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Dietary arginine supplementation altered expression of IGFs and IGF receptors in weaning piglets

Rongjun Chen [1]*, Wence Wang [2,3], Shengbin Liu[1],Jie Pan[2], Tiejun Li[2]* and Yulong Yin[2]

[1]Rice Research Institute of Sichuan Agricultural University, Chengdu, Sichuan 611130, China.
[2]Key Laboratory of Animal Nutritional Physiology and Metabolic Process and Key Laboratory of subtropical Agro-ecology, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, Hunan 410125, China.
[3] College of Animal Science, South China Agricultural University, Guangzhou, China

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Young piglets have a high requirement of arginine for growth and metabolic function, but the sow milk or endogenous synthesis cannot provide enough arginine for maximal growth. Dietary Arginine supplementation can improve the immunity of early-weaned piglets and enhance the skeletal muscle synthesis for growth. The insulin-like growth factor (IGF) signaling pathway is an important regulatory factor in regulating fetal and placental growth, proliferation, differentiation, migration and aggregation, and inhibits apoptosis of mammalian cells. However, how the insulin-like growth factor system expression altered in piglets with dietary arginine supplementation, and whether arginine plays a role in IGF system secretion is little noticed. This study was conducted to investigate the effect of dietary arginine supplementation in modulation IGF system of weanling piglets. Twelve 21-day-old healthy piglets (Landrace×Yorkshire) with a mean body weight (BW) were assigned randomly to two treatments representing diets supplemented with 0.6% L-arginine and 1.23% L-alanine (isonitrogenous control). At 28 days of age, 12 piglets were killed and longissimus muscle, liver and kidney were collected. Components of IGF signaling pathway mRNA expression were examined in three tissues, IGF1 was increased in three tissues of arginine group (P<0.05). IGF2 was increased in muscle of arginine group. Both muscle and liver had a higher level of IGFBP5 with arginine supplementation (P<0.05). These data suggest an important role of arginine in modulation of IGF signal pathway and the involvement of IGF and IGF receptors in the improved growth performance.

Key words: L-Arginine, IGFs, early-weaned piglets.

INTRODUCTION

Arginine is the substrate for important metabolic pathways such as nitric oxide (NO), agmatine, creatine, and urea synthesis, and it displays remarkable metabolic and regulatory versatility in cells (Jobgen WS, 2006; Mieulet Virginie et al., 2010: Zhang et al., 2010). Young mammals (especially piglets) have a particularly high requirement of arginine for growth and metabolic function, but the sow milk or endogenous synthesis cannot provide enough arginine for maximal growth (Wu et al., 2004; Wu et al., 2012; Flynn et al., 2002). Arginine deficiency may causes growth retardation, intestinal and reproductive dysfunction, impaired immune and neurological development, cardiovascular and pulmonary abnormalities, impaired wound healing, hyperammonemia, and even death in animals (Wakabayashi et al., 1994; Wu et al., 2007, 2009; Kim et al., 2007; Morris, 2010). Based on the crucial metabolic roles of arginine, there was growing interest in its biochemistry, nutrition, and physiology in recent years. Arginine was recently found to be stimulator of mammalian target of rapamycin (mTOR) pathway in

*Corresponding author. E-mail: chenrj913@163.com.

Table 1. The ingredients and nutrient levels of experimental diets.

Ingredient	Percentage (%)
Corn (CP 8%, H_2O < 13%)	51.55
Soybean expanded (CP 43%)	24.2
Fish meal (CP 65%)	6
Whey (100%)	9
Cream (EE 50%)	6
Limestone	0.5
Monocalcium phosphate	1
NaCl	0.2
Flavor	0.06
Premix*	1
L-Lysine•.HCl	0.31
Met	0.06
Thr	0.12
Total	100
Nutrient content	
DE (MJ/kg)	14.21
CP (%)	20
Ca (%)	0.71
AP (%)	0.48
L-lysine (%)	1.35
L-methionine (%)	0.42
L-threonine(%)	0.9

