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ORIGINAL ARTICLE

Leucine stimulates ASCT2 amino acid transporter expression in porcine jejunal epithelial cell line (IPEC-J2) through PI3K/Akt/mTOR and ERK signaling pathways

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Abstract Leucine has been shown to influence intestinal protein metabolism, cell proliferation and migration. Furthermore, our previous study demonstrated that branched-chain amino acids could modulate the intestinal amino acid and peptide transporters in vivo. As the possible mechanisms are still largely unknown, in the present work, we studied the transcriptional and translational regulation of leucine on amino acid transporter production in IPEC-J2 cells and the signaling pathways involved. Treatment of IPEC-J2 cells with 7.5 mM leucine enhanced the mRNA expression of the Na^+-neutral AA exchanger 2 (ASCT2) and 4F2 heavy chain (4F2hc) and caused an increase in ASCT2 protein expression. Leucine also activated phosphorylation of 4E-BP1 and eIF4E through the phosphorylation of mTOR, Akt and ERK signaling pathways in IPEC-J2 cells. Pre-treatment of IPEC-J2 cells with inhibitors of mTOR and Akt (rapamycin and wortmannin) or an inhibitor of ERK (PD098059) for 30 min before leucine treatment attenuated the positive effect of leucine in enhancing the protein abundance of ASCT2. These results demonstrate that leucine could up-regulate the expression of the amino acid transporters (ASCT2) through transcriptional and translational regulation by ERK and PI3K/Akt/mTOR activation.

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Abbreviations

ASCT2	Na^+-neutral AA exchanger 2
BCAA	Branched-chain amino acids
4F2hc	4F2 heavy chain
LP	Low protein
mTOR	Mammalian target of rapamycin
RT-PCR	Real-time polymerase chain reaction

Introduction

The intestine is considered an important organ for intestinal barrier, nutrition absorption and innate immunity and has attracted much attention in the field of human and animal nutrition (Johansson et al. 2009; Santaolalla et al. 2011; Huygelen et al. 2012). Recent studies have illustrated that amino acids play critical roles in regulating gut function, such as stimulating cell proliferation, blocking enterocyte apoptosis, regulating cell migration and maintaining intestinal mucosal barrier (Marc Rhoads and Wu 2009; Tan et al. 2010; Wang et al. 2010; Faure et al. 2005). Amino acids in the diet will exert their effects on extra-intestinal tissues after being absorbed by the small intestine, and the transportation of these nutrients is a key regulatory step in utilization of dietary protein by piglets (Baker 2009; He et al. 2013). However, the mechanisms for intestinal amino acid transportation are largely unknown.

The transporters of amino acids in the small intestine can be divided into basic amino acid, neutral amino acid and acidic amino acid systems (Chairoungdua et al. 1999; Kanai and Hediger 1992). For example, rBAT/$b^{0,+}$AT

and 4F2hc/y^+LAT1, encoded by SLC3A1/SLC7A9 and SLC3A2/SLC7A5, are the most important basic amino acid transporters in intestine (Bröer 2008). rBAT/$b^{0,+}$AT plays a vital role for the transportation of basic amino acids from the luminal membrane into enterocytes, while 4F2hc/y^+LAT1 regulates the efflux of basic amino acids across the basolateral membrane (Palacin et al. 2001; Bauch and Verrey 2002). B^0AT1, encoded by SLC6A19, is a major apical neutral amino acid transporter in kidney and intestine (Broer et al. 2004). ASCT2, encoded by SLC1A5, can transport neutral amino acids and help to equalize neutral amino acid composition in epithelial cells, but ASC-like activity is only about 1/10 of system B^0 activity in the intestine (Munck and Munck 1999). EAAT2 and EAAT3, encoded by SLC1A2 and SLC1A1, regulate the absorption of the acidic amino acids, such as aspartate and glutamic acid, in the small intestine (Boehmer et al. 2003).

Many factors have been shown to contribute to the expression of amino acid transporters in the intestine. For instance, during the early suckling period, compared with high body weight littermates, low body weight piglets had lower jejunal expression profiles of both Slc6a19 (B^0AT1) and Slc1a5 (ASCT2) (Yang et al. 2012). Furthermore, amino acid profiles and feed restriction have been demonstrated to influence the expression of nutrient transporter mRNA in the small intestine of broiler chicks, showing that different nutrient levels in the diet may change the expression of amino acid transporters (Gilbert et al. 2008).

Leucine, as one of the branched-chain amino acids (BCAA), plays a critical role in regulatory effects, such as protein synthesis and degradation, insulin secretion and cell autophagy (Sheen et al. 2011; Yang et al. 2010; Yin et al. 2010). Numerous studies have focused on leucine due to its ability to stimulate protein synthesis in muscle through mammalian target of rapamycin (mTOR) signaling pathways with the phosphorylation of S6K1, 4E-BP1 and eIF4E assembly (Escobar et al. 2005, 2006; Torrazza et al. 2010; Yin et al. 2010). Furthermore, leucine has been reported to induce up-regulation of system A amino acid transporters in muscle (Peyrollier et al. 2000). However, at present, little is known about the effect of leucine on the expression of amino acid transporters in the small intestine, which is critical for the supply of amino acids to all tissues and the homeostasis of plasma amino acid levels (Bröer 2008). The effects of leucine have been poorly documented in the intestine (Wu 2009). In recent years, leucine has been shown to influence the protein metabolism, phosphokinase expression, cell proliferation, and may activate the mTOR pathway and cell migration in the intestine (Coeffier et al. 2011; Rhoads et al. 2008; Torrazza et al. 2010), which indicates that it might have a vital role in intestine regulation.

