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Branched-chain amino acid ratios modulate lipid metabolism in adipose tissues of growing pigs

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ABSTRACT

The effects and roles of branched-chain amino acid (BCAAs) ratios in lipid metabolism in adipose tissues of pigs are still unkown. We used pigs (Large White × Landrace, 35 ± 2 d) to investigate the effects of varying BCAA ratios (Leu: Ile: Val = 1:1:1, 1:0.75:0.75, 1:0.51:0.63, 1:0.25:0.25) on growth, carcass traits, and fat metabolism in adipose tissues. Results showed that as the ratio declined, the weight of total fat mass reduced while the adiponectin concentrations increased ($P < .05$), with the lowest/highest values observed in the 1:0.25:0.25 group, respectively. Moreover, varying BCAA ratios modulated the expression of genes related to adipose tissue function ($P < .05$). Concomitant with these changes, the 1:0.25:0.25 group increased/decreased the phosphorylation of AMPKα/mTOR, respectively ($P < .05$). The mRNA abundance of PGC-1α and IL-15 were also increased in diets with BCAA ratios from 1:0.75:0.75 to 1:0.25:0.25. Our data suggest that dietary BCAA ratios in the adequate range, i.e. 1:0.75:0.75–1:0.25:0.25, modulate adipose tissue function including fatty acid synthesis, transport, and oxidation, lipolysis, and adipokine secretion. These effects are partly mediated by AMPK-mTOR pathway and associated with mitochondrial biogenesis, the AMPK-PGC-1α axis, and IL-15 secreted by muscle tissues.

1. Introduction

Adipose tissue is interspersed throughout the body. It has various forms, including white adipose tissue (WAT), brown adipose tissue (BAT), and beige adipose tissue (Ross, 2014). WAT not only serves as an energy storage site, but also plays key roles in glucose and lipid homeostasis via the release of bioenergetic substrates through lipolysis and the storage of excess nutrients in lipid droplets (Green et al., 2016). In addition, WAT can produce a wide range of adipokines and execute numerous functional roles through endocrine and paracrine signaling (Rosen & Spiegelman, 2014). The synthesis and release of lipids and adipokines affect fatty acid metabolism in other tissues such as the liver and skeletal muscle (Green et al., 2016; Yao et al., 2016). However, dysfunction in these pathways can promote the development of insulin resistance (Herman et al., 2012). Moreover, in humans, the increased adiposity is associated with obesity and diabetes, emphasizing the need to prevent and/or treat adipose tissue disturbance that associates with increased risks of developing metabolic disorders (Yao et al., 2016). In

Abbreviations: ACC, Acetyl-CoA carboxylase; AMPKα, AMP-activated protein kinase α; ASA, Abdominal subcutaneous adipose tissue; BAT, Brown adipose tissue; BCAA, Branched-chain amino acid; BM, Biceps femoris muscle; c/EBPα, CCAAT-enhancer-binding-protein α; DSA, Dorsal subcutaneous adipose tissue; FATP-1, Fatty acid transport protein 1; FABP-4, Fatty acid binding protein 4; HSL, Hormone-sensitive lipase; Ile, Isoleucine; L-CPT-1, Liver carnitine palmitoyl transferase-1; Leu, Leucine; LM, Longissimus dorsi muscle; LPL, Lipoprotein lipase; mTOR, Mammalian target of rapamycin; PGC-1α, Peroxisome proliferator-activated receptor gamma co-activator 1-alpha; PM, Psoas major muscle; PPARγ, Peroxisome proliferator-activated receptor γ; PRA, Perirenal adipose tissue; SAT, Subcutaneous adipose tissue; SIRT1, Silent information regulator transcript 1; TF, Transcription factor; UCP3, Uncoupling protein 3; Val, Valine; VAT, Visceral adipose tissue; WAT, White adipose tissue

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the meat industry, body fat content and distribution in growing pigs are of special interest for production efficiency and meat quality (Lebret & Mourot, 1998). Overall, these observations suggest that a better understanding of fat mass development is of great importance for both humans and animals and highlight the need to elucidate the molecular mechanisms underlying the metabolic regulation of adipose tissues.

Branched-chain amino acids (BCAAs), containing leucine (Leu), isoleucine (Ile), and valine (Val), are metabolic signature in obesity and diabetes, and function as a direct-acting nutrient signal and act directly on the adipocytes (the major cellular constituent of adipose tissue) to affect the fat metabolism, favoring adiposity reduction (McAllan, Cotter, Roche, Korpela, & Nilaweera, 2013; Yao et al., 2016). In support of this view, numerous studies have demonstrated that Leu is a promising candidate for the regulation of overall lipid balance (Bai, Greene, Li, Kidd, & Dridi, 2015; Bruckbauer et al., 2012; Chen & Reimer, 2009; Fu, Li et al., 2015; Fu, Bruckbauer et al., 2015; Sun & Zemel, 2007; Zhang et al., 2007). However, we note that most studies have mainly focused on effects of the increasing dietary Leu levels (Zhang et al., 2007). More importantly, some studies have indicated that increasing dietary Leu concentrations exert no effect on lipid metabolism (Nairizi, She, Vary, & Lynch, 2009). One possible explanation for this observation is that dietary BCAA imbalance occurs. Leu, Ile, and Val have similar chemical structures and compete for the same enzymes that catalyze the first two catabolic steps (Langer, Scislowski, Brown, Dewey, & Fuller, 2000). An excessive supply of Leu may increase the catabolism of all BCAAs and further enhance the nutritional needs for Ile and Val (Wiltafsky, Pfaffl, & Roth, 2010). Consequently, the imbalanced BCAA do not have the ability to affect lipid metabolism. Moreover, Val deficiency could induce the reduction of feed intake and growth, and a high level of Leu further aggravates the consequences of Val deficiency (Gloaguen et al., 2011). Therefore, balancing the three BCAA ratio in diets is of enormous nutritional importance. Previous studies used the indicator amino acid oxidation technique to test the ratio of BCAAs during enteral feeding and show that the Leu: Ile: Val ratio of 1.8:1:1.2 is appropriate in neonatal piglets weighing between 1 and 5 kg during enteral feeding since the percentage of phenylalanine oxidation was minimal (Elango, Goonewardene, Pencharz, & Ball, 2004). However, the optimum ratio of BCAA might be different for growing pigs, and this has not been investigated to the authors' knowledge.

Investigation into the effects of BCAA, especially Leu, on fatty acid metabolism has been facilitated by the development of animal models, particularly rats and mice, whereas studies in swine models to date are sparse (Fu, Bruckbauer et al., 2015; Guo, Yu, Hou, & Zhang, 2010; Li, Xu, Lee, He, & Xie, 2012; Zhang et al., 2007). Although rodents are small and thus useful for multivariable experiments, they differ from humans in metabolism and physiology (Arner, 2005; Davis, Cain, Banz, & Peterson, 2013). In contrast, pigs possess many anatomical and physiological similarities to humans, as well as a high sequence and chromosome structure homology (Groenen et al., 2012; Vamathevan et al., 2013). These peculiarities make the pig an interesting model for understanding the role of BCAA in the regulation of fat metabolism in adipose tissues.

Therefore in the present study, a pig model was used to: (1) compare and contrast the effects of dietary BCAA ratios on fat metabolism in different location of adipose tissues, and (2) unraveling the molecular mechanisms of BCAA ratio action of fat metabolism. It was hypothesized that the optimal dietary BCAA ratios could inhibit fatty acid synthesis and elevate fatty acid β-oxidation in the adipose tissue of growing pigs. Insights into the underlying molecular mechanisms of BCAA action of fat metabolism are of great interest not only in animal biology for feed efficiency improvement, but also in molecular nutrition and medicine for potential nutritional supplement optimization and therapeutic perspectives.

Table 1
Composition and nutrient levels of the diets (air-dried basis, %).