*Providing the following per kg diet: $CuSO_4.5H_2O$, 19.8 mg; KI, 0.20 mg; $FeSO_4.7H_2O$, 400 mg; $NaSeO_3$, 0.56 mg; $ZnSO_4.7H_2O$, 359 mg; $MnSO_4.H_2O$, 10.2 mg; vitamin K (menadione), 5 mg; vitamin B1, 2 mg; vitamin B2, 15 mg; vitamin B12, 30 lg; vitamin A ,5,400 IU; vitamin D3, 110 IU; vitamin E, 18 IU; choline chloride, 80 mg; antioxidants, 20 mg; Fungicide, 100 mg

tyrosine protein kinase, binding by IGFs and activating cultured pig intestinal cells (Yao et al., 2008). Dietary arginine supplementation has been proposed as a safe and effective method to enhanced the growth of milk-fed young pigs or the immune status of neonatal pigs and early-weaned piglets, rats or mice (Kim and Wu, 2007; Li et al., 2007; Tan et al 2007; Liu et al., 2008; Zeng et al., 2008). All studies examining the impact of arginine supplementation on early-weaning piglets have focused on effects on growth performance and immune function, and the underlying molecular mechanisms by which arginine may affect the endocrine system in piglets remains unclear. The insulin-like growth factor (IGF) signaling pathway is an important regulatory factor of muscle growth in animals. Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are polypeptides with structural homology to pro-insulin and are important in regulating fetal and placental growth (DeChiara et al., 1990; Liu et al., 1993; Dai et al., 2010; Tang et al., 2005). They promote the proliferation, differentiation, migration and aggregation, and inhibit apoptosis of mammalian cells. Type I IGF receptor (IGF1R) is a kind of ligand-activated the downstream signal pathway, whereas insulin-like growth factor 2 receptor (IGF2R) activates the effect of IGF2 at the cell surface and mannose-6-phosphate (M6P)-tagged proteins in the trans-Golgi network (Hawkes and Kar, 2004; Samantha Gardner et al., 2010). The bio-availability and bio-action of IGFs are regulated by a family of six high-affinity binding proteins (BP) (IGFBP-1 to 6) (Clemmons, 1997). IGFBPs promote or inhibit the actions of IGFs, by competing with the IGF receptors for the peptide (Hiroyasu Kamei et al., 2011). Previous evidence indicates that IGFBP-5 can bind the IGFs with high affinity, inhibiting growth factor activity by preventing interaction with the IGF type 1 receptor (Lassala et al., 2004). More investigations make great efforts on the role of arginine in the mTOR signal pathway, but few studies focused on the relationship of arginine and IGF system in regulating swine growth and alleviating weaning stress. We hypothesized that the expression of insulin-like growth factor system is also altered in weaned piglets with dietary arginine supplementation. This hypothesis was tested in the present study by comparing the expression of IGF1, IGF2, IGFR1, IGFR2 and IGFBP5 in muscle, liver and kidney of weaned piglets with dietary arginine supplementation and basic ration group.

MATERIALS AND METHODS

Animals and tissue collection

We conducted the experiment in accordance with the Chinese guidelines for animal welfare and it was approved by the animal welfare committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (Yin et al., 1993). Twelve, 21-d-old healthy piglets (Landrace×Yorkshire) with a mean body weight (BW) of 5.24 ± 0.24 kg were removed from four sows and were divided into two groups randomly. The test group was supplemented with 0.6% L-arginine, and the control group was fed with 1.23% L-alanine (isonitrogenous control) (dietary composition is summarized in Table 1). At 28 days of age, 12 piglets were killed after anesthesia with an intraperioneal injection of sodium pentobarbital (50 mg/kg BW) followed by quick dissection of muscle, liver and kidney (Yin et al., 2001). Fresh samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis for gene expression.

Total RNA Isolation

Total RNA was prepared from the frozen samples using TRIzol (Invitrogen). RNA concentration was measured on a NanoDrop ND-1000 ultraviolet (UV) spectrophotometer (Isogen, Maarssen, Netherlands). The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm, and RNA integrity was confirmed by agarose gel electrophoresis (Wang et al., 2009).

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of genes expression

RNA was reverse transcribed by priming and reverse transcriptase. An amount of 1 µg total RNA extracted from longissimus muscle,

Table 2. Gene primer Sequence and Accession number.