The study of the regulation of transporters leads to the elucidation of mechanisms that can change transport rates in the intestine (Ferraris 2001; Ferraris and Diamond 1989). Based on the results of an experiment conducted in our lab showing that BCAA can regulate the amino acid and peptide transporters in the small intestine of piglets fed protein reduced diet (Zhang et al. 2013), we hypothesized that the leucine supplementation can regulate the expression of amino acid transporters. Thus, the major aim of this experimental study was to assess the effects of leucine on intestinal amino acid transporters in vitro and elucidate the underlying mechanisms through which it functions.

Materials and methods

Cell culture and treatment

The IPEC-J2 cell line, a porcine IEC line originally derived from the jejunal crypts of a neonatal piglet, was kindly provided by Dr. Guoyao Wu (Texas A & M University). Cells were cultured in six-well plates in DMEM/F12 medium (Thermo, Waltham, MA, Cat: SH30023.0113) supplemented with 5 % (vol/vol) fetal bovine serum (FBS, Gibco, Carlsbad, CA, Cat: 10099-141), 5 μg/L ITS (Sciencell, Carlsbad, CA, USA, Cat: 0803) and 5 μg/L epidermal growth factor (Sciencell, Carlsbad, CA, USA, Cat: 105-04). After reaching 90 % confluence, cells were starved for 2 h in an amino acid-deprived medium with Earle's Balanced Salt Solution (EBSS) (Sigma, St. Louis, MO, USA, Cat: E2888) and a vitamin mixture (Sigma, St. Louis, MO, USA, Cat: R7256), according to established protocols (Nishikawa et al. 2007). After starvation, cells were used for the following experiments.

Experiment I

IPEC-J2 cells were cultured in the presence of 0, 1, 2.5, 5, 7.5 or 10 mM leucine, isoleucine or valine, respectively. After 1, 2, 6, 8 or 10 h, IPEC-J2 cells were collected to examine the expression of CAT-1, 4F2hc, rBAT, ASCT2, y^+LAT, $B^{0,+}$AT, B^0AT and PepT-1.

Experiment II

IPEC-J2 cells were cultured in the presence of 7.5 mM leucine. After 0-, 5-, 10-, 30-, 40- or 60-min incubation, the IPEC-J2 cells were collected to examine the phosphorylation of mTOR, Akt and MAPK (ERK, P38 and JNK). The phosphorylation state of 4EBP-1 and eIF4E was tested after incubation periods of different durations (0, 1, 2, 4 and 8 h).

Table 1 Primers used for real-time PCR

Genes	Primers	Sequences (5′–3′)	Size (bp)	Tm (°C)	Accession no.
ASCT2	Forward	GCCAGCAAGATTGTGGAGAT	206	60	DQ231578
	Reverse	GAGCTGGATGAGGTTCCAAA			
B^0AT1	Forward	CACAACAACTGCGAGAAGGA	155	60	DQ231579
	Reverse	CCGTTGATAAGCGTCAGGAT			
CAT-1	Forward	TGCCCATACTTCCCGTCC	192	59	NM_001012613
	Reverse	GGTCCAGGTTACCGTCAG			
$b^{0,+}AT$	Forward	ATCGGTCTGGCGTTTTAT	144	59	NM_001110171
	Reverse	GGATGTAGCACCCTGTCA			
y^+LAT1	Forward	GCCCATTGTCACCATCATC	216	59	NM_001110421
	Reverse	GAGCCCACAAAGAAAAGC			
4F2hc	Forward	CTCGAACCCACCAAGGAC	174	59	XM_003361818
	Reverse	GAGGTGAGACGGCACAGAG			
Pept-1	Forward	CCCAGGCTTGCTACCCAC	144	60	NM_214347
	Reverse	ACCCGATGCACTTGACGA			
rBAT	Forward	TTTCCGCAATCCTGATGTTC	146	59	NM_001123042
	Reverse	GGGTCTTATTCACTTGGGTC			
β-Actin	Forward	TGCGGGACATCAAGGAGAAG	216	60	XM_003357928
	Reverse	AGTTGAAGGTGGTCTCGTGG			

ASCT2 Na^+-neutral AA exchanger, *B^0AT1* system B^0 neutral AA transporter, *CAT-1* cationic amino acid transporter 1, *$b^{0,+}AT$* related to $b^{0,+}$ amino acid transporter, *y^+LAT1* y^+ L amino acid transporter-1, *4F2hc* 4F2 heavy chain, *Pept-1* intestinal peptide transporter, *rBAT* basic amino acid transporter

Experiment III

IPEC-J2 cells were pre-treated for 30 min with either 20 nM rapamycin (Sigma, St. Louis, MO, Cat: R8781), or 10 μM wortmannin (Sigma, St. Louis, MO, Cat: W1628), or 10 μM PD098059 (Sigma, St. Louis, MO, Cat: P215-1MG). Then, IPEC-J2 cells were cultured in the presence of 7.5 mM leucine. After 8 h, IPEC-J2 cells were collected to examine the mRNA expression and protein abundance of 4F2hc and ASCT2.

