Animal Nutrition 6 (2020) 513–520

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

A selectively suppressing amino acid transporter: Sodium-coupled neutral amino acid transporter 2 inhibits cell growth and mammalian target of rapamycin complex 1 pathway in skeletal muscle cells

Lingyu Zhang [a, b, c], Yehui Duan [a, b], Qiuping Guo [a, b, c], Wenlong Wang [a, b, d], Fengna Li [a, b, *]

[a] Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process, Key Laboratory of Agro-ecological Processes in Subtropical Region, Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, Hunan 410125, China

[b] Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha, Hunan 410125, China

[c] University of Chinese Academy of Sciences, Beijing, 100039, China

[d] Laboratory of Animal Nutrition and Human Health, School of Biology, Hunan Normal University, Changsha, 410018, China

ARTICLE INFO

Article history:
Received 3 December 2019
Received in revised form
11 March 2020
Accepted 13 March 2020
Available online 26 June 2020

Keywords:
α-methylaminoisobutyric acid
Sodium-coupled neutral amino acid transporter 2 suppression
Cell growth
Mammalian target of rapamycin
Muscle cell

ABSTRACT

Sodium-coupled neutral amino acid transporter 2 (*SNAT2*), also known as solute carrier family 38 member 2 (*SLC38A2*), is expressed in the skeletal muscle. Our research previously indicated that *SNAT2* mRNA expression level in the skeletal muscle was modulated by genotype and dietary protein. The aim of this study was to investigate the key role of the amino acid transporter SNAT2 in muscle cell growth, differentiation, and related signaling pathways via *SNAT2* suppression using the inhibitor α-methyl-aminoisobutyric acid (MeAIB). The results showed that *SNAT2* suppression down-regulated both the mRNA and protein expression levels of SNAT2 in C2C12 cells, inhibited cell viability and differentiation of the cell, and regulated the cell distribution in G0/G1 and S phases ($P < 0.05$). Meanwhile, most of the intercellular amino acid content of the cells after MeAIB co-culturing was significantly lower ($P < 0.05$). Furthermore, the mRNA expression levels of system L amino acid transporter 1 (*LAT1*), silent information regulator 1, and peroxisome proliferator-activated receptor-gamma co-activator 1 alpha, as well as the protein expression levels of amino acid transporters LAT1 and vacuolar protein sorting 34, were all down-regulated. The phosphorylated protein expression levels of mammalian target of rapamycin (mTOR), regulatory-associated protein of mTOR, 4E binding protein 1, and ribosomal protein S6 kinase 1 after MeAIB treatment were also significantly down-regulated ($P < 0.05$), which could contribute to the importance of SNAT2 in amino acid transportation and skeletal muscle cell sensing. In conclusion, SNAT2 suppression inhibited C2C12 cell growth and differentiation, as well as the availability of free amino acids. Although the mTOR complex 1 signaling pathway was found to be involved, its response to different nutrients requires further study.

1. Introduction

The marked gains in skeletal muscle mass in adolescents are due to a high rate of protein synthesis regulated by an enhanced sensitivity to hormones (such as insulin) and nutrients (such as protein or amino acids). In addition to their role as major substrates for energy production and protein synthesis, amino acids also have the capacity to transduce signals to metabolic pathways (e.g. mammalian target of rapamycin, mTOR) via transporters (Yin et al., 2010; Li et al., 2011; Duan et al., 2015). These transporters,

* Corresponding author.
E-mail address: lifengna@isa.ac.cn (F. Li).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2020.03.010

also known as "transceptors," possess dual functions, first as transporters and also as sensors, capable of sensing amino acid availability from the upstream of the signaling pathways (Goberdhan et al., 2016). Transceptors include system L amino acid transporter 1 (LAT1), proton assisted amino acid transporter (PAT), and sodium-coupled neural amino acid transporter 2 (SNAT2) (Suryawan and Davis, 2011; Poncet and Taylor, 2013; Taylor, 2014). The synthetic amino acid analogue α-methyl-aminoisobutyric acid (MeAIB) selectively suppresses the increase in SNAT2 expression (Gazzola et al., 2001; Lopez-Fontanals et al., 2003; Tang et al., 2018).

The mTOR signaling pathway plays a key role in cell metabolism, growth stimulation, and skeletal muscle protein accretion. It includes 2 independently regulated complexes: mammalian target of rapamycin complex 1 (mTORC1) and complex 2 (mTORC2). However, only mTORC1 is involved in amino acid sensing. The mTORC1 consists of mTOR, regulatory-associated protein of mTOR (Raptor), DEP domain-containing mTOR-interacting protein (DEPTOR), proline-rich Akt substrate of 40 kDa (PRAS40), and the G protein β-subunit-like protein (GβL) (Yoon and Chen, 2013; Goberdhan et al., 2016).

SNAT2 is expressed in most extraneural tissues, including skeletal muscle (Mackenzie and Erickson, 2004) and exhibits functional and regulatory properties of the classically defined system A transporter. The inhibition of SNAT2 using RNA interference (RNAi) strongly impairs signaling through mTOR, ribosomal protein S6 kinase 1 (S6K1), and 4E binding protein 1 (4EBP1), resulting in the impairment of protein synthesis (Evans et al., 2007). Cellular amino acid withdrawal induces an adaptive increase in the expression, stability, and function of the transporter SNAT2. During amino acid withdrawal, the c-Jun-N-terminal kinase signaling pathway is activated and induces the expression of SNAT2 in L6 myotubes (Hyde et al., 2007). The activity of SNAT2 is upregulated by insulin, osmotic shock, and amino acid deprivation (Kashiwagi et al., 2009). The expression of SNAT2 is also modulated by nutrients. Dietary protein-to-carbohydrate ratio influences SNAT2 abundance in mammary glands during lactation (Velázquez-Villegas et al., 2015), and the increase in SNAT2 function is restrained in cells subjected to prior incubation with linoleic acid (Nardi et al., 2015).

