Nutrition 29 (2013) 284–290

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Upregulation of amino acid transporter expression induced by L-leucine availability in L6 myotubes is associated with ATF4 signaling through mTORC1-dependent mechanism

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ARTICLE INFO

Article history:
Received 29 March 2012
Accepted 10 May 2012

Keywords:
Amino acid transporter
L-leucine
Mammalian target of rapamycin
Protein metabolism

ABSTRACT

Objective: Essential amino acids, especially L-leucine, initiate the signaling of the mammalian target of rapamycin complex-1 (mTORC1) and protein synthesis in skeletal muscle. Current information on the relation between amino acid transporter mechanisms and mTORC1 signaling is sparse. The objectives of this study were to determine whether an increase in leucine availability upregulates the gene transcription and translation of amino acid transporters and other amino acid members in an mTORC1-dependent pathway that control amino acid use (general control non-repressed-2 and activating transcription factor-4) and to measure the factors related to protein synthesis and proteolysis.

Methods: L6 skeletal muscle cells that had been treated with L-leucine (0.105 g/L) were incubated for 30 min to stimulate the transcription of L-type amino acid transporter-1, CD98, and sodium-coupled neutral amino acid transporter-2 and increase activating transcription factor-4 protein, which is dependent on the mTORC1 signaling pathway.

Results: A rapid, high level of p70 S6 kinase-1 phosphorylation was detected but was suppressed by rapamycin ($P < 0.05$). The addition of leucine decreased the atrogin-1 transcription abundance in an insulin-involved manner ($P < 0.05$), which could not be completely blocked by rapamycin ($P = 0.055$).

Conclusions: Our findings indicate that the mTOR is a component of the nutrient signaling pathway, which regulates system A and L amino acid transporters, the initiation factors involved in mRNA translation, and is downstream of forkhead box-O in L6 myotubes.

Introduction

It is well established that essential amino acids (EAAs), in particular the branched-chain AAs (i.e., L-leucine), stimulate skeletal muscle protein synthesis by the activation of the mammalian target of rapamycin complex-1 (mTORC1) signaling pathway, which regulates translation initiation and elongation [1–4]. Very recent work has shown that an increase in EAA availability exerts a link to AA transporter expression in human skeletal muscle [5] and L6 rat muscle cells [6], and that this mechanism is dependent on protein synthesis. Although the mTORC1 pathway has been the focus of attention in studies of the skeletal muscle of humans and rats [2,7–9], whether the AA signaling increase of AA transporter expression in skeletal muscle is sensitive to rapamycin is not fully understood.

There is evidence in the literature showing that the integration of active transport mechanisms may account for the molecular mechanisms influencing the transmembrane distribution of AAs and cellular protein metabolism regulation. One group of AA transporters, system A type (i.e., sodium-coupled neutral amino acid transporter-2 [SNAT2]/*SLC38A2*), called *secondary active transporters*, generate the net movement of AAs from the extracellular to the intracellular pool, whereas tertiary active transporters transport through exchangers such as system L type (L-type amino acid transporter-1 [LAT1]/*SLC7A5* and CD98/*SLC3A2*), another group of AA transporters, which allows for the redistribution of individual branched-chain AAs without affecting total pool sizes [10]. These two systems of transporters have been highlighted in the present work because these active transporters function by coupled transport processes at the cell surface [11]. Changes in AA availability are sensed by these transporters and, hence, have a profound effect on signaling by the highly

The funding of this research is supported by Earmarked Fund for China Agriculture Research System (CARA-36).

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http://dx.doi.org/10.1016/j.nut.2012.05.008

Table 1
Primers used for the real-time polymerase chain reaction analysis

Primer	Sequence 5′ to 3′	Product size (bp)	Accession number
Atrogin-1	ccatcaggagaagtggatctatgtt gcttcccccaaagtgcagta	73	AY059628
MuRF1	tgttctggtaggtcgtttccg atgccggtccatgatcactt	355	AY059627
LAT1	gaccctgatgtacgccttct gcaggccaggataaagaaca	181	AB015432
CD98	acttggctgagtggcagaat agatcgctggtggattcaag	118	NM_019283
SNAT2	aatggaatccttgggctttc gccagaccgtatgccttatg	176	NM_181090
ATF4	gctatggatgggttggtcag agctcatctggcatggtttc	149	BC158588
GCN2	agaactcaatcacgccaaca ataaagaggcaccgggtctt	123	NM_019335
β-actin	tcgtaccactggcattgtgat cgaagtctagggcaacatagca	233	NM_031144

ATF4, activating transcription factor-4; GCN2, general control non-repressed-2; LAT1, L-type amino acid transporter-1; MuRF1, muscle ring finger 1; SNAT2, sodium-coupled neutral amino acid transporter-2

conserved general control non-repressed (GCN) and TOR pathways, which play an important role in the dynamic control of protein synthesis and cell growth [12–14]. In consequence, the activation of activating transcription factor-4 (ATF4)/cAMP response element-binding protein 2 (CREB2), regulated downstream of the TORC1 or GCN2 pathway, crucially supports the expression of genes encoding AA transporters and AA biosynthetic enzymes [12,13,15], maintaining a cellular adaptation to an altered nutrient state [15,16]. Because of the possible role mediated by AA transporters/transceptors in the signal regulation of global protein synthesis and cell growth, it is important to consider the interactions between the AA transporters and the nutrient-responsive signaling pathways leading to protein metabolism.