Gene name		Primer	
X94251	GADPH	Sense	5-aaggagtaagagcccctgga-3
		Anti-sense	5- tctgggatggncgctggaa -3
M31175	IGF1	Sense	5-tcttctacttggccctgtgctt-3
		Anti-sense	5-ccagctcagccccacaga-3
X56094	IGF2	Sense	5-acctccccatgtcaggctagt-3
		Anti-sense	5-gggagatacagaccaagccaat-3
AB003362	IGFR1	Sense	5-aacaacattgcctcggagcta-3
		Anti-sense	5-tgggagtggcggatcttc-3
AF339885	IGFR2	Sense	5-gccccccagcaggaatc-3
		Anti-sense	5-acgtgacttgggaaattgcat-3
U41340	IGFBP5	Sense	5-aagaagctgacccagtccaagt-3
		Anti-sense	5-ctcatctcaggcgccaagat-3

Table 3. Performance of weanling piglets fed diets containing 0.6% L-arginine or 1.23% L-alanine (control).

Item	Control group	L-arginine group
Initial weight (g)	5370±53	5230±203
Finish weight (g)	5870±210	6503±208*
Weight gain (g/d)	70±23	167±31*
Feed intake (g/d)	183±12	187±21
Feed conversion	2.61±0.47	1.07± 0.18*

Data are means ± SEM, n = 6.

liver or kidney sample was incubated with 0.5 µg random primer (Gibco BRL) for 10 min at 70°C in 12 µl. Samples were then cooled on ice, and the following components were added: 1 µl deoxyribonucleotides (each 10 mM), 4 µl RT buffer (supplied with the reverse transcriptase kit), 1.5 µl $MgCl_2$ (25 mM), 2 µl dithiothreitol (100 mM), 0.5 µl RNase inhibitor (20 IU/µl) and 1 µl reverse transcriptase (200 U/µl; SuperScript II, Gibco BRL). Samples were incubated for 1 h at 42°C and the reaction stopped by heating to 70°C for 15 min. After cooling for 10 min at 4°C, 1 µl RNase (2 U/µl, RNase H, Gibco BRL) was added and the solution was then heated at 37°C for 20 min.

The cDNA was stored at -20°C until used (Wu et al., 2010). Five biological replications were used for real-time qRT-PCR. All primers (Table 2) were designed by software Primer Expression 3.0 (Applied Biosystems). *GADPH* gene was used as internal control. Real time PCR primer sequences for selected insulin-like growth factor (IGF) and *GADPH* genes were designed using the sequences provided by the given GenBank accession numbers. Before the qRT-PCR, the primers were tested by conventional RT-PCR and agarose gel electrophoresis. All qRT-PCR assays were carried out on ABI7900 using QuantiTect SYBR Green RT-PCR Kit (Cat. No.204243, QIAGEN). Each 10 µl reaction mixture contained 5 µl Master Mix (2x), 0.2 µl reverse transcriptase, 0.5 µl of each primer (10 mM), 2 µl of template RNA sample (40 ng), and 2.3 µl RNase-free water.

The thermal cycling profile was performed with three-step cycle: 48°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 40 s and 72°C for 20 s. The relative expression levels for all genes tested in this study were normalized to *GADPH* gene used two-fold dilutions of cDNAs and delta Ct method. The target gene changes were compared with control group (Tan et al., 2011).

Statistical analysis

Values are presented as the mean ± standard error of mean (SEM). The difference among experimental groups was compared by unpaired *t*-test or one-way ANOVA followed by Student-Newman-Keuls post-hoc test. $P < 0.05$ was considered as statistically significant.

RESULTS

Growth performance of piglets fed diets containing L-arginine and L-alanine (control)

The growth performance of piglets fed diets containing 0.6% L-arginine or 1.23% L-alanine (control) is summarized in Table 3. It is noticeable that the arginine group had a higher weight gain and feed conversion than the control group ($P < 0.05$). Feed intake was not affected ($P = 0.76$) by arginine.

mRNA expression of IGF system genes between arginine group and control group of piglets

The mRNA expression of selected genes of the IGF system was investigated in the muscle, liver and kidney of arginine and control piglets (Figures 1 to 3). In all three tissues, the expression level of *IGF1* and *IGFBP5* genes