In our previous studies using pigs, a well-recognized animal model for human, we cloned the *SNAT2* gene and investigated the characterization of its expression (Li et al., 2015). Another study of us showed that *SNAT2* mRNA expression level in the skeletal muscle is markedly modulated by the pig breed and dietary protein level (Liu et al., 2016). To further study the role of SNAT2 in skeletal muscle cells, in the present study, we determined the cell cycle, differentiation, and expression levels of key genes related to amino acid transport, myokines, and energy metabolism, as well as the activation of mTOR signaling pathway.

2. Materials and methods

2.1. Cell culture

Mouse C2C12 cell lines sourced from American Type Culture Collection (ATCC) were cultured in high glucose (25 mmol/L) Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) in a 5% CO_2 incubator at 37 °C. Once the myoblasts reached about 90% confluency, the growth media was changed to differentiation media supplemented with 2% (vol/vol) horse serum (Gibco, AR, USA) for 4 to 6 d. MeAIB (Sigma, MO, USA) was co-cultured with the cells during proliferation and proliferation.

2.2. Cell growth characteristics

C2C12 myoblasts were plated in 96-well plates. The proliferation of the cells was measured using a commercial cell counting kit (CCK-8, Dojindo, Osaka, Japan), following the manufacturer's specifications. Absorbance was detected at a wavelength of 450 nm (BioTek Instruments, Winooski, USA) and the results were expressed as the optical density (OD_{450}).

2.3. Cell cycle analysis by flow cytometry

After treatment, about 1×10^6 cells were rinsed and harvested then re-suspended in a phosphate-buffered saline (PBS)/ethanol mixture (30:70, vol/vol) and incubated in PBS containing 200 μg/mL propidium iodide (PI) (Sigma, MO, USA) and 1 mg/mL RNase (Sigma, MO, USA). The samples were then analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, USA). The fluorescence intensities of the cells stained with PI were monitored at 630 nm.

2.4. Free amino acid profile

Myotubes were rinsed twice using PBS then harvested and dissolved in water with methanol (1:1, vol/vol) at 4 °C for 30 min, followed by centrifuging at 10,000 × *g* for 10 min. The resulting supernatant was filtered through a glass wool column and stored at −80 °C until further analysis. After centrifuging to separate the soluble from insoluble material, 40 μL of the supernatant was labeled with iTRAQ reagents using an AA 45/32 kit (Applied Biosystems, Life Technology, Forrest City, CA, USA), according to the manufacturer's instructions. The supernatant was then analyzed using an Applied Biosystems 3200 Q TRAP LC/MS/MS system equipped with a RP-C18-column (150-mm length, 4.6-mm diameter, 5-μm particle size).

2.5. Real-time PCR analysis

C2C12 myotubes were harvested and total RNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality and quantity of RNA was determined by ultraviolet spectroscopy using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE). Thereafter, about 1 μg of total RNA samples were processed into cDNA using a reverse transcription -PCR system (Promega, Madison, WI, USA). The primer sequences for the selected genes are shown in Table 1. Real-time PCR was carried out using SYBR Green I as the PCR core reagents in a final volume of 20 μL using an ABI7900HT PCR system (Applied systems, Forrest City, CA, USA). The mRNA expression levels of the target genes in arbitrary units were calculated from the value of the threshold cycle (Ct) of real-time PCR compared to that of the internal control using the comparative Ct method: $2^{\Delta Ct}$ ($Ct = Ct_{\beta\text{-actin}} - Ct_{\text{gene of interest}}$). The house-keeping gene β-actin was used as the internal control against which target gene expression was normalized.

2.6. Western blotting analysis

The cells were rinsed twice using PBS, harvested, pelleted by centrifugation, and lysed in radio immunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mmol/L Tris–HCl at pH 7.4), combined with a protease inhibitor cocktail and phosphatase inhibitors. Soluble proteins (20 to 30 μg) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) then transferred to a polyvinylidene fluoride (PVDF) membrane, blocked with 5% non-fat milk in tris-buffered saline (TBS) with 0.05% Tween-20 for 1 h, and incubated overnight with primary antibodies, followed by horseradish peroxidase-linked secondary antibodies. The protein bands

Table 1
Primers used for real-time PCR analysis.