Ingredients (%)	Leu:Ile:Val			
	1:1:1	1:0.75:0.75	1:0.51:0.63	1:0.25:0.25
Corn	70.26	70.26	70.26	70.26
Soybean meal	12.40	12.40	12.40	12.40
Whey powder	4.30	4.30	4.30	4.30
Fish meal	4.00	4.00	4.00	4.00
Soybean oil	2.80	2.80	2.80	2.80
L-Lysine HCl	0.80	0.80	0.80	0.80
DL-Methionine	0.25	0.25	0.25	0.25
L-Threonine	0.29	0.29	0.29	0.29
L-Tryptophan	0.08	0.08	0.08	0.08
L-Leucine	0.09	0.34	0.60	1.34
L-Isoleucine	0.76	0.64	0.40	0.14
L-Valine	0.70	0.57	0.55	0.07
Dicalcium phosphate	0.74	0.74	0.74	0.74
Limestone	0.70	0.70	0.70	0.70
Salt	0.30	0.30	0.30	0.30
Premix[a]	1.00	1.00	1.00	1.00
Nutritional content, %				
Digestible energy (MJ/kg)[b]	14.23	14.23	14.23	14.23
Ether extract	5.06	5.01	5.13	4.92
Crude protein	16.91	16.88	17.01	17.05
Lysine	1.05	1.00	1.10	1.01
Methionine	0.41	0.41	0.37	0.39
Threonine	0.73	0.77	0.76	0.74
Tryptophan	0.21	0.20	0.22	0.23
Leucine	1.21	1.44	1.65	2.35
Isoleucine	1.15	1.12	0.81	0.56
Valine	1.29	1.07	0.99	0.59
Leu:Ile:Val	1:0.95:1.06	1:0.78:0.74	1:0.49:0.60	1:0.24:0.25

[a] Supplied per kg of diet: $CuSO_4 \cdot 5H_2O$ 19.8 mg; KI 0.20 mg; $FeSO_4 \cdot 7H_2O$ 400 mg; $NaSeO_3$ 0.56 mg; $ZnSO_4 \cdot 7H_2O$ 359 mg; $MnSO_4 \cdot H_2O$ 10.2 mg; Vitamin K (menadione) 5 mg; Vitamin B_1 2 mg; Vitamin B_2 15 mg; Vitamin B_{12} 30 μg; Vitamin A 5400 IU; Vitamin D_3 110 IU; Vitamin E 18 IU; choline chloride 80 mg; antioxidants 20 mg; fungicide 100 mg.

[b] Digestible energy was calculated values.

2. Materials and methods

2.1. Animals and experimental diets

The present study was performed following the Chinese guidelines for experimental protocols and animal welfare, and approved by the committee on animal care of the Institute of Subtropical Agriculture, the Chinese Academy of Sciences.

A total of 32 pigs (Large White × Landrace, 35 ± 2 d, barrow) with a mean initial weight of 9.85 ± 0.35 kg were chosen and divided into four groups using a randomized complete block design based on body weight, with eight pigs per group. The pigs in the four groups were fed one of the four isoenergetic diets with Leu: Ile: Val ratios of 1:1:1, 1:0.75:0.75, 1:0.51:0.63, and 1:0.25:0.25. The diet composition is presented in Table 1. All the experimental diets meet the nutritional requirements for growing pigs. The experiment lasted for 45 d. The clean water and food were freely available during the whole experiment period.

2.2. Sample collection

Pigs were weighed at the beginning and the end of the experiment respectively, and feed consumption was recorded on a daily basis. Blood samples were collected into 10 ml tubes from the jugular vein puncture and centrifuged at 3000*g* and 4 °C for 15 min. Then, the serum supernatants were stored at −80 °C until analysis. At the end of the experiment, pigs were slaughtered by electrically stunning (250 V, 0.5 A, for 5–6 s) and exsanguinating after blood sampling. Perirenal fat

and total fat weights were recorded. Samples of WAT including subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were rapidly excised from the right side of the carcass. In particular, in the present study, SAT refers to dorsal subcutaneous adipose tissue (DSA) and abdominal subcutaneous adipose tissue (ASA), and VAT refers to perirenal adipose tissue (PRA). Meanwhile, skeletal muscle samples including biceps femoris muscle (BM), longissimus dorsi muscle (LM), and psoas major muscle (PM) were also rapidly excised from the right side of the carcass. Adipose and muscle tissue samples were quickly frozen in liquid nitrogen and then stored at −80 °C until further analysis.

2.3. Blood chemical parameters

The concentrations of serum leptin, adiponectin, interleukin-15 (IL-15), and insulin were measured using commercial ELISA kits (Cusabio Life Science Inc., Wuhan, China). Circulating glucose concentrations were determined using commercial kit from CIBA Corning (OH, USA). The serum concentrations of nonesterified fatty acids (NEFAs) were analyzed using a biochemical analytical instrument (Beckman CX4; Beckman Coulter, Germany) and commercial kits (Leadman Biotech Limited, Beijing, China).

2.4. BCAA levels in serum, skeletal muscle and adipose tissues of growing pigs

BCAA concentrations in serum and in the selected muscle and adipose tissues of growing pigs were measured on an Applied Biosystems 3200 Q TRAP LC/MS/MS system equipped with an RP-C18-column (150 mm length, 4.6 mm diameter, 5 mm particle size), as previously described (Li et al., 2015).

2.5. Reverse transcription and real-time quantitative PCR

The quantitative RT-PCR analysis was conducted as previously described (Duan, Duan et al., 2016; Duan et al., 2015). Briefly, total RNA was extracted from muscle and adipose tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Primers for the selected genes were designed using the Oligo 6.0 software (Table 2). The house-keeping gene β-actin was used as internal control to normalize the expression of target genes. The relative quantification of gene amplification by RT-PCR was performed using the value of the threshold cycle (Ct). Relative expressions of target genes were determined by the $2^{-\triangle\triangle Ct}$ method (Duan, Duan et al., 2016).

2.6. Western blot analysis

Western blot analysis was performed according to our previous studies (Duan et al., 2014; Li et al., 2014, 2016). The polyclonal antibodies used were as follows: anti-phospho (p)-the mammalian target of rapamycin (mTOR) and total (t)-mTOR, anti-p-regulatory associated protein of mTOR (Raptor) and t-Raptor, anti-p-AMP-activated protein kinase α (AMPKα) and t-AMPKα, anti-peroxisome proliferator-activated receptor gamma (PPARγ), anti-the CCAAT-enhancer-binding-protein α (C/EBPα), and anti-β-actin. All the primary antibodies were purchased from Cell Signaling Technology (Danvers, MA) except anti-p/t-Raptor from Santa Cruz Biotechnology. The bands of the protein were visualized using a chemiluminescent reagent (Pierce, Rockford, USA) with a ChemiDoc XRS system (Bio-Rad, Philadelphia, PA, USA). We quantified the resultant signals using Alpha Imager 2200 software (Alpha Innotech Corporation, CA, USA) and normalized the data with the value of the inner control β-actin or the corresponding total protein.

2.7. Statistical analysis

All data in this study was analyzed by the one-way analysis of variance (ANOVA) using SAS 8.2 software (Cary, NC, USA) followed by a Duncan's multiple comparison test. Results are presented as means with standard errors. Differences between significant means were considered as statistically different at $P < .05$.

Table 2
Primers used for real-time quantitative PCR.