RNA isolation and RT-PCR analysis

Total RNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany, Cat: 74014) according to the manufacturer's protocol. 1 μg RNA was reverse-transcribed to complementary DNA (cDNA) using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Ostu, Japan, Cat: 6110A) according to the manufacturer's protocol. Primers for the selected genes were designed using Oligo 7.0 Software (Table 1). Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Singapore) with SYBR Green PCR Master Mix (Takara, Ostu, Japan, Cat: RR820A), containing $MgCl_2$, dNTP, and Hotstar Taq polymerase. Quantification of target mRNA was conducted using a relative standard curve generated by a serial dilution ($1:10^7$–1:1) of amplification products. The PCR system consisted of 5.0 μL of SYBR Green qPCR mix, 1.0 μL of cDNA, 3.6 μL of double distilled water, and 0.4 μL of primer pairs (25 μM forward and 25 μM reverse) in a total volume of 10 μL. The protocols for all genes included a denaturation program (1 min at 95 °C), amplification and quantification program repeated for 35 cycles (5 s at 95 °C, 30 s at 58 °C), followed by the melting curve program at 60–95 °C with a heating rate of 0.1 °C per second and continuous fluorescence measurement. Each sample was measured in triplicate.

Western blot analysis

The total proteins and membrane proteins were extracted from IPEC-J2 cells. To extract the total protein, the IPEC-J2 cells were lysed in RIPA buffer (150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris–HCl at pH 7.4, plus a protease inhibitor cocktail purchased from Applygene, Beijing, China, Cat: HX18622). The membrane proteins of IPEC-J2 cells were extracted by Mem-PER Eukaryotic Membrane Protein Extraction Kit (Pierce, Rockford, IL, USA, Cat: 89826) and proteins were deglycosylated by the PNGase F (New England Biolabs, Ipswich, MA, USA, Cat: P0704S) according to the instruction. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA, Cat: 23227). Equal amounts of proteins (30 mg) were electrophoresed on SDS polyacrylamide gels. Prestained protein markers (Fermentas, Waltham, MA, USA, Cat: 26616) were used in each gel. Proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA, Cat: IPVH00010) and blocked with 5 % nonfat dry milk overnight at 4 °C. The transfer efficiency was assessed by gel staining with Coomassie Blue. Samples were incubated with corresponding primary

antibodies (1:500 dilution for 2 h at room temperature or overnight at 4 °C) against ASCT2 (Santa Cruz Biotechnology, Cat: sc-130963), 4F2hc (Santa Cruz Biotechnology, Cat: sc-31249), p-4E-BP1 (Thr^{70}) (Cell Signaling Technology, Cat: 13396), 4E-BP1 (Cell Signaling Technology, Cat: 9644P), p-eIF4E (Ser^{209}) (Cell Signaling Technology, Cat: 9741), eIF4E (Cell Signaling Technology, Cat: 2067), p-mTOR (Ser^{2448}) (Cell Signaling Technology, Cat: 5536), mTOR (Cell Signaling Technology, Cat: 2983), p-Akt (Ser^{473}) (Cell Signaling Technology, Cat: 4058), Akt (Cell Signaling Technology, Cat: 4691), p-ERK (Thr^{202}/Tyr^{204}) (Cell Signaling Technology, Cat: 4377), ERK (Cell Signaling Technology, Cat: 4695), p-p38 (Thr^{180}/Tyr^{182}) (Cell Signaling Technology, Cat: 9215), p-38 (Cell Signaling Technology, Cat: 8690), p-JNK (Thr^{183}/Tyr^{185}) (Cell Signaling Technology, Cat: 4668), JNK (Cell Signaling Technology, Cat: 9258) and Na^+/K^+ ATPase (Millipore, Billerica, MA, USA) β-actin (Cell Signaling Technology, Cat: 4970). After being washed with Tris-Tween-20 buffer (pH 7.4), membranes were incubated with a secondary antibody (Horseradish Peroxidase-Conjugated Goat Anti-Rabbit IgG) (Zhongsan Gold Bridge, Beijing, China, Cat: ZDR-5306) at a ratio of 1:7,000 dilution for 1 h at room temperature. The membrane was exposed by AlphaImager 2200 (Alpha Innotech, San Leandro, CA, USA) automatically. Band densities were detected with the Western Blot Luminance Reagent (Applygene, Beijing, China, Cat: HX1868) and quantified using AlphaImager 2200 (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

Statistical analysis was performed using the statistical software SAS Version 9.2. Data were analyzed by ANOVA according to the GLM procedure of SAS. Means were separated by Student–Newman–Keuls multiple range test. Differences at $P < 0.05$ were considered significant.

Results

Leucine induced the mRNA and protein expression of 4F2hc and ASCT2 amino acid transporters in IPEC-J2 cells

Cells were treated with various concentrations of leucine, isoleucine or valine (0, 1, 2.5, 5, 7.5 or 10 mM) for 1, 2, 6, 8 or 10 h. In this experiment, the mRNA expression of B^0AT and rBAT amino acid transporters was too weak to be detected in the IPEC-J2 cells. By contrast, the mRNA expression of 4F2hc and ASCT2 amino acid transporters was significantly affected by leucine for 2, 6, 8 and 10 h treatments ($P < 0.05$) (Fig. 1). However, isoleucine and valine did not affect the mRNA expression of any amino acid transporters for any time or dose treatments ($P > 0.05$) (Fig. 2 shows the effect of 7.5 mM leucine, isoleucine or