Item	Primer sequences (5′-3′)	Product size, bp	Tm, °C
Myogenin	F: ACCTTCCTGTCCACCTTCAG	168	60
	R: CAGACTTCCTCTTACACACC		
SNAT2	F: TGAAGAAGACCGAAATGGGA	96	58
	R: TGGTGGGGTATGAGTAGTTG		
LAT1	F: GCGGGAGAAGATGTTGGAG	136	62
	R: GAAGATGCCAGAGCCGATGA		
IL-15	F: TCCATCTCGTGCTACTTGTG	365	56
	R: CAGCCAGATTCTGCTACAT		
Myostatin	F: CAGACCCGTCAAGACTCCTACA	120	62
	R: CAGTGCCTGGGCTCATGTCAAG		
FOXO1	F: CTGGGTGTCAGGCTAAGAGT	104	60
	R: GGGGTGAAGGGCATCTTT		
UCP3	F: ACTGCTGCTGACACGAGA	266	62
	R: GACAACACTGTGGGCTGA		
AMPKα	F: TGAAGATCGGCCACTACATCCT	71	60
	R: CTTGCCCACCTTCACTTTCC		
SIRT1	F: ACCTCCCAGACCCTCAAG	145	58
	R: TTCCTTCCTTATCTGACAAAGC		
PCG-1α	F: AAACCACACCCACAGGATCAG	72	62
	R: TCTTCGCTTTATTGCTCCATGA		
β-actin	F: GACCTCTATGCCAACACA	270	64
	R: TCAGTAACAGTCCGCCTA		

Tm = annealing temperature; F = forward; R = reverse; *SNAT2* = sodium-coupled neutral amino acid transporter 2; *LAT1* = system L amino acid transporter 1; *IL-15* = interleukin 15; *FOXO1* = fork head transcription factor 1; *UCP3* = uncoupling protein 3; *AMPKα* = AMP-activated protein kinase α; *SIRT1* = silent information regulator transcript 1; *PCG-1α* = peroxisome proliferator-activated receptor γ coactivator-1α.

were visualized using a chemiluminescent reagent (Pierce, Rockford, IL, USA) with a digital luminescent image analyzer LAS-1000 (Fujifilm, Japan). The resultant signals were quantified using Alpha Imager 2200 software (Alpha Innotech Corporation, San Leandro, CA, USA), and normalized against the internal control.

2.7. Statistical analysis

All the experiments were repeated independently 3 times. The data obtained were analyzed using the Student's *t*-test with the aid of the SAS Software package (SAS version 8.2; Cary, North Carolina, US). Differences were considered statistically significant at $P < 0.05$. The results were expressed as the mean ± standard error (SE).

3. Results

3.1. MeAIB inhibits the proliferation of C2C12 myoblasts

As presented in Fig. 1, MeAIB was diluted to different concentrations (0, 0.5, 1, 2, 5, and 10 mmol/L) and treated with C2C12 cells for 1, 2, 4, 8, 12, 24, and 48 h. The cell viability (OD_{450} value) showed that different concentrations of MeAIB from 1 to 12 h had no effect on cell growth. However, an effect was observed after 24 h of treatment, and a similar tendency was observed between 24 and 48 h. The OD_{450} value decreased with an increased MeAIB concentration. In particular, 5 mmol/L MeAIB was found to significantly reduce C2C12 myoblasts after 48 h compared to the blank and 0.5 to 2 mmol/L MeAIB ($P < 0.05$). Therefore, the treatment of myoblast cells with 5 mmol/L MeAIB for 48 h was used for subsequent analysis.

3.2. MeAIB regulates the cell cycle of C2C12 myoblasts

To determine the effect of MeAIB on the cell cycle, we treated the myoblasts with or without MeAIB (5 mmol/L) for 48 h after serum starvation. The results showed that after treatment, there were more cells distributed in G0/G1 phase, and less cells distributed in S phase compared to the blank ($P < 0.05$). Meanwhile, no significant difference was noted in G2/M phase between the 2 treatments (Fig. 2).

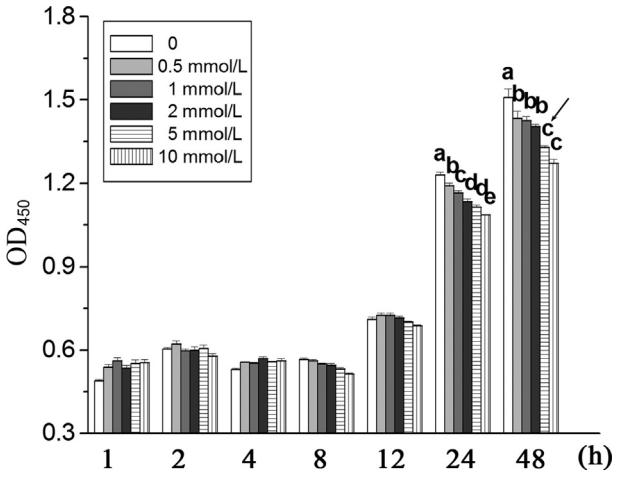

Fig. 1. MeAIB regulates the cell viability of C2C12 myoblasts. The cells were treated with various MeAIB concentrations for different time periods (1, 2, 4, 8, 12, 24 and 48 h) before determining the OD_{450} value. Data are represented as the mean ± SE ($n = 6$). MeAIB = α-methylaminoisobutyric acid. Data columns with different letters (a, b, c, d, e) are significantly different ($P < 0.05$).

3.3. Differentiation marker gene expression level of C2C12 cells

To determine the effect of MeAIB on C2C12 myoblasts differentiated into myotubes, the mRNA and protein expression levels of a marker gene, myogenin, was investigated (Fig. 3). The results indicated that after treatment with MeAIB (5 mmol/L), the expression level of myogenin was down-regulated significantly ($P < 0.05$).

3.4. Free amino acid profile of myotubes

The free amino acid profile of the myotubes after treatment with MeAIB is shown in Table 2. The results clearly indicated that the concentrations of all the essential and non-essential amino acids in the C2C12 myotubes were remarkably reduced, except for cysteine and L-serine ($P < 0.05$).