Genes	Primers	Sequences (5′-3′)	Size (bp)
ACC	Forward	ATCCCTCCTTGCCTCTCCTA	208
	Reverse	ACTTCCCGTTCAGATTTCCG	
LPL	Forward	CTCGTGCTCAGATGCCCTAC	148
	Reverse	GGCAGGGTGAAAGGGATGTT	
HSL	Forward	GCAGCATCTTCTTCCGCACA	195
	Reverse	AGCCCTTGCGTAGAGTGACA	
FATP-1	Reverse	GGAGTAGAGGGCAAAGCAGG	208
	Forward	AGGTCTGGCGTGGGTCAAAG	
FABP-4	Reverse	TGGAAACTTGTCTCCAGTG	147
	Forward	GGTACTTTCTGATCTAATGGTG	
Sirt1	Reverse	GGTTTGAAGAATGTTGCCTG	114
	Forward	CCGTTTACTAATCTGCTCCT	
PGC-1α	Reverse	GCCCAGTCTGCGGCTATTT	265
	Forward	GTTCAGCTCGGCTCGGATTT	
L-CPT-1	Reverse	GCATTTGTCCCATCTTTCGT	198
	Forward	GCACTGGTCCTTCTGGGATA	
UCP3	Reverse	GAGATGGTGACCTATGATGT	260
	Forward	CGCAAAAAGGAAGGTGTGAA	
TNF-α	Reverse	CCACGTTGTAGCCAATGTCA	395
	Forward	CAGCAAAGTCCAGATAGTCG	
IL-15	Reverse	GCATCCAGTGCTACTTGTGT	118
	Forward	TGCCAGGTTGCTTCTGTTTT	
Irisin	Reverse	GTTGTGACTGCTGAATGCCT	284
	Forward	GTCTCTCCTCCTTCCTTGTG	
Prdm16	Reverse	GGACAGACACCTCAAGAAGC	306
	Forward	TCCTCCTCCTCCTCCTCCTC	
PPARα	Reverse	TTCGGAGAACCATTCGGCTAAAG	302
	Forward	AATTCCGAGGATCTGCTGGACAGT	
β-actin	Forward	TGCGGGACATCAAGGAGAAG	292
	Reverse	AGTTGAAGGTGGTCTCGTGG	

3. Results

3.1. Growth performance and carcass traits

As presented in Table 3, the daily weight gain in the 1:0.75:0.75 and 1:0.51:0.63 groups were significantly higher than that in the 1:0.25:0.25 group, with an intermediate value in the 1:1:1 group ($P < .05$). The feed conversion rate in the 1:1:1 group tended to be higher than that in the 1:0.75:0.75 group, with intermediate values in the 1:0.51:0.63 and 1:0.25:0.25 groups ($P = .09$). No difference in average daily feed intake was observed among all groups. Next, we evaluated the weight of perirenal fat and total fat. The weight of perirenal fat were highest in the 1:1:1 group, followed by the 1:0.75:0.75 group, and the lowest value was observed in the 1:0.51:0.63 and 1:0.25:0.25 groups ($P < .05$). Likewise, the weight of total fat mass was highest in the 1:1:1 group and lowest in the 1:0.25:0.25 group, with an intermediate value in the 1:0.75:0.75 and 1:0.51:0.63 groups ($P < .05$).

3.2. Serum levels of adipokines and biochemical parameters

As shown in Table 3, the concentrations of serum leptin were highest in the 1:1:1 group and lowest in the 1:0.25:0.25 group, with an intermediate value in the other two groups ($P < .05$). However, the concentrations of both adiponectin and IL-15 increased greatly with an increase of BCAA ratio, and were highest in the 1:0.25:0.25 group ($P < .05$). The serum insulin concentration in the 1:1:1 group was significantly lower than that in other groups ($P < .05$). The serum concentrations of NEFAs and glucose were not affected by diets ($P > .05$).

Table 3
Effects of BCAA ratios on the growth performance, carcass traits, and serum adipokines and biochemical parameters of growing pigs.

	Leu:Ile:Val				SEM	*P*-value
	1:1:1	1:0.75:0.75	1:0.51:0.63	1:0.25:0.25		
Initial body weight (kg)	9.85	9.69	9.84	9.85	0.35	0.96
Final body weight (kg)	33.35^{ab}	35.56^{a}	36.64^{a}	31.24^{b}	0.66	0.02
Daily weight gain (kg)	0.55^{ab}	0.60^{a}	0.63^{a}	0.50^{b}	0.03	0.01
Feed conversion rate	2.12^{a}	1.74^{b}	1.84^{ab}	2.01^{ab}	0.18	0.09
Daily feed intake (kg/d)	1.12	1.06	1.16	0.99	0.14	0.18
Weight of lean mass (kg)	13.34	13.25	13.60	12.09	0.43	0.21
Lean percentage (%)	58.41	57.83	57.37	58.31	0.55	0.93
Weight of perirenal fat, g	146.31^{a}	135.45^{ab}	116.54^{b}	120.98^{b}	2.25	0.02
Weight of total fat mass, kg	2.62^{a}	2.12^{abc}	2.30^{ab}	1.62^{c}	0.29	0.02
Leptin, ng/mL	1.29^{a}	0.92^{ab}	0.74^{ab}	0.51^{b}	0.07	0.03
Adiponectin, μg/mL	3.89^{b}	4.22^{b}	4.53^{b}	5.97^{a}	0.38	0.02
IL-15, pg/mL	0.82^{b}	0.96^{b}	1.33^{ab}	1.72^{a}	0.24	0.03
NEFA, μmol/L	525.58	524.61	655.82	742.42	5.53	0.37
Glucose, mmol/L	5.62	6.22	5.24	6.69	0.41	0.20
Insulin, IU/mL	9.60^{b}	17.01^{a}	20.28^{a}	18.57^{a}	1.70	0.04

^{a,b,c} Values within a row with different superscripts differ significantly ($P < .05$).

3.3. BCAA levels in serum, skeletal muscle and adipose tissues of growing pigs

As presented in Table 4, BCAA levels in serum and selected adipose tissues of growing pigs were measured. There was no difference in serum Leu concentrations among the groups. The serum Ile concentrations were highest in the 1:1:1 and 1:0.75:0.75 groups, followed by the 1:0.51:0.63 group, and lowest in the 1:0.25:0.25 group ($P < .05$). Similarly, the serum Val concentrations were highest in the 1:1:1 group and lowest in the 1:0.25:0.25 group, with an intermediate value in the 1:0.75:0.75 and 1:0.51:0.63 groups ($P < .05$).

BCAA concentrations were also measured in the selected muscle and adipose tissues. No difference was detected in the Ile concentration of BM, LM, and PM among the groups. The Leu concentration in BM was highest in the 1:0.25:0.25 group, followed by the 1:1:1 and 1:0.51:0.63 groups, and lowest in the 1:0.75:0.75 group ($P < .05$). The Leu levels in PM were highest in the 1:0.25:0.25 and 1:0.51:0.63 groups and lowest in the 1:1:1 group, with an intermediate value in the 1:0.75:0.75 group ($P < .05$). No difference was detected in the concentrations of Leu in LM. The Val concentration in BM, LM, and PM was highest in the 1:0.25:0.25 group and lowest in the 1:1:1 and 1:0.75:0.75 groups ($P < .05$). In DSA, the concentrations of Leu, Ile, and Val were highest in the 1:1:1 group, and decreased gradually with a decrease in the ratio ($P < .05$). Similar results were obtained in ASA and PRA ($P < .05$).

3.4. Transcript level of genes related to lipid metabolism in adipose tissues

As shown in Fig. 1, the mRNA abundance of genes related to

Table 4
Dietary BCAA ratios affected BCAA levels in serum, skeletal muscle, and adipose tissues of growing pigs.