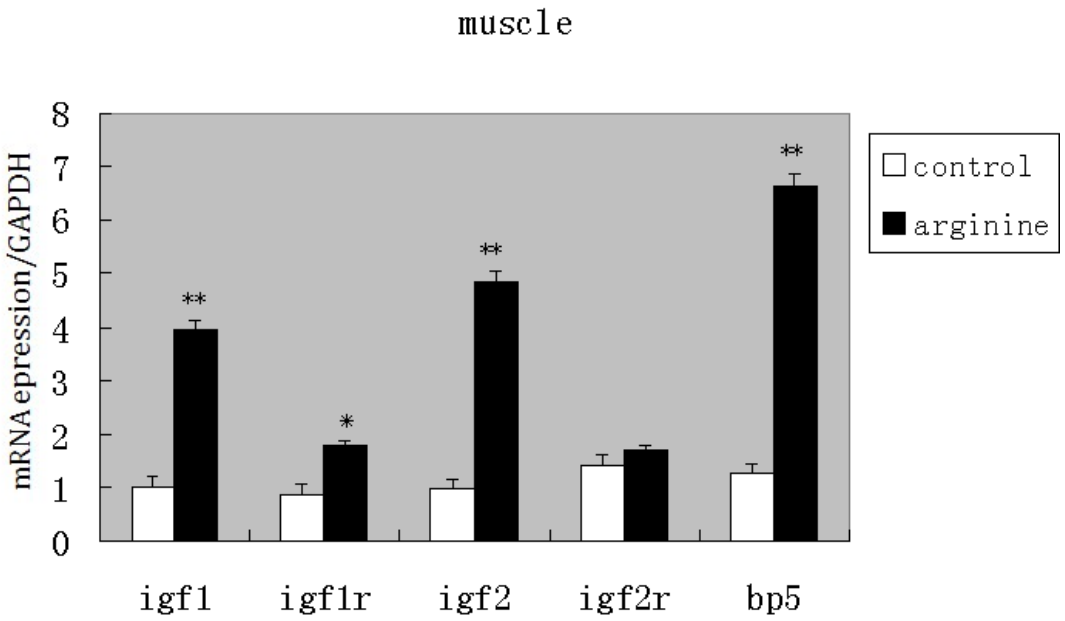

Figure 1. Relative mRNA expression of IGF1, IGF2, IGF1R, IGF2R and IGFBP5 in muscle. *P <0.05; **P < 0.01. Values are means with pooled SEM (n=6).

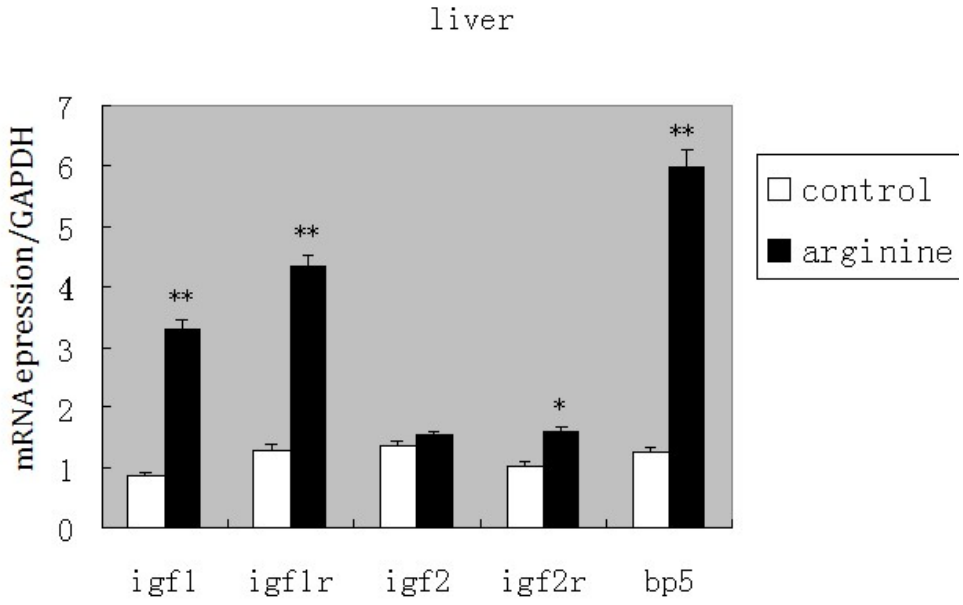

Figure 2. Relative mRNA expression of IGF1, IGF2, IGF1R, IGF2R and IGFBP5 in liver.*P <0.05; **P < 0.01. Values are means with pooled SEM (n=6).

in arginine piglets was significantly higher than that of control piglets (p<0.05). In muscle and liver, *IGFBP5* level was higher than in kidney. IGF1R mRNA abundance was higher in the muscle and liver of the arginine group. *IGF2* gene with high mRNA level was only in muscle of arginine group (p<0.05). As to *IGF2R* gene, it was not much change in muscle of arginine group, which is different from other two tissues.