3.5. mRNA expression levels of key genes

The mRNA expression levels of key genes related to amino acid transporters, myokines, and energy metabolisms are shown in Fig. 4. The results indicated that MeAIB down-regulated the mRNA expression levels of the transporters *SNAT2* and *LAT1*, as well as the myokine interleukin-15 (*IL-15*) ($P < 0.05$). The value of silent information regulator 1 (*SIRT1*) and peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (*PGC-1α*) were both decreased significantly ($P < 0.05$). However, no difference was observed in the expression levels of myostatin, fork head box protein O1 (*FOXO1*), uncoupling protein 3 (*UCP3*), or AMP-activated protein kinase α (*AMPKα*) ($P > 0.05$).

3.6. Protein expression levels of transporters and mTOR/Raptor signaling pathway

The protein expression levels of the amino acid transporters are shown in Fig. 5. The results illustrated that the transporters SNAT2, LAT1, and vacuolar protein sorting 34 (hVps34) were all significantly down-regulated after treatment with MeAIB ($P < 0.05$). However, no difference was noted in the value of mitogen-activated protein kinase kinase kinase kinase-3 (MAP4K3) ($P > 0.05$).

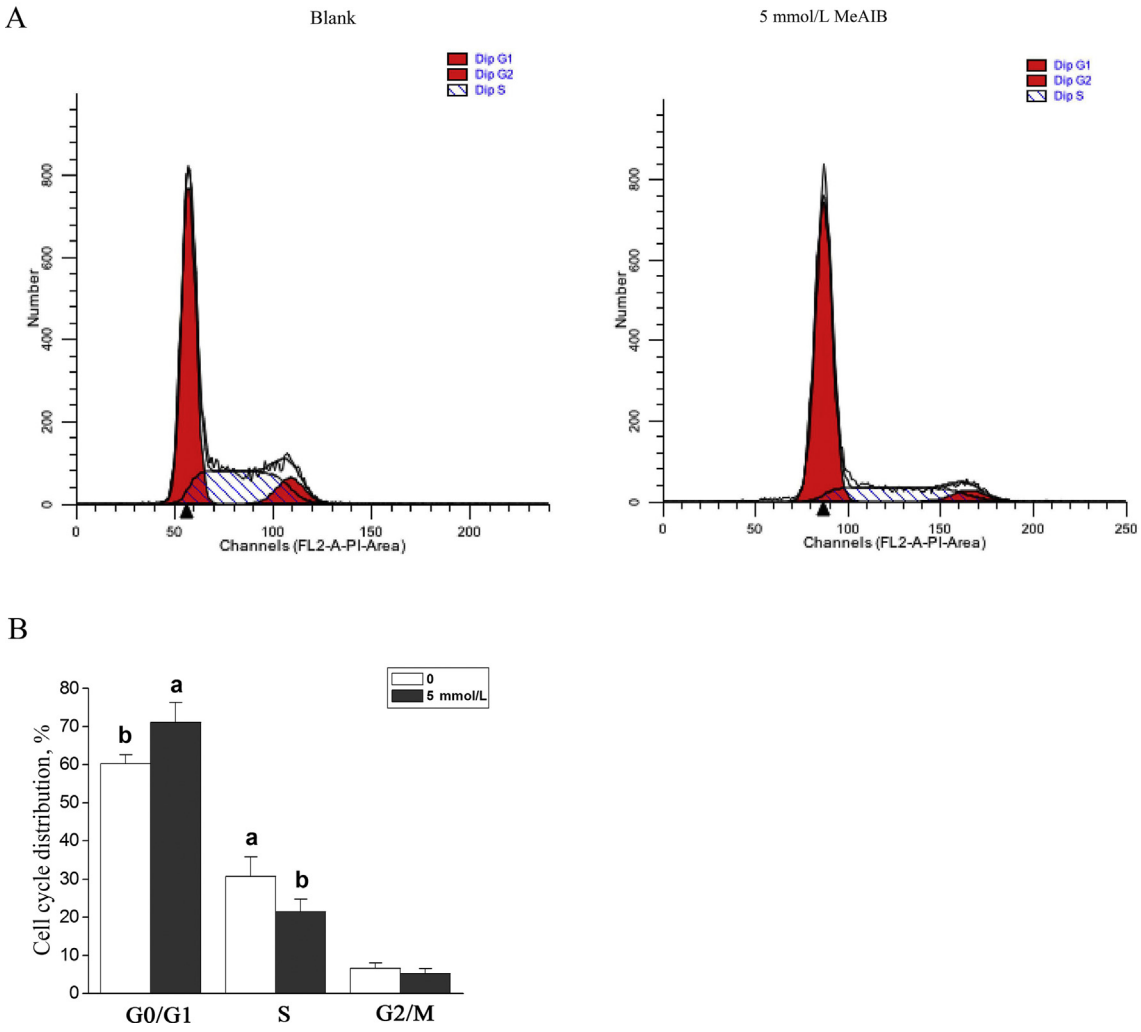

Fig. 2. MeAIB action on cell number (A) and cell cycle distribution (G0/G1, S, and G2/M) (B) of C2C12 cells. The cells were synchronized by serum deprivation and then treated with (blank) or without MeAIB (5 mmol/L) for 48 h. Data are represented as the mean ± SE ($n = 6$). MeAIB = α-methylaminoisobutyric acid; FL2-A-PI = fluorescence area-propidium iodide. Data columns with different letters (a, b) are significantly different ($P < 0.05$).