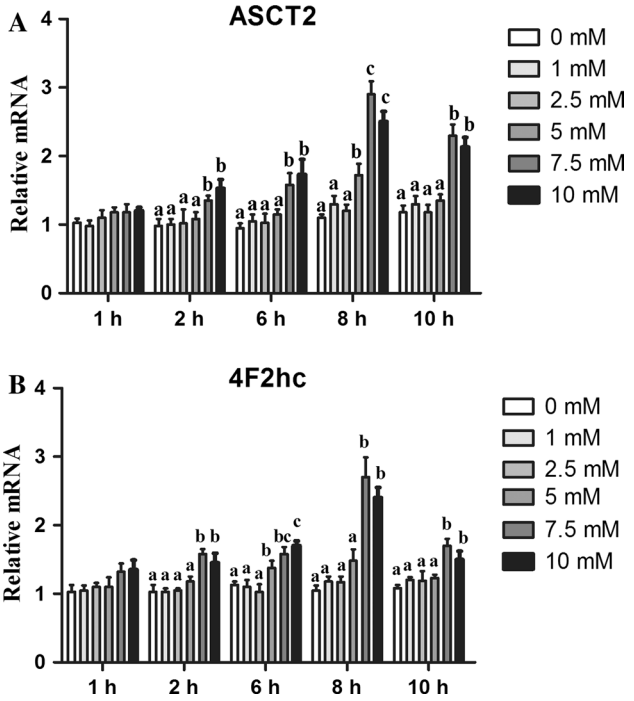

Fig. 1 The relative mRNA expression of ASCT2 (**a**) and 4F2hc (**b**) is stimulated by leucine in IPEC-J2 cells ($n = 4$). Cells were treated with 0, 1, 2.5 5, 7.5 or 10 mM leucine for the time points indicated in the figure. The mRNA expression of ASCT2 (**a**) and 4F2hc (**b**) was detected by real-time PCR. β-Actin was used as an internal standard for normalization. Means followed by same or no letter did not differ ($P > 0.05$)

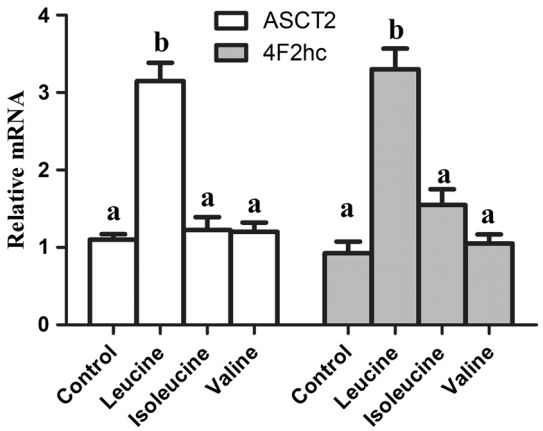

Fig. 2 The relative mRNA expression of ASCT2 and 4F2hc is stimulated by 7.5 mM leucine, not isoleucine or valine in IPEC-J2 cells after 8 h treatment ($n = 4$). Cells were treated with 7.5 mM of leucine, valine or isoleucine for 8 h. The mRNA expression of ASCT2 and 4F2hc was detected by real-time PCR. β-Actin was used as an internal standard to normalization. Means followed by same letter did not differ ($P > 0.05$)

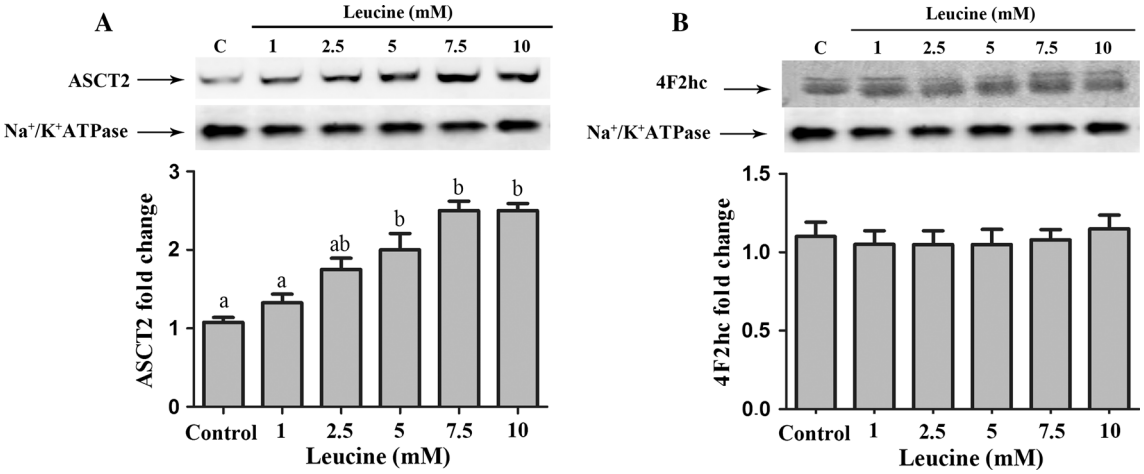

Fig. 3 The protein abundance of amino acid transporter ASCT2 (**a**) and 4F2hc (**b**) was analyzed after 8 h of treatment with leucine in IPEC-J2 cells ($n = 4$). Cells were treated with 0, 1, 2.5 5, 7.5 or 10 mM leucine for 8 h. The protein abundance of ASCT2 (**a**) and 4F2hc (**b**) was detected by Western Blot. Na^+/K^+ ATPase was used as an internal standard to normalization. Means followed by same or no letter did not differ ($P > 0.05$)

