hypertrophy and differentiation in skeletal muscle. To define the physiological roles of leucine on skeletal muscle metabolism from multiple points of view, the following studies were performed.

5.1 Direct effects of leucine on slow-fiber and mitochondria related genes expression in skeletal muscle through mTOR signaling

Previous study reported that supplementation of chronic BCAAs leads to the

characteristic of slow-twitch muscle fibers and increased mitochondrial biogenesis in both cardiac and skeletal muscles of middle-aged mice through the activation of mTOR signaling. However, it has remained unclear whether leucine alone can directly affect the muscle fiber-type and mitochondrial biogenesis in young animal. To elucidate the effect of leucine on muscle fiber-type and mitochondrial biogenesis and its mechanism, animal and cultured cell experiments were performed. As a result, it was demonstrated that acute oral administration of leucine (135 mg/100 g body weight) could alter the gene expression related to fiber type and metabolism in rat skeletal muscle through the activation of mTOR signaling. The finding suggests the direct and acute effect of leucine on skeletal muscle metabolic characteristics and should contribute the development of novel functional foods and supplements.

5.2 Leucyl-tRNA synthetase is required for the myogenic differentiation of C2C12 myoblasts, but not for hypertrophy or metabolic alteration of myotubes

Recent studies have identified the intracellular sensors of amino acids (e.g. Leucyl-tRNA synthetase, Sestrin2) required for the activation of mTOR signalling. However, it has remained unclear whether Leucyl-tRNA synthetase (Lars) is involved in the activation of mTOR signalling and skeletal muscle physiology, including myogenic differentiation, hypertrophy and energy metabolism. To determine the role of Lars, small interfering (si)-RNA mediated knockdown was performed against C2C12 myoblasts and differentiated myotube. Knockdown of *Lars* decreased phosphorylated p70 S6 kinase, a crucial downstream target of mTOR signaling, in C2C12 myoblasts, suggesting that Lars is involved in the activation of mTOR signaling. Also, *Lars* knockdown inhibited the differentiation of C2C12 myoblasts into myotubes, and this was

accompanied with decreased level of Insulin-like growth factor 2 (*Igf2*) expression. The results suggested the link between the mTOR–IGF2 axis and Lars in myogenic differentiation. However, *Lars* knockdown did not affect the hypertrophy of myotubes and energy metabolism (glycolysis and mitochondrial respiration) of myotubes. The results demonstrated for the first time that Lars is essential for the activation of mTOR signaling in skeletal muscle cells and myogenic differentiation through the induction of *Igf2* expression.

5.3 Activation of BCAAs catabolism inhibited the proliferation and differentiation of C2C12 myoblast

It is well known about the positive effects of BCAAs in the regulation of skeletal muscle protein metabolism, much less is known about the effects of catabolism of BCAAs in regulating myogenesis. Suzuki et al. (from our lab) have reported that increased catabolism of BCAAs by BCKDK inhibitor BT2 treatment affected muscle metabolism in C2C12 differentiated myotubes. Since the expression of BCAAs metabolic enzymes (*Bcat2, Bckdha* and *Bckdk*) were confirmed in both undifferentiated myoblasts and differentiated myotubes by qRT-PCR, the catabolism of BCAAs were promoted by the BCKDK inhibitor BT2 in C2C12 myoblasts. The activation of BCAAs catabolism by BT2 impaired C2C12 myoblasts proliferation and differentiation. The results suggested the possibility that increased BCAAs catabolism inhibits myoblasts proliferation and differentiation. Although the levels of branched-chain keto acids or other metabolites of BCAAs were not quantified in this study, not only leucine but also metabolites of leucine might also have important effects on myoblast differentiation.

Conclusion

To summarize the findings, (1) the acute administration of leucine alone can up-regulate mitochondrial genes and slow-fiber related gene expression through mTOR signaling. The results suggested the possibility that leucine can alter fiber-type muscle cells and regulate metabolism in skeletal muscle. (2) Lars is essential for the activation of mTOR signaling in skeletal muscle cells and myogenic differentiation thought the induction of *Igf2* expression. The fact that Lars knockdown did not affect the activation of mTOR signaling, myotube hypertrophy, or metabolic alteration suggests a compensatory effect of leucine sensing by another sensor.

(3) Increased BCAAs catabolism inhibits myoblasts proliferation and differentiation.

The findings should be important in the fields of nutrition, skeletal muscle physiology and metabolic disease, and contributes to the development of novel functional foods and supplements.

List of publication related to this doctoral thesis:

1. **Yoriko Sato**, Yusuke Sato, Kodwo Amuzuah Obeng, Fumiaki Yoshizawa

   Acute oral administration of L-leucine upregulates slow-fiber– and mitochondria-related genes in skeletal muscle of rats

   Nutrition Research 57 36-44 (2018)

2. **Yoriko Sato**, Yusuke Sato, Reiko Suzuki, Kodwo Amuzuah Obeng, Fumiaki Yoshizawa

   Leucyl-tRNA synthetase is required for the myogenic differentiation of C2C12 myoblasts, but not for hypertrophy or metabolic alteration of myotubes

   Experimental Cell Research 364 (2) 184-190 (2018)

3. **Yoriko Sato**, Hayato Tate, Fumiaki Yoshizawa, Yusuke Sato

   Data on the proliferation and differentiation of C2C12 myoblast treated with branched-chain ketoacid dehydrogenase kinase inhibitor

   Data in Brief 31 105766 (2020)

Publications not included in this thesis:

1. Yaohui Nie, **Yoriko Sato**, Chao Wang, Feng Yue, Shihuan Kuang, Timothy P. Gavin

   Impaired exercise tolerance, mitochondrial biogenesis, and muscle fiber maintenance in miR-133a–deficient mice

   The FASEB Journal 30 (11) 3745-3758 (2016)

2. Yusuke Sato, Hideaki Ohtsubo, Naohiro Nihei, Takane Kaneko, **Yoriko Sato**, Shin-Ichi Adachi, Shinji Kondo, Mako Nakamura, Wataru Mizunoya, Hiroshi Iida, Ryuichi Tatsumi, Cristina Rada, Fumiaki Yoshizawa

Apobec2 deficiency causes mitochondrial defects and mitophagy in skeletal muscle

The FASEB Journal 32 (3) 1428-1439 (2018)

3. Yaohui Nie ,**Yoriko Sato**, Ron T. Garner, Christopher Kargl, Chao Wang, Shihuan Kuang, Christopher J. Gilpin, Timothy P. Gavin

   Skeletal muscle-derived exosomes regulate endothelial cell functions via reactive oxygen species-activated nuclear factor-κB signaling

   Experimental Physiology 104 (8) 1262-1273 (2019)

4. Reiko Suzuki, **Yoriko Sato**, Kodwo Amuzuah Obeng, Daisuke Suzuki, Yusuke Komiya, Shin-ichi Adachi, Fumiaki Yoshizawa, Yusuke Sato

   Energy metabolism profile of the effects of amino acid treatment on skeletal muscle cells: Leucine inhibits glycolysis of myotubes

   Nutrition 77 110794 (2020)

5. Reiko Suzuki, **Yoriko Sato**, Misato Fukaya, Daisuke Suzuki, Fumiaki Yoshizawa, Yusuke Sato

   Energy metabolism profile of the effects of amino acid treatment on hepatocytes: Phenylalanine and phenylpyruvate inhibits glycolysis of hepatocytes.

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