Items	Leu:Ile:Val				SEM	*P*-value
	1:1:1	1:0.75:0.75	1:0.51:0.63	1:0.25:0.25		
Serum (nmol/mL)						
L-leucine	179.14	161.68	202.71	188.35	2.59	0.70
L-isoleucine	145.82^{a}	127.03^{a}	111.16^{ab}	67.59^{b}	2.34	0.02
L-valine	474.81^{a}	331.76^{b}	302.17^{b}	131.63^{c}	3.54	< 0.0001
Longissimus dorsi muscle (air-dried basis, mg/g)						
L-leucine	0.29	0.30	0.29	0.36	0.10	0.31
L-isoleucine	0.17	0.18	0.16	0.18	0.08	0.75
L-valine	0.17^{b}	0.21^{ab}	0.22^{ab}	0.24^{a}	0.08	0.02
Biceps femoris muscle (air-dried basis, mg/g)						
L-leucine	0.30^{ab}	0.26^{b}	0.29^{ab}	0.34^{a}	0.10	0.01
L-isoleucine	0.16	0.15	0.16	0.17	0.07	0.27
L-valine	0.20^{b}	0.21^{b}	0.23^{ab}	0.27^{a}	0.09	0.02
Psoas major muscle (air-dried basis, mg/g)						
L-leucine	0.26^{b}	0.32^{ab}	0.35^{a}	0.35^{a}	0.09	< 0.0001
L-isoleucine	0.17	0.16	0.16	0.17	0.07	0.29
L-valine	0.17^{c}	0.23^{b}	0.21^{b}	0.27^{a}	0.07	< 0.0001
Dorsal subcutaneous adipose tissue (ug/g)						
L-leucine	3.34^{a}	3.41^{a}	2.24^{b}	2.31^{b}	0.42	0.03
L-isoleucine	1.57^{a}	1.25^{ab}	0.98^{b}	1.00^{b}	0.25	0.04
L-valine	2.34^{a}	1.71^{ab}	1.39^{b}	1.43^{b}	0.32	0.04
Abdominal subcutaneous adipose tissue (ug/g)						
L-leucine	6.49^{a}	6.26^{a}	5.05^{b}	5.28^{b}	0.52	0.02
L-isoleucine	3.35^{a}	2.70^{ab}	2.30^{b}	2.52^{b}	0.37	0.04
L-valine	5.39^{a}	3.68^{b}	3.63^{b}	3.40^{b}	0.49	0.03
Perirenal adipose tissue (ug/g)						
L-leucine	7.89^{a}	6.23^{b}	5.37^{bc}	4.43^{c}	0.60	0.02
L-isoleucine	3.33^{a}	2.23^{b}	1.99^{b}	2.11^{b}	0.38	0.03
L-valine	5.74^{a}	3.77^{b}	3.10^{b}	3.34^{b}	0.49	0.04

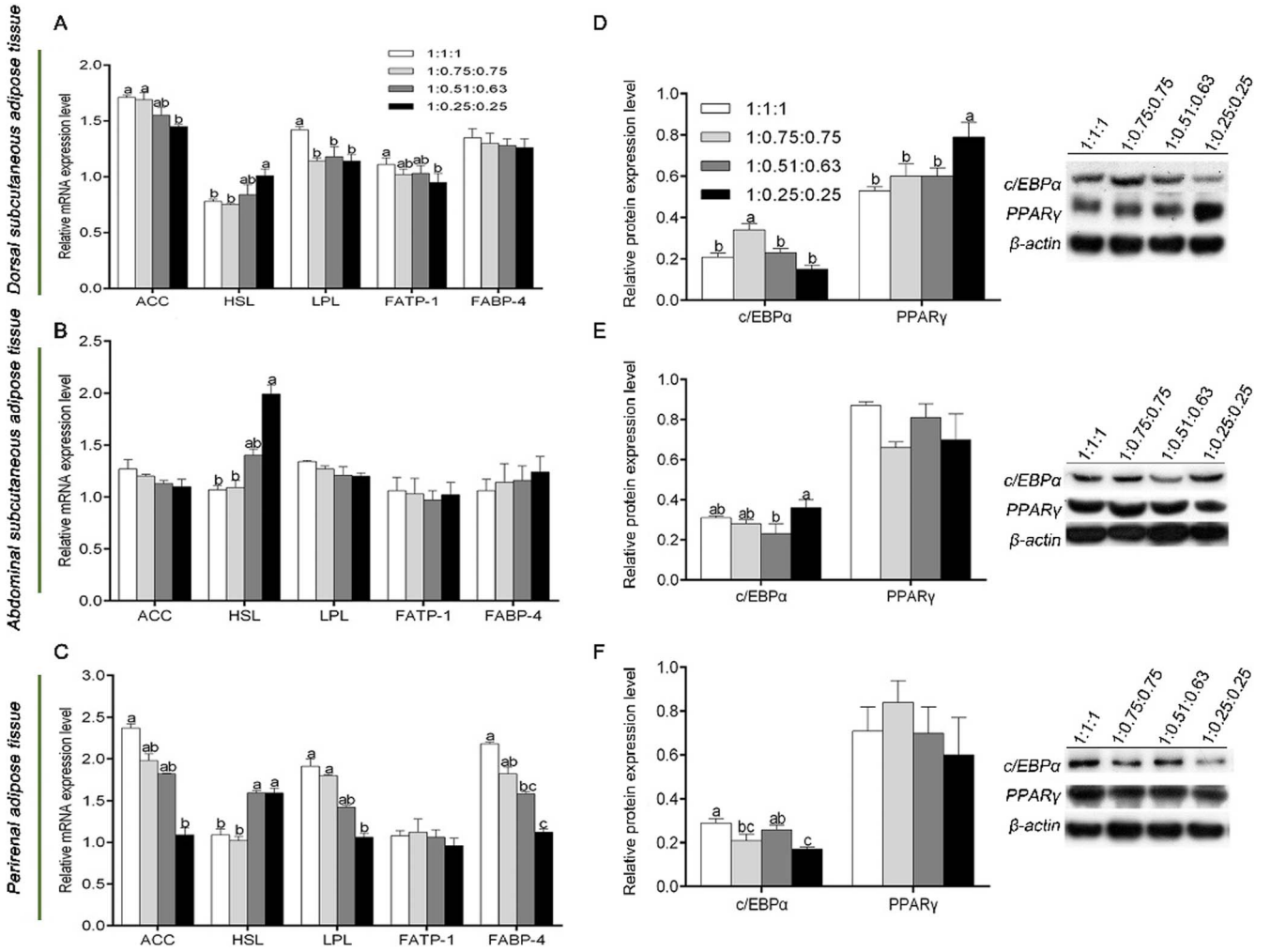

Fig. 1. The mRNA and protein expression of genes related to lipid metabolism in adipose tissues of pigs fed varying BCAA ratios. Values are means, with their standard errors represented by vertical bars (n = 8). [a,b]Mean values with different letters were considered to be significantly different ($P < .05$).

lipogenesis (acetyl-CoA carboxylase, ACC; lipoprotein lipase, LPL), lipolysis (hormone-sensitive lipase, HSL), and lipid transport (fatty acid transport protein, FATP-1; fatty acid binding protein 4, FABP4) were measured in DSA, ASA, and PRA. In DSA (Fig. 1A), the mRNA expression levels of ACC, LPL, and FATP-1 in the 1:1:1 group were higher than those in the 1:0.25:0.25 group, with intermediate values in the other two groups ($P < .05$). Conversely, the HSL mRNA expression was highest in the 1:0.25:0.25 group and lowest in the 1:1:1 and 1:0.75:0.75 groups, with an intermediate value in the 1:0.51:0.63 group ($P < .05$). Diet treatments did not influence the FABP-4 mRNA expression ($P > .05$). In ASA (Fig. 1B), similar alterations were seen for the HSL mRNA expression ($P < .05$). The difference was not statistically significant for the mRNA expression of ACC, LPL, FATP-1, and FABP-4 among all groups ($P > .05$). In PRA (Fig. 1C), similar observations about the mRNA levels of ACC, LPL, and FABP-4 occurred in response to diet treatments, with a marked upregulation and downregulation in the 1:1:1 and 1:0.25:0.25 group, respectively ($P < .05$). Similar alterations for the HSL mRNA expression to those in DSA were also seen ($P < .05$).