valine on the mRNA expression of the ASCT2 and 4F2hc amino acid transporters). Culture of IPEC-J2 at both 7.5 and 10 mM leucine for 2, 6, 8 or 10 h significantly enhanced the mRNA expression of the ASCT2 amino acid transporter ($P < 0.05$), and 5 mM leucine treatment for 8 h also significantly increased the mRNA abundance of the ASCT2 amino acid transporter ($P < 0.05$) (Fig. 1a). For the 4F2hc amino acid transporter, there was a significant increase in the mRNA expression for both 7.5 and 10 mM leucine treatments at 2, 8 and 10 h ($P < 0.05$). The increase in mRNA expression of the 4F2hc amino acid transporter was also observed for 5, 7.5 and 10 mM leucine supplementation in comparison with the control at 6 h ($P < 0.05$) (Fig. 1). The protein abundance of the ASCT2 amino acid transporter was significantly increased for 5, 7.5 and 10 mM leucine supplementation after 8 h, compared with the control (Fig. 3a, $P < 0.05$). However, leucine supplementation had no effect on the protein expression of the 4F2hc amino acid transporter (Fig. 3b, $P > 0.05$).

Phosphorylation of 4E-BP1, eIF4E, mTOR, Akt, ERK, P38 and JNK in response to leucine supplementation

7.5 mM leucine supplementation markedly enhanced the phosphorylation of 4E-BP1 during the 1 h culture (Fig. 4). The phosphorylation level of eIF4E was also increased with 7.5 mM leucine supplementation in IPEC-J2 cells for 1–8 h culture (Fig. 4). The phosphorylation of mTOR was induced by 7.5 mM leucine treatment in the 5 min culture, reaching higher levels at 30, 40 and 60 min (Fig. 5). The phosphorylation level of Akt was also induced by 7.5 mM leucine in the 5 min culture and reaching higher levels at 40 and 60 min (Fig. 5). The increase in ERK phosphorylation was observed after 5 min treatment ($P < 0.05$), but no significant difference of P38 and JNK phosphorylation was observed at any tested time point (Fig. 6).

The mRNA expression and protein abundance of the ASCT2 and 4F2hc amino acid transporters in response to leucine supplementation in the presence of the inhibitors of mTOR, Akt, and ERK

Compared with leucine supplementation alone, rapamycin and wortmannin pre-treatment before leucine supplementation markedly attenuated the positive induction of the mRNA expression of the ASCT2 and 4F2hc ($P < 0.05$) (Fig. 7). In contrast, PD098059 pre-treatment before leucine supplementation had no such effect (Fig. 7). The protein abundance of the ASCT2 was all attenuated by pre-treatment of these three inhibitors before leucine supplementation ($P < 0.05$) (Fig. 8).

Discussion

Several amino acids have been demonstrated to differentially modulate the mRNA expression of different amino acid transporters in different species. For instance, dietary lysine supplementation up-regulated mRNA expression of intestinal $b^{0,+}$-like and y^+-like transport systems in chicken (Torras-Llort et al. 1998). The mRNA expression of $b^{0,+}$AT, 4F2hc and PepT-1 amino acid and peptide transporters was up-regulated in the ileum of growing pigs when the lysine level increased from 0.6 to 0.8 % in the diet (Wang et al. 2012). Leucine and Isoleucine, alone or in combination, significantly decreased expression of $b^{0,+}$