The expression levels of the phosphorylated proteins of the key molecules in the mTOR/Raptor signaling pathway were also measured and the results are shown in Fig. 6. The results indicated that the protein expression levels of phosphorylated mTOR, Raptor, 4EBP1, and S6K1 were all down-regulated in myotubes after treatment with MeAIB ($P < 0.05$).

4. Discussion

The C2C12 cell line is extensively used to determine the proliferation, differentiation, and protein synthesis of skeletal muscle cells (Li et al., 2014a, b; Duan et al., 2017). Amino acid transceptors are nutrient sensors, which are now established as integral components of nutrient-regulated signaling pathways, modulating the cell growth, proliferation, and metabolic rate (Kimball and Jefferson, 2010).

System A transporter SNAT2 exhibits functions as a hybrid transporter-receptor, and the structural changes during the transport cycle may be transduced to the signaling pathway (Donaton et al., 2003; Wu et al., 2006). Transporters of the SNAT family except for SNAT2 are expressed at low levels in the muscle (Yao et al., 2000; Hyde et al., 2001). The acute inhibition of the flux could be conducted by treating with MeAIB, an amino acid analogue (Evans et al., 2007).

In the current study, we found that the inhibition of SNAT2 using MeAIB largely decreased the viability of myoblasts. Meanwhile, the relative expression levels of *SNAT2* mRNA and protein were found to decrease significantly. Therefore, C2C12 cells treated with MeAIB effectively inhibited the activity of SNAT2. On the other hand, our results showed that MeAIB drove more cells from S phase to G0/G1 phase, indicating that most cells were blocked at G0/G1 phase, but did not proliferate into S phase. The results of cell viability and cell cycle both suggested that the suppression of the amino acid transceptor SNAT2 inhibits C2C12 myoblast growth.

Skeletal muscle differentiation is an integrative and complex process involving the exit of myoblasts from cell cycle to form multinucleated myotubes. Myogenin plays a critical role in the regulation of the final stage of differentiation (Lassar et al., 1994; Antoniou et al., 2014). Lacking the myogenin gene, mice die soon after birth due to myoblasts being unable to fuse into multinucleated myofibers (Venuti et al., 1995). Our results indicated that SNAT2 suppression decreases the relative mRNA and protein expression levels of the marker gene myogenin after pre-incubating with MeAIB. These findings suggest that the amino acid transceptor SNAT2 plays a key role in skeletal muscle cell differentiation.

SNAT2 is a key determinant of the intracellular concentration of amino acids, exerting a protein anabolic effect in skeletal muscle (such as branched amino acids). One function of amino acid

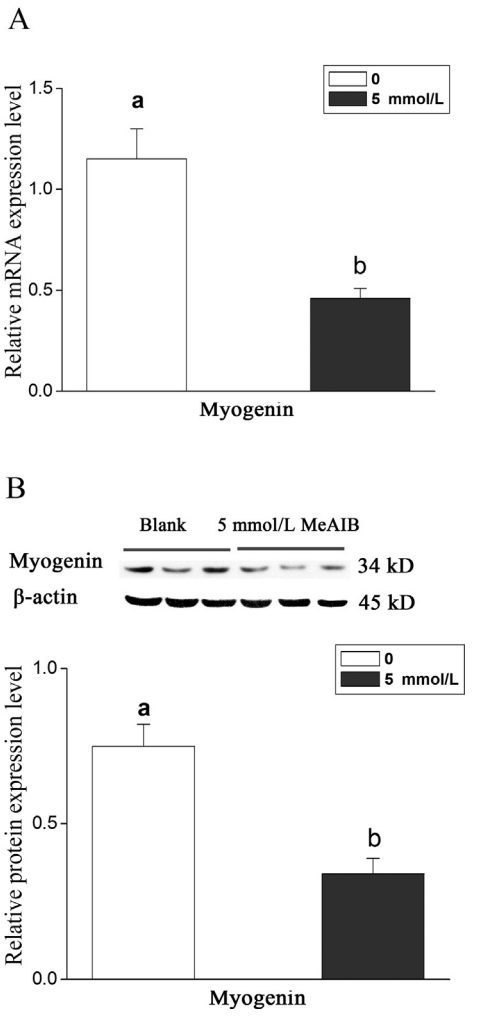

Fig. 3. The mRNA (A) and protein (B) expression levels of C2C12 differentiation mark gene myogenin. The expression levels were normalized using β-actin as an internal control using real-time PCR. Data are represented as the mean ± SE ($n = 6$). MeAIB = α-methylaminoisobutyric acid. Data columns with different letters (a, b) are significantly different ($P < 0.05$).

transporters, located on the cell membrane, is transporting amino acids across the plasma membrane. SNAT2 inhibition depletes intracellular glutamine and other anabolic amino acids, leading to the impairment of protein synthesis depending on mTOR signaling (Evans et al., 2007). Our results of the intracellular amino acid concentration were consistent with the previous study, wherein the concentrations of essential and non-essential amino acids were largely reduced after SNAT2 inhibition, except for cysteine and serine, which are both non-essential amino acids and could be synthesized mainly from methionine and glycine, respectively (Jahoor, 2012; Shigemi et al., 2010). It is surprising that the suppression of a single transporter protein, SNAT2, led to a marked depletion in intracellular amino acids in cultured skeletal muscle cells. These results suggest that SNAT2 might play an important role in free amino acid transport and availability, and consequently in protein synthesis, based on the amounts of amino acids in the skeletal muscle cells, which is consistent with previous studies (Tang et al., 2018; Kashiwagi et al., 2009).