Since the transcription of the above-mentioned genes is under the control of several transcription factors (TFs), we next measured the protein expression of critical TFs CCAAT/enhancer binding protein-alpha (C/EBPα) and peroxisome proliferator-activated receptor alpha and gamma (PPARγ). In DSA, the expression of C/EBPα and PPARγ proteins was higher in the 1:0.75:0.75 and 1:0.25:0.25 groups ($P < .05$, Fig. 1D), respectively. In ASA, the expression of C/EBPα protein was highest in the 1:0.25:0.25 group and lowest in the 1:0.51:0.63 group, with an intermediate value in the 1:1:1 and 1:0.75:0.75 groups ($P < .05$, Fig. 1E). In PRA, the expression of C/EBPα protein was highest in the 1:1:1 group and lowest in the 1:0.25:0.25 group, with an intermediate value in the 1:0.75:0.75 and 1:0.51:0.63 groups ($P < .05$, Fig. 1F). No difference in the expression of PPARγ protein were observed among the groups in the ASA and PRA of pigs (Fig. 1E and F).

3.5. The mRNA expression of mitochondrial β-oxidation- and biogenesis-related genes in adipose tissues

As shown in Fig. 2, the mRNA abundance of key genes related to mitochondrial β-oxidation (liver carnitine palmitoyl transferase-1, L-CPT-1; uncoupling protein 3, UCP3) and biogenesis (silent information regulator transcript 1, Sirt1; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC-1α) was measured in selected adipose tissues. The dietary treatments did not change the relative mRNA expression of Sirt1 in ASA and PRA and of UCP3 in DSA of pigs ($P > .05$). However, the mRNA expression of Sirt1 in DSA, UCP3 in ASA and PRA, and of PGC-1α and L-CPT-1 in all selected WAT were lowest in the 1:1:1 group, and increased greatly in response to a reduction of BCAA ratio ($P < .05$).

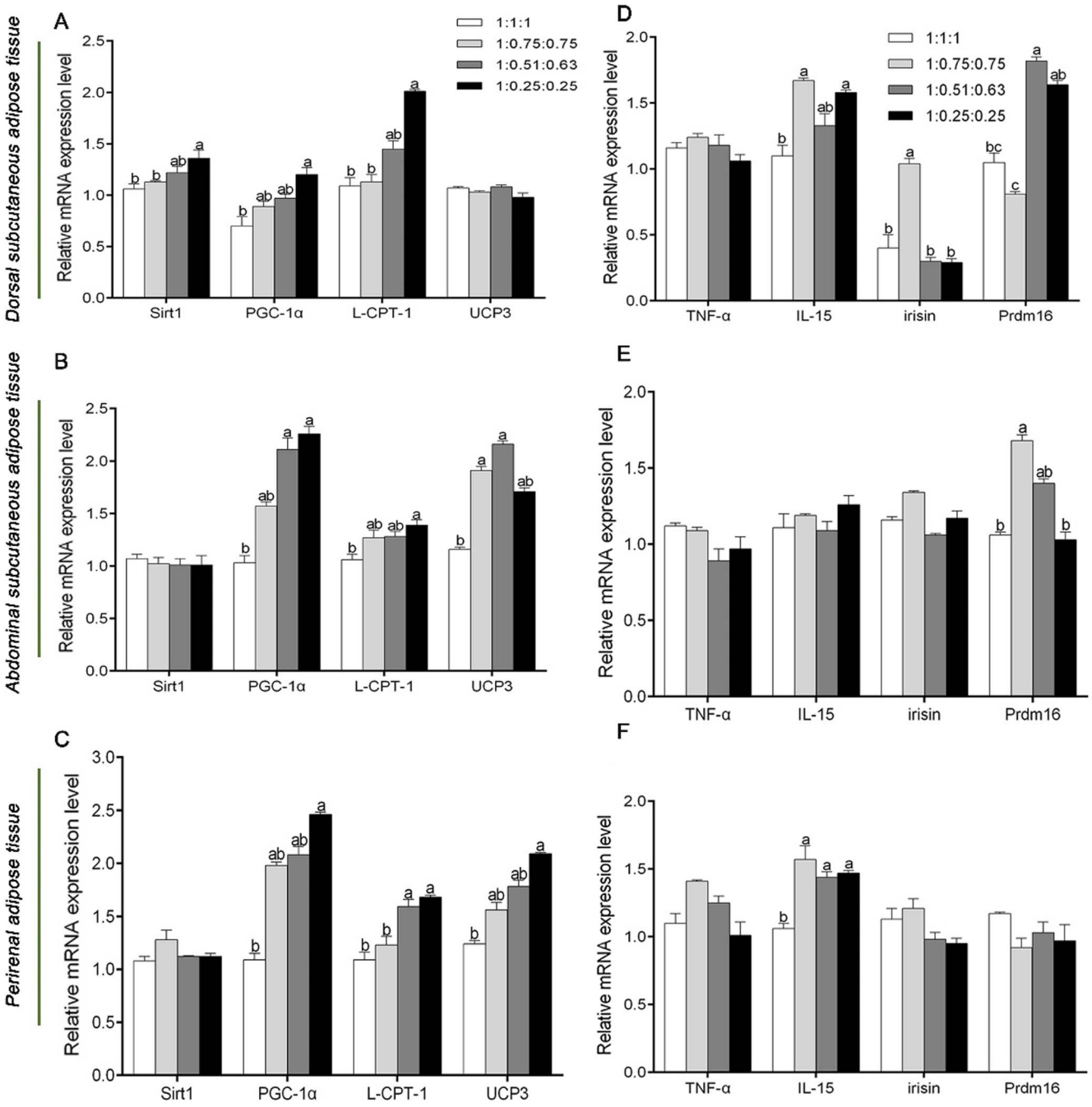

Fig. 2. Effects of BCAA ratios on the mRNA abundances of genes related to mitochondrial biogenesis (Sirt1 and PGC-1α) and fatty acid oxidation (L-CPT-1 and UCP3), as well as cytokines (TNF-α, IL-15, and irisin) and Prdm16 in adipose tissues of pigs. Values are means, with their standard errors represented by vertical bars (n = 8). [a,b]Mean values with different letters were considered to be significantly different ($P < .05$).

3.6. The mRNA expression of cytokines in adipose tissues

The mRNA abundance of cytokines (tumor necrosis factor-α, TNF-α; IL-15; and irisin) was measured in adipose tissues. In DSA, the expression levels of TNF-α mRNA was lower in the 1:0.25:0.25 group than those in other groups ($P < .05$, Fig. 2D). The expression levels of IL-15 was highest in 1:0.75:0.75 and 1:0.25:0.25 groups and lowest in the 1:1:1 group, with an intermediate value in the 1:0.51:0.63 group ($P < .05$, Fig. 2D). The 1:0.75:0.75 group exhibited the highest expression levels of irisin mRNA ($P < .05$, Fig. 2D). In ASA, no difference was detected in the mRNA abundance of TNF-α, IL-15, and irisin (Fig. 2E). In PRA, the relative expression level of IL-15 was higher in 1:0.75:0.75, 1:0.51:0.63, and 1:0.25:0.25 groups than that in the 1:1:1 group ($P < .05$, Fig. 2F), and no difference was observed for the expression level of TNF-α and irisin among the groups (Fig. 2F).

3.7. The mRNA expression of PR domain containing 16 (Prdm16) in adipose tissues

The mRNA abundance of Prdm16 was highest in DSA of pigs in the 1:0.51:0.63 group and in ASA of pigs in the 1:0.75:0.75 group ($P < .05$, Fig. 2), and no difference was observed in PRA of pigs among the groups (Fig. 2).