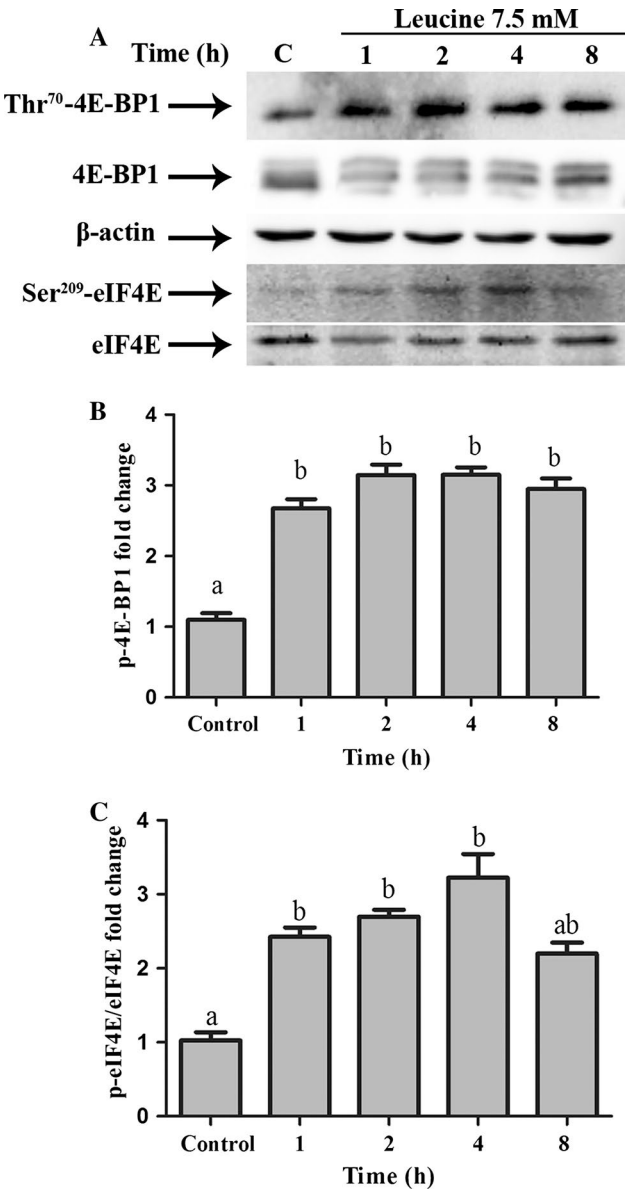

Fig. 4 Analysis of the phosphorylation statue of 4E-BP1 and eIF4E signaling pathways in IPEC-J2 cells treated with 7.5 mM leucine ($n = 4$). Cells were treated with 7.5 mM leucine for the time points indicated in the figure. The phosphorylated forms of 4E-BP1 (Thr^{70}) and eIF4E (Ser^{209}) were detected by Western Blot. **a** Represented the immunoblots of phosphorylation forms of 4E-BP1 and eIF4E. (**b**, **c**) Represented the quantitation of the phosphorylation fold changes of p-4E-BP1 and p-eIF4E, respectively. Means followed by same letter did not differ ($P > 0.05$)

AT amino acid transporter and significantly increased the CAT-1 amino acid transporter in the jejunum of piglets (Cervantes-Ramirez et al. 2013). Furthermore, our previous data demonstrated that BCAA up-regulated the small intestinal amino acid and peptide transporters (CAT-1, 4F2hc, rBAT and PepT-1) in piglets (Zhang et al. 2013). In this study, we confirmed that leucine strongly induced the mRNA expression of the ASCT2 and 4F2hc amino acid transporters in porcine IPEC-J2 cells.

In addition to species-specific regulation of amino acid transporters by different amino acids, gene-specific and cell-specific regulation patterns by leucine were evident. First, among all the amino acid transporters that were analyzed, the mRNA expressions of CAT-1, EAAT1 and PepT-1 were not inducible by leucine, isoleucine or valine in IPEC-J2 cells. Second, the expression of rBAT and B^0AT, existing in the small intestine, was not detected in the IPEC-J2 cells. Similarly, there is evidence that system-B^0 specific amino acid uptake activity was absent in rat crypt-like cell line IEC-17 but was higher in less differentiated cells such as a Caco-2 cell line (Moreno et al. 1996; Costa et al. 2000). In addition, the expression of B^0AT1 was demonstrated in enterocyte-specific pattern, but not in any other type of intestinal epithelial cells (Bröer et al. 2004). Third, for those genes that were inducible by leucine, each was differentially regulated, with a different magnitude of induction. For example, ASCT2 was induced nearly threefold, whereas 4F2hc was induced by almost 2.5-fold in Porcine IPEC-J2 cells in response to 7.5 mM leucine supplementation. Therefore, it is important to verify the expression patterns of amino acid transporters by leucine stimulation in different cell types.

The intestinal AA exchanger ASCT2 (SLC1A5) is responsible for the exchange of neutral AA across the apical membrane (Torres-Zamorano et al. 1998). Epidermal growth factor and nitric oxide were demonstrated to up-regulate the transport activity and mRNA and protein expression of ASCT2 (Uchiyama et al. 2005). Furthermore, the intestinal AA exchanger 4F2hc (SLC3A2), a transmembrane glycoprotein, not only acted as an amino acid transport, but also regulated multiple cellular functions, including extracellular signaling, epithelial cell adhesion/polarity, and cell–cell interactions (Yan et al. 2008; Shennan et al. 2004). There is evidence that 4F2hc expression was rapidly and transiently up-regulated following essential amino acid ingestion in humans (Drummond et al. 2010). In this study, we found that only leucine, not isoleucine or valine, can up-regulate the expression levels of ASCT2 (mRNA and protein) and 4F2hc (mRNA), indicating that ASCT2 and 4F2hc expression may be regulated by leucine as its special role in signaling pathway regulation. Our data provide novel information about translational up-regulation of ASCT2 production induced by leucine in IPEC-J2 cells.

Based on previous reports, mTOR, Akt, and MAPK signaling pathways may participate in leucine-mediated induction of amino acid transports (ASCT2 and 4F2hc). Leucine was proved to modulate protein synthesis directly through mTOR/4E-BP1/eIF4E (Hara et al. 1998; Kimball et al. 1999) or indirectly through PI3K/Akt/mTOR/4E-BP1/eIF4E signaling pathways (Sekulić et al. 2000;