To date, little is known about the mechanism of AA transporter expression in cultured L6 rat muscle cells in response to an increase in L-leucine availability. Thus, the primary purpose of this study was to determine the expression levels of L-type (LAT1/*SLC7A5* and CD98/*SLC3A2*) and A-type (SNAT2/*SLC38A2*) AA transporters in relation to leucine kinetics in L6 rat muscle cells after adding L-leucine to the test media. Second, we aimed to determine the response induced by leucine availability associated with members of the general AA control pathway (GCN2 and ATF4). It was hypothesized that an increase in leucine availability would stimulate ATF4 and AA transporter expressions and the mechanism on which these depend in the mTOR signaling pathway.

Materials and methods

Materials

Rat L6 skeletal muscle cells were kindly provided by the American Type Culture Collection (Manassas, VA, USA). Cell culture medium (Dulbecco's Modified Eagle's Medium [DMEM]), DMEM lacking leucine, and fetal bovine serum were purchased from Thermo Scientific Hyclone, Inc (Logan, UT, USA). One percent (v/v) penicillin-streptomycin and 0.25% sterile trypsin–ethylenediaminetetraacetic acid solution were obtained from Gibco-BRL (Paisley, Renfrewshire, UK). The signaling pathway inhibitor rapamycin, L-leucine, and bovine insulin were obtained from Sigma-Aldrich (Poole, Dorset, UK). Protein assay and western blotting reagents were from Bio-Rad Laboratories (Hercules, CA, USA). The antibodies (rabbit polyclonal) against phospho-specific S6 kinase-1 (S6k1; Thr389) and ATF4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc., respectively. Horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (Cell Signaling Technology, Bedford, ME, USA) was used as a secondary antibody. Enhanced chemiluminescence detection reagents were obtained from Amersham Pharmacia Biotech (UK Ltd., Little Chalfont, UK). Reagents for RNA isolation and quantitative real-time polymerase chain reaction (PCR) were purchased from Invitrogen, Inc. (Carlsbad, CA, USA) and Takara, Inc. (Shiga, Japan).

Cell culture

Monolayers of L6 muscle cells were grown and maintained in 100-mm dishes with DMEM containing 10% fetal bovine serum, penicillin 100 000 IU/L, and streptomycin 100 μg/mL at 37°C under humidified 95% air/5% CO_2. When the cells were approximately 80% confluent, they were seeded in 6-well culture plates (2.5×10^4 cells/well) for the measurement of mRNA levels and western blot analysis. Although the cells achieved confluence, the concentration of fetal bovine serum was decreased to 2% to induce a differentiation into myotubes. The myoblast fusion to form myotubes was then allowed to continue for another 2 d before commencing the experiments, and the cells were washed twice with phosphate buffered saline. Serum-free DMEM without leucine was then added to the plates, and the cells were starved and incubated for 6 h. At that time, the cells were randomly divided into two groups. Rapamycin was added to the plates in one group to a final concentration of 100 nmol/L. Leucine at a final concentration equivalent to that present in the complete DMEM and/or insulin (100 nmol/L) was added 30 min after treatment with rapamycin. The cells were returned to the incubator, and 30 min later, myotubes were harvested and lysed in ice-cold lysis buffer as described in detail previously [17]. Subsequent additions to the cells were made at the times and concentrations described in detail in the figure captions.

Protein immunoblot analysis

The protein content of the cell lysates for western blotting was assayed using the Bio-Rad detergent-compatible protein assay kit (no. 500-0116). The protein content was determined using the Bradford assay [18]. Aliquots of cell homogenate containing 50 μg of protein were combined with an equal volume of sodium dodecylsulfate sample buffer and then subjected to sodium dodecylsulfate/polyacrylamide gel electrophoresis and immunoblotting as described previously [19]. Briefly, the proteins were separated by electrophoresis and transferred to polyvinyl difluoride membranes. The membranes were blocked with Tris-buffered saline (pH 7.0) containing 5% bovine serum albumin and 0.05% (v/v) Tween-20. The membranes were probed with anti-phospho Thr389 S6k1 (1:1000) or ATF4 (1:3000) antibodies. After the primary antibody incubation, the

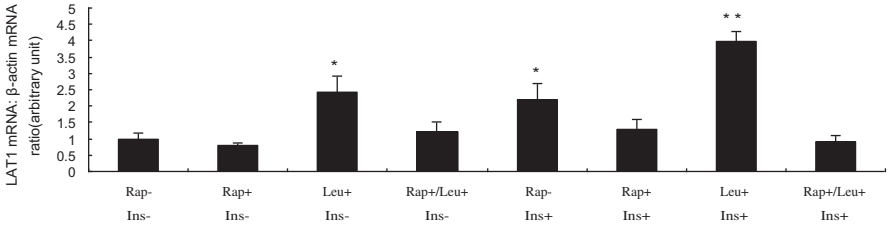

Fig. 1. Effect of leucine availability on mRNA levels of LAT1 in cultured L6 myotubes. Myotubes were cultured for 30 min in the absence or presence of bovine insulin 100 nmol/L bovine insulin with or without rapamycin 100 nmol/L. The mRNA level of LAT1 was determined by measuring the lysates of cells with a real-time polymerase chain reaction assay as described in MATERIALS AND METHODS. Results are presented as mean ± SEM of three experiments, each performed in triplicate. Asterisks indicate a statistically significant change from the control value (obtained from unstimulated cells) by the Duncan multiple-range test ($P < 0.05$). Ins+, with insulin; Ins−, without insulin; LAT1, L-type amino acid transporter-1; Leu+, with leucine; Rap+, with rapamycin; Rap−, without rapamycin.