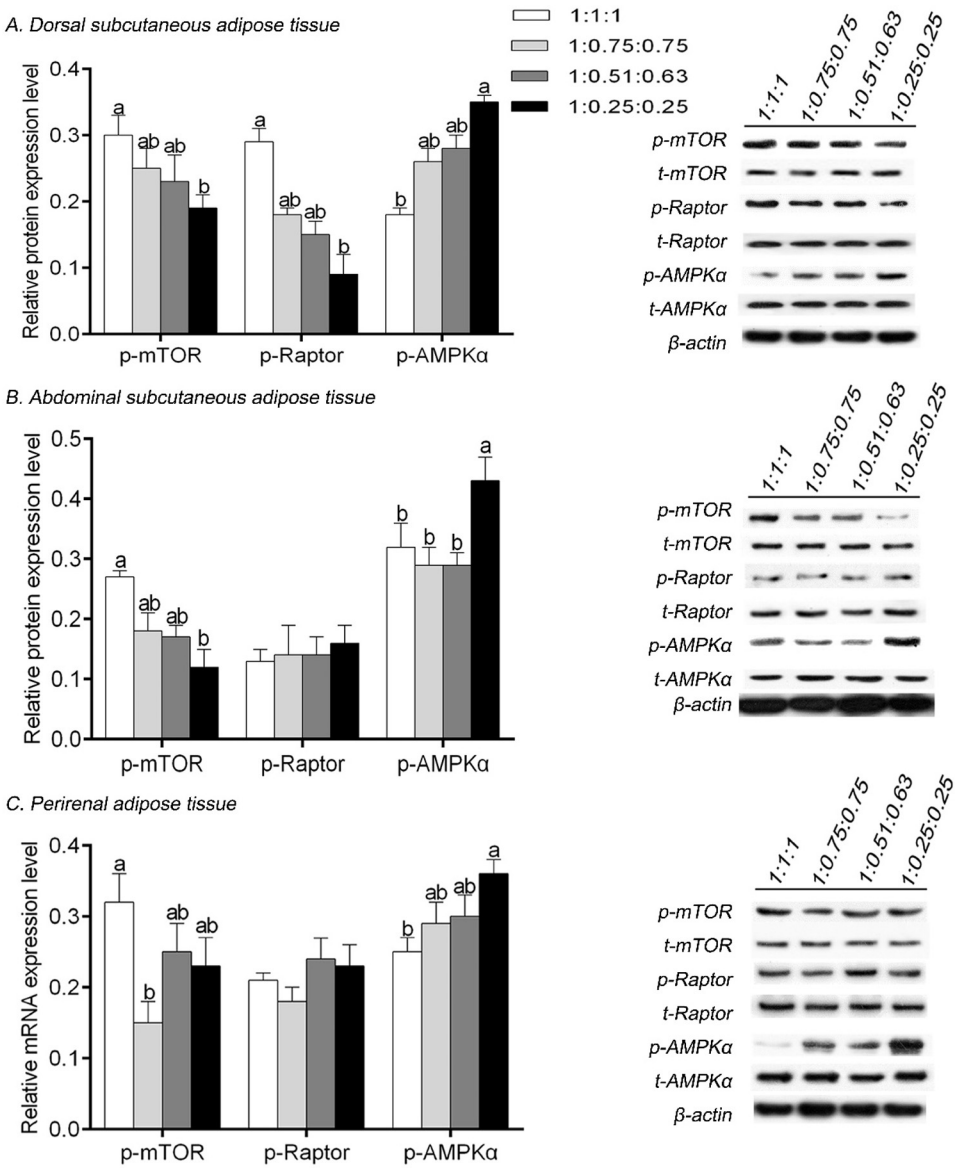

Fig. 3. Western blot analysis of mTOR, Raptor, and AMPKα phosphorylation in adipose tissues of pigs fed varying BCAA ratios. Values are means, with their standard errors represented by vertical bars (n = 8). [a,b]Mean values with different letters were considered to be significantly different ($P < .05$).

3.8. Protein expression of key molecules related to AMPK-mTOR pathway in adipose tissues

As shown in Fig. 3, the protein expression levels of key molecules related to AMPK-mTOR pathway were determined in selected WAT. In DSA, the 1:1:1 group significantly induced the phosphorylation of mTOR and raptor and decreased AMPKα phosphorylation compared to those of other groups, and the 1:0.25:0.25 group exhibited the highest and lowest expression levels of p-AMPKα and p-mTOR, respectively ($P < .05$, Fig. 3A). Similar results were obtained for the phosphorylation of mTOR in ASA and of AMPKα in ASA and PRA ($P < .05$, Fig. 3B and C). In PRA, the mTOR phosphorylation was lowest in the 1:0.75:0.75 group and highest in the 1:1:1 group, with an intermediate value in the 1:0.51:0.63 and 1:0.25:0.25 groups ($P < .05$, Fig. 3C). No difference was observed in the raptor phosphorylation in ASA and PRA of pigs (Fig. 3B and C).

3.9. The mRNA expression of PPARα in muscle tissues

The mRNA expression levels of PPARα in LM of pigs in the 1:0.75:0.75 group were significantly higher than those in the 1:1:1 and 1:0.51:0.63 groups, but was not statistically different from those in the 1:0.25:0.25 groups ($P < .05$, Fig. 4A). The mRNA expression level of PPARα in BM of pigs in the 1:1:1 group was significantly higher than that in the other groups, and there was no difference among these groups ($P < .05$, Fig. 4B). The mRNA expression level of PPARα in PM of pigs was highest in the 1:0.25:0.25 group and lowest in the 1:1:1 and 1:0.75:0.75 groups, with an intermediate value observed in the 1:0.51:0.63 group ($P < .05$, Fig. 4C).

4. Discussion

Here, we used growing pigs as model of choice to investigate the effect of different BCAA ratios on fat metabolism. We found that variation of dietary BCAA ratios affected or even improved pig growth performances as evidenced by increased average daily gain and decreased feed:gain. In contrast, some other studies exhibited a different pattern of responses (no changes or depression of these parameters) (Bai et al., 2015; Burnham, Emmans, & Gous, 1992; Iwasa et al., 2013; Lordelo, Gaspar, Le Bellego, & Freire, 2008). This discrepancy between these findings might be attributed to differences in the experimental approaches (animal models, diet composition, and BCAA ratios). For example, it has been reported that BCAA excess and/or deficiency did not improve feed intake or growth rate (Lordelo et al., 2008). In

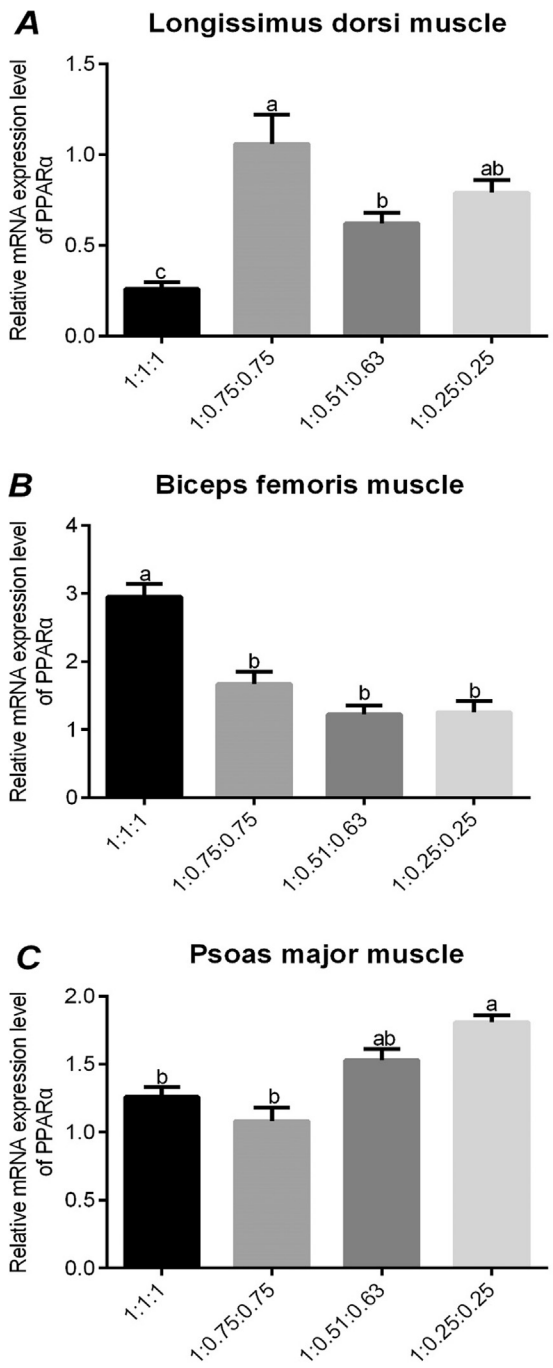

Fig. 4. The mRNA expression of PPARα in skeletal muscle of pigs fed varying BCAA ratios. Values are means, with their standard errors represented by vertical bars (n = 8). [a,b]Mean values with different letters were considered to be significantly different ($P < .05$).