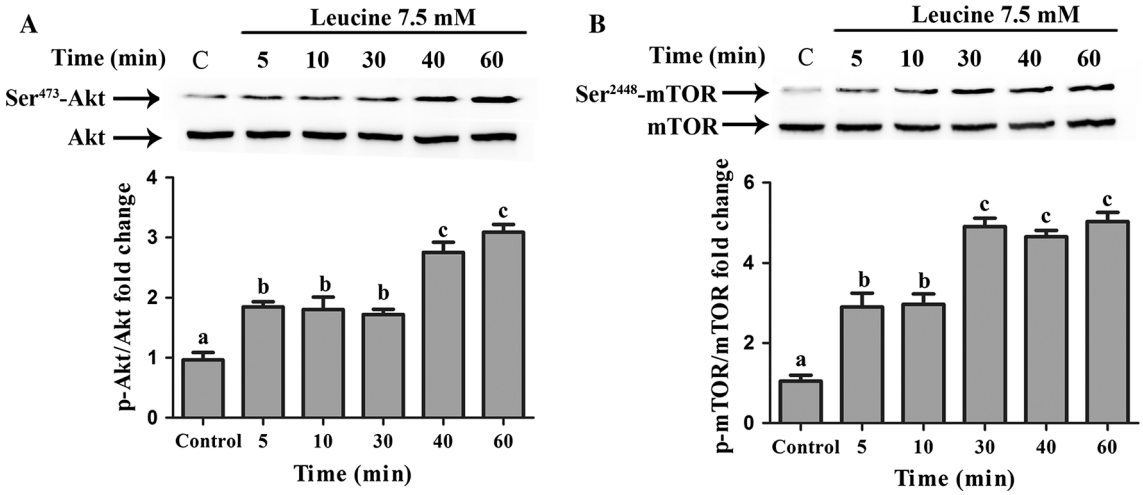

Fig. 5 Analysis of PI3K-Akt and mTOR signaling pathways in IPEC-J2 cells treated with leucine ($n = 4$). Cells were treated with leucine 7.5 mM for the time points indicated in the figure. The phosphorylated forms of phospho-Ser^{473} Akt (**a**) and phospho-Ser^{2448} mTOR (**b**) were detected by Western Blot. Representative immunoblots and quantitation of the phosphorylation fold changes are also shown in **a** and **b**. Means followed by same letter did not differ ($P > 0.05$)

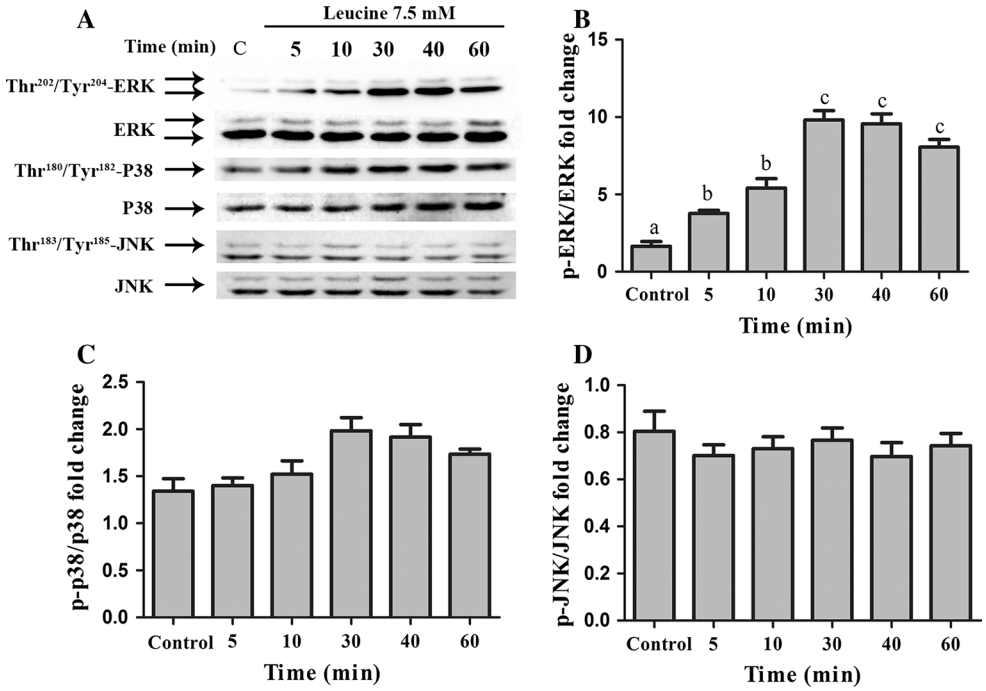

Fig. 6 Analysis of ERK (p42 and p44), p38, and JNK signaling pathways in IPEC-J2 cells treated with leucine ($n = 4$). IPEC-J2 cells were treated with 7.5 mM leucine for the time points indicated in the figure and the phosphorylated forms of ERK (Thr^{202}/Tyr^{204}), p38 MAPK (Thr^{180}/Tyr^{182}), and JNK (Thr^{183}/Tyr^{185}) were detected by Western Blot analysis. **a** Represents the immunoblots of phosphorylation forms of ERK, p38 MAPK, and JNK. **b**–**d** Represent the quantitation of the phosphorylation fold changes of p-ERK, p-38 and p-JNK, respectively. Means followed by same or no letter did not differ ($P > 0.05$)

Rommel et al. 2001). The phosphorylation of 4E-BP1 and the release of the initiation factor eIF4E could activate translation via the formation of the initiation complex (Fox et al. 1998; Anthony et al. 2000). Our results showed that phosphorylation of Akt, mTOR, 4E-BP1 and eIF4E was induced by leucine stimulation in IPEC-J2 cells. However, it was also demonstrated that mTOR phosphorylation induced by leucine was independent of the PI3K/Akt signaling pathway (Lang and Frost 2005). The activation of PI3K signaling pathway could also directly affect the mRNA expression (Park et al. 2010; Pore et al. 2003). A previous study found that both PI3K and MAPK inhibition had a negative effect on the glucose transporter (GLUT1) mRNA and protein expression, which means that PI3K signaling pathway might participate in the glucose uptake regulation (Chen et al. 2001). However, it is unknown whether amino acid transporter expression was affected directly by PI3K or through the PI3K/Akt/mTOR