LAT1 is primarily isolated from C6 rat glioma cells, sub-serving the amino acid transport system L (Kanai et al., 1998) and expressed in the skeletal muscle and any fast-growing cells (Uchino et al., 2002). It transports large neutral amino acids, including leucine, isoleucine, valine, phenylalanine, tryptophan, and

Table 2
Effects of sodium-coupled neutral amino acid transporter 2 (SNAT2) suppression on the free amino acid profile of the C2C12 myotubes (μmol/L).

Item	Control	MeAIB	SEM	*P*-value
Essential amino acids				
L-isoleucine	11.31 ± 0.61[a]	7.78 ± 0.45[b]	0.47	<0.01
L-leucine	11.92 ± 0.53[a]	8.34 ± 0.35[b]	0.39	<0.01
L-lysine	13.39 ± 0.15[a]	7.21 ± 0.46[b]	0.29	<0.01
L-methionine	3.25 ± 0.20[a]	2.17 ± 0.03[b]	0.12	<0.01
L-phenylalanine	7.96 ± 0.12[a]	5.16 ± 0.26[b]	0.18	<0.01
L-threonine	43.25 ± 1.04[a]	14.38 ± 0.64[b]	0.75	<0.01
L-tryptophan	2.29 ± 0.05[a]	1.43 ± 0.04[b]	0.04	<0.01
L-valine	15.41 ± 0.62[a]	10.00 ± 0.45[b]	0.47	<0.01
Non-essential amino acids				
Cysteine	0.50 ± 0.05	0.29 ± 0.15	0.10	0.26
Glycine	288.10 ± 10.65[a]	178.30 ± 4.07[b]	6.98	<0.01
L-alanine	29.55 ± 0.90[a]	18.48 ± 0.32[b]	0.58	<0.01
L-arginine	7.35 ± 0.28[a]	4.63 ± 0.10[b]	0.18	<0.01
L-asparagine	3.60 ± 0.17[a]	1.31 ± 0.04[b]	0.11	<0.01
L-aspartic acid	7.23 ± 0.28[a]	2.49 ± 0.08[b]	0.18	<0.01
L-glutamic acid	139.19 ± 12.69[a]	96.63 ± 2.26[b]	7.90	0.03
L-glutamine	103.07 ± 1.47[a]	17.50 ± 0.26[b]	0.92	<0.01
L-histidine	3.92 ± 0.40[a]	2.56 ± 0.12[b]	0.26	0.03
L-proline	62.83 ± 2.15[a]	10.45 ± 0.61[b]	1.37	<0.01
L-serine	10.20 ± 0.31	8.35 ± 0.65	0.44	0.06
L-tyrosine	8.62 ± 0.41[a]	5.18 ± 0.11[b]	0.26	<0.01
Other amino acids				
1-methyl-L-histidine	0.91 ± 0.02[a]	0.42 ± 0.02[b]	0.02	<0.01
Ethanolamine	10.52 ± 0.07	12.02 ± 0.58	0.36	0.06
Hydroxy-L-proline	1.28 ± 0.10[a]	0.67 ± 0.04[b]	0.07	<0.01
L-carnosine	5.11 ± 0.05[a]	1.43 ± 0.04[b]	0.09	<0.01
L-ornithine	1.02 ± 0.04[a]	0.57 ± 0.05[b]	0.04	<0.01
Taurine	204.17 ± 3.13[a]	182.39 ± 3.36[b]	2.81	<0.01
β-alanine	33.50 ± 0.57[a]	25.69 ± 0.36[b]	0.41	<0.01
γ-amino-n-butyric acid	0.75 ± 0.01[a]	0.44 ± 0.02[b]	0.02	<0.01

MeAIB = α-methylaminoisobutyric acid.
[a, b] Means within the same row with different superscripts are significantly different ($P < 0.05$).

histidine, among others (Kühne et al., 2007). Our previous study indicated that LAT1 mRNA expression was up-regulated by dietary protein restriction (Li et al., 2016). In the present experiment, both LAT1 mRNA and protein expression was found to be decreased when SNAT2 was suppressed. Moreover, the protein expression of hVps34 was also reduced, although not for MAP4K3. hVps34 and MAP4K3, both of which act as important sensors mediating amino acid sensing to mTORC1 (Nobukuni et al., 2005; Yan and Backer, 2007; Gulati and Thomas, 2007; Findlay et al., 2007). These results suggest that SNAT2 inhibition might impair the function of other amino acid sensors, such as LAT1 and hVps34, when the availability of intracellular free amino acids is reduced.

Skeletal muscle is the largest organ capable of secreting cytokines, such as the newly identified member IL-15 (Febbraio and Pedersen, 2005; Pedersen and Edward, 2009). These cytokines play a key role in the crosstalk between the skeletal muscle and adipose tissue (Bilski et al., 2013; Li et al., 2014a, b). Myostatin or growth differentiation factor 8 is also a myokine (Matsakas et al., 2009) and is a key mediator between the energy metabolism and endurance capacity of skeletal muscle (Mouisel et al., 2014). Here, we determined the expression levels of genes encoding *IL-15* and myostatin and found that *IL-15* expression was down-regulated by SNAT2 inhibition. However, this did not occur in myostatin. These results suggest that SNAT2 may be associated with myokine secretion.