addition, amino acid imbalance may reduce feed intake, particularly when associated with an amino acid deficiency (Harper, Benevenga, & Wohlheuter, 1970). For instance, the intake of excessive Leu, which means a BCAA imbalance, may result in a reduction in feed intake in pigs (Langer et al., 2000). Interestingly, no significant difference in the daily feed intake was detected in response to various BCAA ratios in the present study. However, we observed that the weight of perirenal fat and total fat mass was raised in pigs fed the dietary Leu:Ile:Val ratio of 1:1:1. Excessive amounts of body fat has been viewed as detrimental to carcass quality and the health of human consumers (Allee, Ohea, Leveille, & Baker, 1971). Moreover, the highest BCAA levels in adipose tissue and the lowest BCAA levels in skeletal muscles of the 1:1:1 group indicate that circulating BCAAs are preferentially absorbed by adipose tissues in this group. In recent years, BCAAs especially Leu have been reported to inhibit lipogenesis and to promote lipolysis and fatty acid oxidation in adipocytes, favoring adiposity reduction (Yao et al., 2016). Therefore, we hypothesized that the larger amount of body fat mass in the 1:1:1 group was associated with the effects of BCAA on the decrease in the inhibition of lipogenesis and/or in the promotion of lipolysis in adipose tissues. Intriguingly, the molecular signature in the transcripts/proteins assessed (discussed below) supported this hypothesis. Therefore, from an overall live performance viewpoint, the BCAA ratios used in our experimental conditions seemed to be balanced, but the ratio of 1:1:1 may be not optimal.

Adipose tissues are not only an energy storage site, but exert key roles in lipid metabolism, and produce a wide range of cytokines (Yao et al., 2016). Two important fat-derived cytokines known to significantly regulate energy expenditure are leptin and adiponectin (Curry, 2014). Circulating Leptin is directly proportional to body fat mass (Mantzoros et al., 2011), while circulating adiponectin concentrations are inversely proportional to fat mass (Galic, Oakhill, & Steinberg, 2010). Our current results fit well with these previous studies, showing that with a decrease of Leu: Ile: Val ratio, serum leptin and adiponectin concentrations gradually decreased and increased respectively, which matched with the change trend of body fat mass. Therefore, the data presented here constitute evidence that the ratio of 1:1:1 may be not optimal.

Next, we measured the effects of BCAA ratios on expression levels of genes related to lipid metabolism in adipose tissues. When lipogenesis occurs, LPL controls the entry of exogenous fatty acids into the adipose tissues (Mersmann, 1998), and ACC is the key enzyme responsible for catalyzing the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step for both synthesis and elongation of fatty acid synthesis (Koonen, Glatz, Bonen, & Luiken, 2005). HSL is the rate-limiting enzyme known to hydrolyze the diacylglycerol in mammalian adipose tissue (Zimmermann et al., 2004). Of note, fatty acid transporters facilitate and regulate cellular fatty acid uptake. Several fatty acid transporters have been identified, including FATP-1 and FABP-4 (Schwenk, Holloway, Luiken, Bonen, & Glatz, 2010). FATP-1 is predominantly expressed in WAT, and overexpression of FATP-1 promote cellular fatty acid uptake and acyl-CoA synthetase in adipocytes (Zhan, Poppelreuther, Ehehalt, & Fullekrug, 2012). In this study, the variation of dietary BCAA ratios significantly affected markers of fatty acid synthesis, transport, and catabolism in SAT (especially in DSA) and VAT of pigs, as evidenced by upregulated expression of ACC, LPL, and FATP-1 as well as downregulated expression of HSL in the 1:1:1 group, thus increasing lipid stores. In addition, our results show that pigs fed the protein-reduced diet with 1:1:1 of Leu:Ile:Val had downregulated mRNA expression of PPARα in LM and PM. PPARα regulates various target genes, including those implicated in fatty acid oxidation and lipid metabolism. Once activated, PPARα could augment fatty acid oxidation in skeletal muscle (Ferre, 2004; Fokko & Plutzky, 2007). From these data together with results of carcass traits, we suggest that the largest mass of body fat weight in the 1:1:1 group may be partly attributed to the increased lipogenesis and decreased lipolysis in muscle and adipose tissues.

The expression of target genes relative to lipogenesis and lipolysis is controlled by a complex network of TFs (Schreurs, Kuipers, & van der Leij, 2010). C/EBPα and PPARγ are at the center of this network and oversee the entire terminal process of adipocyte differentiation (Farmer, 2006). Unexpectedly, the modulation of these TFs appeared to be in disagreement with that of lipogenic genes in our *in vivo* experimental conditions. To our knowledge, *in vivo* the networks between TFs and their target gene responses are extremely complicated and distinctly modulated in a context- and cell type-dependent fashion (Gardner, Allis, & Strahl, 2011). Hence, the same TF can exert different effects, relying on the nature of the co-regulator (coactivator or corepressor) to which they are bound (Mottis, Mouchiroud, & Auwerx,

2013). Further *in vitro* studies determining the interaction of these TF with co-regulators in 3 T3-L1 cell lines upon varying BCAA ratios will provide insight into these mechanisms.

Since the TF profile is not conclusive, and since the AMPK-mTOR pathway has been reported to regulate the effect of BCAA in muscle tissue, we sought to measure the involvement of AMPK-mTOR signaling pathway in adipose tissues (Duan, Guo et al., 2016; Efeyan, Zoncu, & Sabatini, 2012). Activation of AMPK promotes the oxidation of long chain fatty acid in muscle tissues and impairs mTOR signaling (Inoki, Zhu, & Guan, 2003; Koonen et al., 2005). mTOR can promote adipogenesis (Cai, Dong, & Liu, 2016). Intriguingly, although mTOR signaling pathway is required for white adipocyte differentiation, it is only essential for the first stage of brown adipogenesis differentiation. Accordingly, subsequent suppression of this pathway by AMPK has been reported to be indispensable for brown adipocyte differentiation (Fernandez-Veledo, Vazquez-Carballo, Vila-Bedmar, Ceperuelo-Mallafre, & Vendrell, 2013; Vila-Bedmar, Lorenzo, & Fernandez-Veledo, 2010). Previous studies have revealed that Leu can enhance the secretion of adiponectin from adipocytes (Blumer et al., 2008; Sun & Zemel, 2007), and may stimulate AMPK indirectly by increasing adiponectin levels (Macotela et al., 2011). In agreement with these results, the induction of AMPK phosphorylation in adipose tissues and the increased concentrations of serum adiponectin occurs in the 1:0.25:0.25 group, with a concurrent decrease of the phosphorylation of mTOR and raptor. These data suggest that the 1:0.25:0.25 group activates AMPK indirectly through increasing adiponectin levels, subsequently leading to the inhibition of mTOR signaling in adipose tissues. Inhibition of mTOR signaling by AMPK may suppress fatty acid synthesis or promote lipolysis, leading to the reduction of total fat weight. More importantly, inhibition of mTOR signaling may promote brown adipocyte differentiation in SAT, as evidenced by the upregulation of Prdm16 at mRNA levels in DSA and ASA. Prdm16, a key transcriptional regulatory factor involved in the brown adipogenic program, can bind and co-regulate PPARγ and PGC-α to promote brown fat-specific gene induction (Timmons et al., 2007; Virtanen et al., 2009). More detailed studies will be needed to dissect the mechanism(s) mediating the effects of BCAA ratio on the browning of WAT and lipid metabolism in adipose tissues.