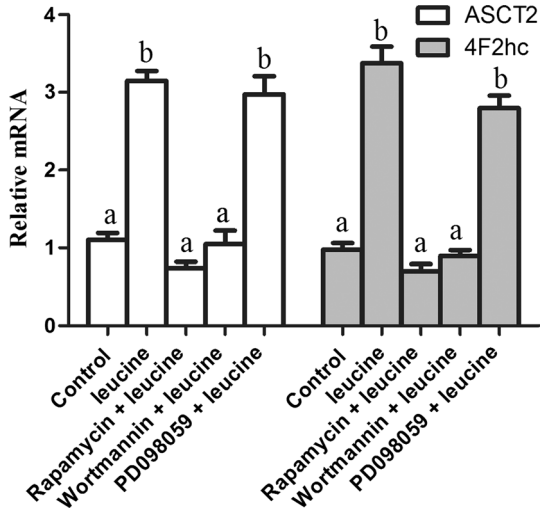

Fig. 7 The mRNA abundance of amino acid transporter ASCT2 with leucine stimulation in IPEC-J2 cells pre-treated with inhibitors ($n = 4$). Cells were pre-treated for 30 min with either 20 nM rapamycin (mTOR inhibitor), 10 μM wortmannin (PI3K inhibitor), or 10 μM PD098059 (MEK1inhibitor) prior to incubation with 7.5 mM leucine for 8 h and the mRNA expression of ASCT2 and 4F2hc was detected by real-time PCR. β-Actin was used as an internal standard to normalization. Means followed by same letter did not differ ($P > 0.05$)

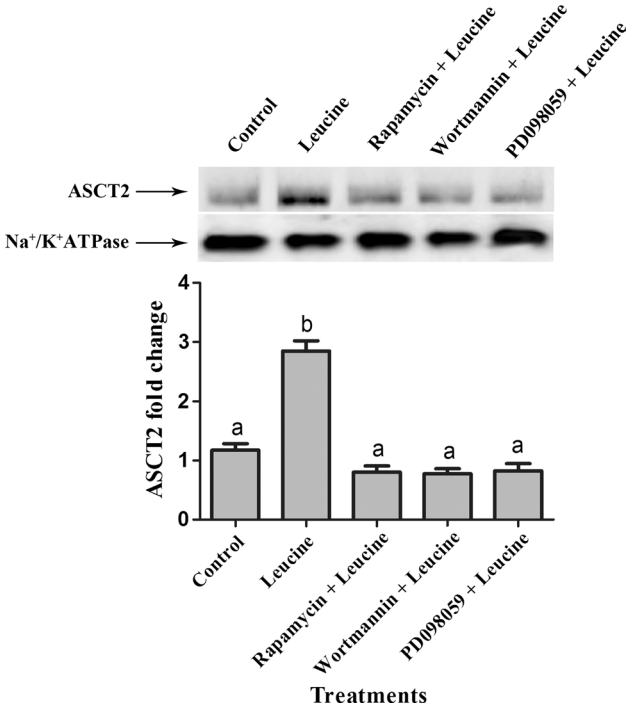

Fig. 8 The protein abundance of amino acid transporter ASCT2 with leucine stimulation in IPEC-J2 cells pre-treated with inhibitors ($n = 4$). Cells were pre-treated for 30 min with either 20 nM rapamycin (mTOR inhibitor), 10 μM wortmannin (PI3K inhibitor), or 10 μM PD098059 (MEK1inhibitor) prior to incubation with 7.5 mM leucine for 8 h. The protein abundance of ASCT2 was detected by Western Blot. Na^+/K^+ ATPase was used as an internal standard to normalization. Means followed by same letter did not differ ($P > 0.05$)

signaling pathway in this paper. Besides mTOR signaling pathway, eIF4E phosphorylation could be regulated by members of the mitogen-activated protein kinase (MAPK) superfamily like ERK and p38 (Pyronnet 2000; Walsh and Mohr 2004). In this study, only the phosphorylation of ERK but not JNK or p38, was regulated by leucine, which was similar to a previous experiment showing that leucine regulates the phosphorylation of ERK in hepatic stellate cells (de Obanos et al. 2006). Inhibition of mTOR and PI3K attenuated leucine-mediated induction of mRNA expression of amino acid transporter ASCT2, but inhibition of ERK did not. Similarly, it was demonstrated that the PI3K/Akt/mTOR signaling pathway could regulate the mRNA expression of glucose and amino acid transporters in muscle and placenta (Yu et al. 2012; Roos et al. 2009). However, some studies found that the inhibition of ERK signaling pathway can down-regulate the target mRNA expression (Sun et al. 2010). This may be related to the different function of ERK signaling pathway on different target mRNA expression. Inhibition of mTOR, PI3K or ERK attenuated leucine-mediated induction of protein expression of amino acid transporter ASCT2. This indicates that PI3K/Akt/mTOR and ERK signaling pathways play a critical role in modulating expression of amino acid transporters. Therefore, leucine-mediated induction of amino acid transport (ASCT2 and 4F2hc) was possibly mediated through PI3K/Akt/mTOR and ERK signaling pathways.

In conclusion, leucine could up-regulate the mRNA abundance of amino acid transporters (4F2ch and ASCT2) and protein expression of amino acid transporter ASCT2 in IPEC-J2 cells, but isoleucine or valine could not. Leucine possibly modulated the expression of amino acid transporters through the PI3K/Akt/mTOR and ERK signaling pathways. These findings have important implications that leucine may be beneficial for regulating neutral amino acid transporter expression in the small intestine.

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Conflict of interest All the authors declare that they have no competing interests in the present work.

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