Myogenin is an important factor and regulator of the muscle metabolism and energy utilization in adult muscles (Flynn et al., 2010). As expressed above, the expression of the differentiation marker gene myogenin was reduced by *SNAT2* suppression. Thus, the expression levels of the genes related to skeletal muscle energy metabolism were measured. Our experiment found that *SNAT2* inhibition decreased the expression levels of *SIRT1* and *PGC-1α*, but

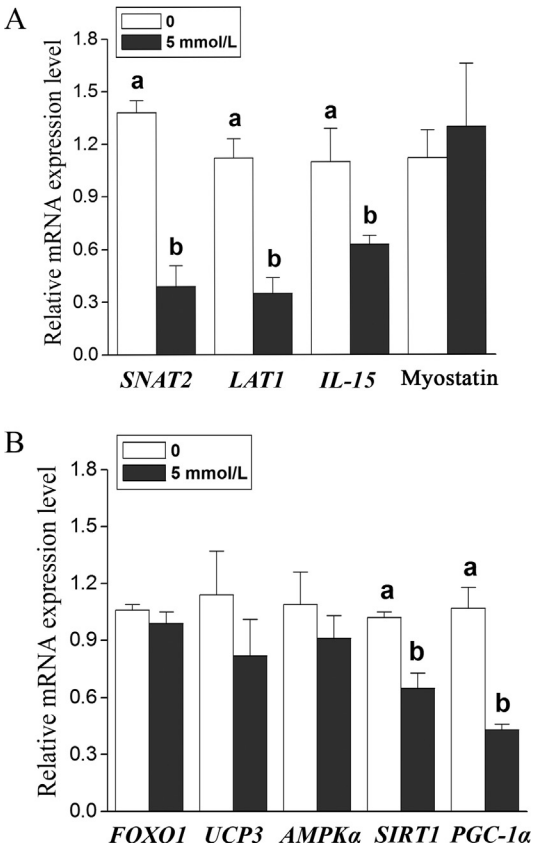

Fig. 4. The mRNA expression levels of the key genes related to amino acid transporters, myokines (A), and energy metabolisms (B). The expression level was normalized using β-actin as an internal control using real-time PCR. Data are represented as the mean ± SE ($n = 6$). *SNAT2* = sodium-coupled neutral amino acid transporter 2; *LAT1* = system L amino acid transporter 1; *IL-15* = interleukin 15; *FOXO1* = fork head transcription factor 1; *UCP3* = uncoupling protein 3; *AMPKα* = AMP-activated protein kinase α; *SIRT1* = silent information regulator transcript 1; *PGC-1α* = peroxisome proliferator-activated receptor γ coactivator-1α. Data columns with different letters (a, b) are significantly different ($P < 0.05$).

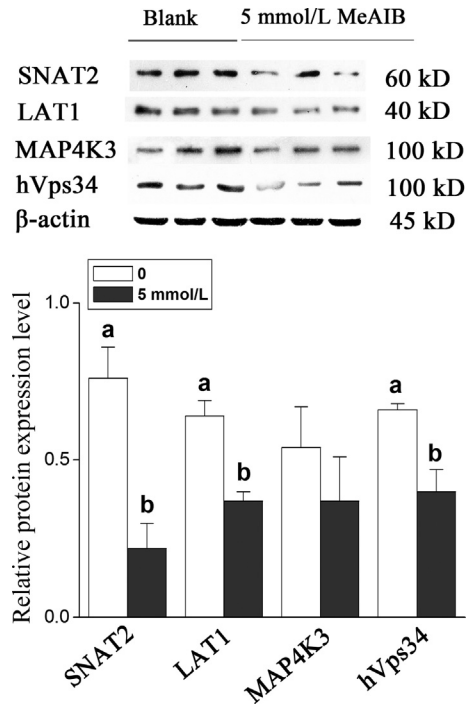

Fig. 5. The effect of MeAIB action on the protein expression levels of the key amino acid transporters of C2C12 myotubes. The expression level was normalized using β-actin as an internal control. Data are represented as the mean ± SE ($n = 6$). MeAIB = α-methylaminoisobutyric acid; SNAT2 = sodium-coupled neutral amino acid transporter 2; LAT1 = system L amino acid transporter 1; MAP4K3 = mitogen-activated protein kinase kinase kinase kinase-3; hVps34 = vacuolar protein sorting 34. Data columns with different letters (a, b) are significantly different ($P < 0.05$).

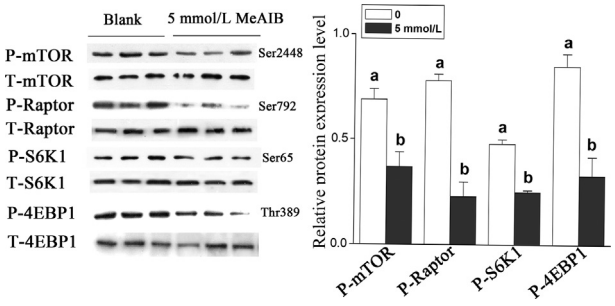

Fig. 6. Phosphorylated protein expression levels of key molecules in mTOR/Raptor signaling pathway (mTOR, Raptor, 4E-BP1, S6K1) are regulated by MeAIB action. The data were normalized to the value of corresponding total protein and represented as the mean ± SE ($n = 6$). MeAIB = α-methylaminoisobutyric acid; P = phosphorylation; mTOR = mammalian target of rapamycin; T = total; S6K1 = ribosomal protein S6 kinase 1; 4EBP1 = 4E binding protein 1. Data columns with different letters (a, b) are significantly different ($P < 0.05$).

no significant difference was observed in the expression of *FOXO1*, *UCP3*, or *AMPKα*. The signaling pathway AMPKα/SIRT1/PGC-1α is an energy sensing network that controls energy expenditure through modulating mitochondrial biogenesis and function. We deduced that SNAT2 is not only involved in amino acid availability and sensing, but also in the energy metabolism of skeletal muscle cells, maintaining energy hemostasis in the whole body.