As discussed above, both energy /nutrient sensors AMPK and mTOR were influenced in response to varying BCAA ratios, suggesting an increased energy demand. Therefore, we hypothesize that varying BCAA ratios might exert an effect on mitochondrial biogenesis and fatty acid β-oxidation. The upregulation of the expression of PGC-α, L-CPT-1, and UCP3 in adipose tissues of pigs fed diets with the Leu: Ile: Val ratio ranging from 1:0.75:0.75 to 1:0.25:0.25 supports our hypothesis. PGC-α is a metabolic coactivator that induces mitochondrial biogenesis and respiration by interacting with TFs (Ventura-Clapier, Garnier, & Veksler, 2008). Moreover, interaction of PGC-α with PPARα TFs potently stimulates the expression of genes related to fatty acid oxidation such as CPT-1 (Gerhart-Hines et al., 2007). UCP3 is a mitochondrial component gene and is utilized to indicate a change in mitochondrial number (Sun & Zemel, 2007, 2009). It is also an uncoupling protein involved in the modulation of fatty acid oxidation (Himms-Hagen & Harper, 2001). Previous studies have revealed that Leu treatment can activate Sirt1, which subsequently induces the phosphorylation (activation) of AMPK. Once activated, AMPK can induce PGC-1α phosphorylation and activation, thus upregulating mitochondrial biogenesis and enhancing fatty acid oxidation in murine myotubes (Stancliffe, 2012; Sun & Zemel, 2007, 2009). In line with these findings, our data indicate that the optimal BCAA ratio may regulate mitochondrial biogenesis and fatty acid oxidation via the Sirt1/AMPK/PGC-1α axis in both SAT and VAT.

SAT and VAT are pathogenic fat depots associated with metabolic syndrome (Rosenquist et al., 2013). In particular, PRA has received increased attention in recent years, for a good correlation between its amount and central obesity and metabolic syndrome (Lim & Meigs, 2013). A key factor linking obesity and metabolic syndrome is the activity of the adipose tissue, an endocrine organ that secrets pro-inflammatory cytokines (such as TNF-α), leading to a chronic low-grade inflammation and insulin resistance (Yao et al., 2016). In the present study, no difference was observed in the expression of TNF-α at mRNA levels upon BCAA ratio treatment. These data indicate that in our experimental conditions varying BCAA ratios may not overproduce TNF-α that contributes to inflammation. Since skeletal muscles share the same characteristics as adipose tissues, that is, secreting cytokines that have endocrine or paracrine functions, and since crosstalk between myogenic cells and adipocytes may exert a pivotal role in lipid metabolism (Li et al., 2017), the expression of myokines (especially, IL-15 and irisin) at mRNA levels was measured. There is mounting evidence indicating that IL-15 plays a key role in reducing adipose tissue mass (reviewed in Duan et al. (2017)), and irisin can increase adipocyte energy expenditure and inhibit lipid accumulation (reviewed in Li et al. (2017)). Previous studies have reported that explanted porcine adipocytes can express IL-15 mRNA when stimulated by interferon gamma (Ajuwon, Jacobi, Kuske, & Spurlock, 2004). In this study, serum IL-15 varied in parallel with IL-15 mRNA in DSA and PRA in response to the dietary treatments. Moreover, the upregulation of IL-15 and irisin mRNA in DSA and PRA of pigs fed diets with the Leu: Ile: Val ratio ranging from 1:0.75:0.75 to 1:0.25:0.25 indicates that adipose tissue mass may be reduced in these groups, which matches with the change of body fat mass. Additionally, the lack of alteration in lean body mass of pigs in the current study shows that declines in adipose tissue mass were due neither to enhancements in metabolic rate caused by massive lean tissue growth nor to generalized cachexia. Previous studies have reported that overexpression of IL-15 in muscle tissue led to no differences in body composition unless the IL-15 was released into the circulation (Quinn, Anderson, Strait-Bodey, Stroud, & Argiles, 2009). Notably, the reasons why in the 1:0.25:0.25 group circulating IL-15 tends to be increased remain to be addressed. One possible explanation is the “hunger-like effects” induced by various BCAA feeding regiments. Therefore, the data presented here constitute evidence that varying BCAA ratios may affect adipose tissue mass via the crosstalk between skeletal muscle and adipose tissue.

In conclusion, dietary BCAA ratios in the adequate range, i.e. 1:0.75:0.75–1:0.25:0.25, modulated adipose tissue function, including fatty acid synthesis, transport, and oxidation, lipolysis, and adipokine synthesis and/or secretion. These effects were likely mediated by AMPK-mTOR pathway and associated with mitochondrial biogenesis and the Sirt1-AMPK-PGC-1α axis. In addition, dietary BCAA ratio in the adequate range may regulate the fat deposition in adipose tissue via IL-15, which is secreted by skeletal muscle tissue (Fig. 5). Future studies are needed to explore the impact of optimal Leu: Ile: Val ratio on the crosstalk between skeletal muscle cells and adipocytes using cell lines. Unraveling the molecular mechanisms of BCAA ratio action of lipid metabolism in adipose tissues may have broader implications not only in animal biology for feed efficiency improvement, but also in molecular nutrition and medicine for potential nutritional supplement optimization and therapeutic perspectives.

Acknowledgments

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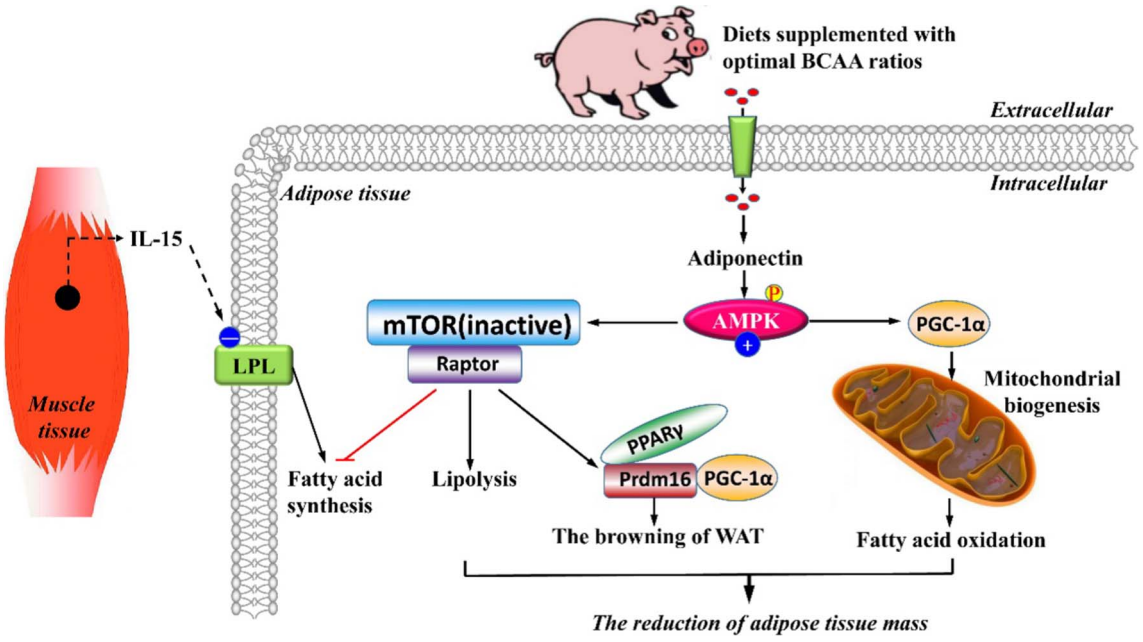

Fig. 5. Schematic representation of hypothesized mechanism of BCAA ratio effects on lipid metabolism in adipose tissues of pigs.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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