Mammalian target of rapamycin complex 1 consists of mTOR, Raptor, and G protein unit-like protein (mLST8/GβL). Raptor is an important adaptor in the recruitment of substrates to mTOR. After activation, such as amino acid sensing, mTORC1 will phosphorylate the 2 best characterized downstream targets, S6K1 and 4EBP1, subsequently enhancing translation initiation and protein synthesis (Wang and Proud, 2006; Wullschleger et al., 2006).

Amino acid transporters in system A (such as SNAT2) and system L (such as LAT1) are most closely related to the mTORC1 signaling pathway. SNAT2 concentrates amino acids within the cell by coupling the influx of amino acids with that of Na + via secondary active transport (Goberdhan et al., 2016; Dickinson and Rasmussen, 2011). This transceptor also activates mTORC1 in the presence of the non-metabolizable amino acid analog MeAIB. SNAT2 inhibition strongly impaires the signaling pathway via mTOR/S6K1/4 EBP1, leading to the impairment of protein synthesis (Evans et al., 2007). The partial silencing of SNAT2 expression in myoblasts and myotubes via siRNA stimulates proteolysis through phosphatidylinositol 3-kinase (PI3K) (Evans et al., 2008). In accordance with previous studies, the results presented here indicate that SNAT2 suppression using MeAIB markedly reduces the phosphorylation of key proteins mTOR, Raptor, S6K1, and 4EBP1, suggesting that the mTORC1 signaling pathway is inhibited.

This study highlights the important function of SNAT2 in skeletal muscle as a nutrient receptor, tasked with sensing nutrients, as opposed to a transporter. Intracellular amino acid availability decreased with reduced expression of the amino acid transceptors LAT1 and hVps34, as well as key genes related to the energy metabolism and cytokines. Meanwhile, the mTORC1 signaling pathway was largely inhibited (Fig. 7). To determine the role and precise contribution of SNAT2 to the modulation of cell growth, differentiation, and metabolism in skeletal muscle cells, further investigations

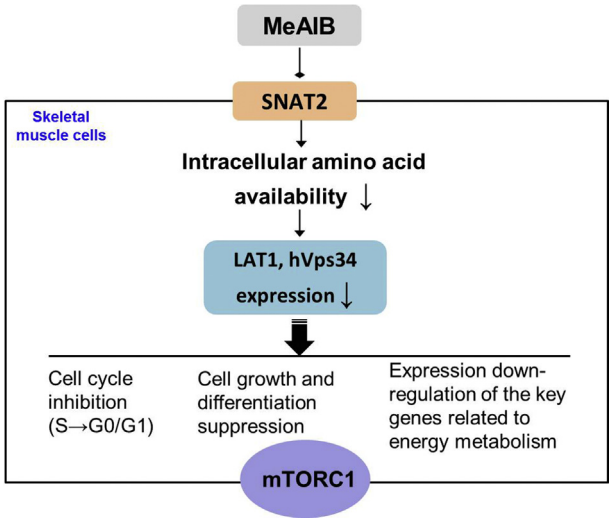

Fig. 7. Amino acid transceptor SNAT2 suppression using MeAIB impaired skeletal muscle cell growth and differentiation by modulating cell viability and the cell cycle. MeAIB = α-methylaminoisobutyric acid; SNAT2 = sodium-coupled neutral amino acid transporter 2; LAT1 = system L amino acid transporter 1; hVps34 = vacuolar protein sorting 34; mTORC1 = mammalian target of rapamycin complex 1.

are needed. However, the specific amino acids involved in the impact of SNAT2 on the mTORC1 signaling pathway and protein metabolism remains unclear. As such, it would be interesting to determine: 1) whether other signaling pathways are impaired by the acute suppression of SNAT2; 2) whether other nutrients would modulate the expression of SNAT2 and the related signaling pathway, in addition to proteins (amino acids), unsaturated fatty acids (Nardi et al., 2015), and carbohydrates (Velázquez-Villegas et al., 2015).

5. Conclusion

In conclusion, the suppression of SNAT2, an amino acid transceptor, using MeAIB impaired the growth and differentiation of skeletal muscle cells by modulating cell viability and the cell cycle. The intracellular amino acid availability decreased with a reduction in the expression levels of 2 other amino acid transceptors, LAT1 and hVps34, as well as key genes related to the energy metabolism and cytokines. Meanwhile, the mTORC1 signaling pathway was largely inhibited. Our results suggest that SNAT2 might play an important role in skeletal muscle tissue development. Although the mTORC1 pathway was found to be involved, its exact function and response to different nutrients (amino acids or proteins) need to be investigated further in future studies.

Conflict of Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

This study was financially supported by the National Key R&D Program (2018YFD0500405), the Science and technology projects of Changsha City (kq1801059), the STS regional key projects of Chinese Academy of Sciences (KFJ-STS-QYZD-052), the Youth Innovation Promotion Association CAS (2016326), and the Earmarked Fund for China Agriculture Research System (CARS-35).

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