DISCUSSION

Although it is well established that arginine participates in multiple pathways with nutritional and physiological importance in young mammals, the endocrine influence of arginine and the underlying of its effect on IGF system have remained obscure. Here, we investigated the mRNA expression of IGF1, IGF2, IGF1R, IGF2R, and IGFBP5

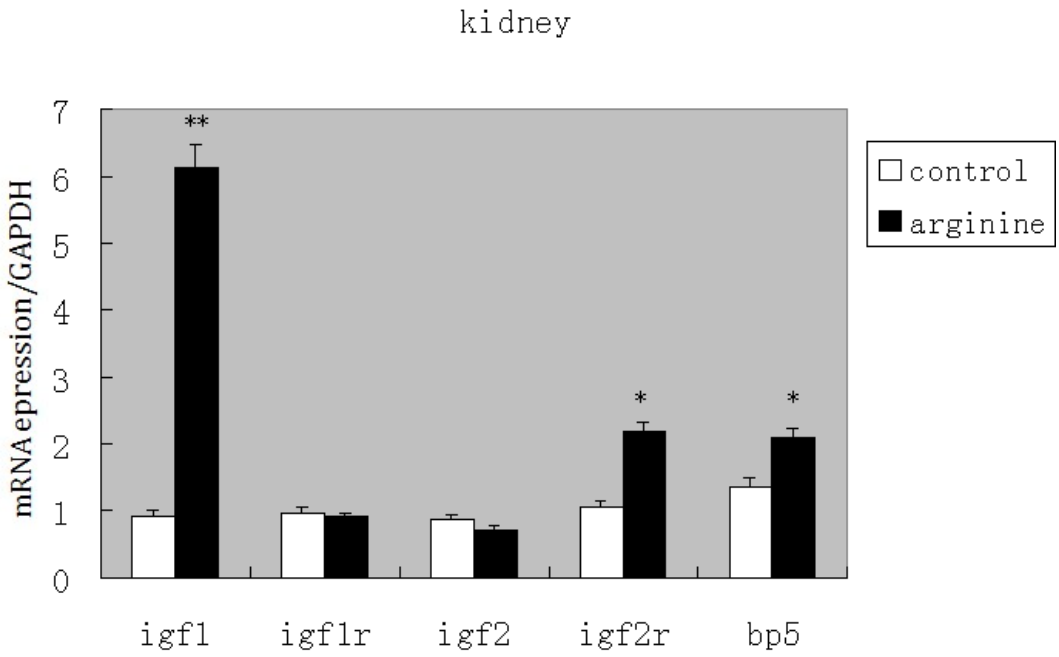

Figure 3. Relative mRNA expression of IGF1, IGF2, IGF1R, IGF2R and IGFBP5 in kidney.*P <0.05; **P < 0.01. Values are means with pooled SEM (n = 6).

with dietary supplementation of 0.6% arginine in muscle, kidney and liver of weaning piglets. Moreover, we showed the protein expression of these five factors in liver and kidney. In these tissues, the abundance of IGF-1 in arginine group was remarkably higher than in control group. The mRNA expression of IGF-1 receptor (IGF1R) in arginine group was lower than IGF1 abundance in muscle but much higher than IGF1 level in liver, as well as the protein expression in liver, but in kidney, there was no obvious change between these two groups. In contrast to the mRNA expression of IGF1, IGF2 was only highly expressed in muscle of arginine group. As to IGFBP5, it was significantly expressed in muscle and liver in arginine group (P< 0.05), but have no difference in kidney between arginine and control groups.

It has been demonstrated that insulin-like growth factors (IGFs) and associated signaling pathways play an important role in physiological development and maintaining homeostasis of mammals (Stewart and Rotwein, 1996; LeRoith et al., 2001; Duan et al., 2010). Starvation and malnutrition can reduce circulating levels of IGF-1, due to a reduction in IGF1 production by the liver, the primary IGF-1 secretory organ (Thissen et al., 1994; Bornfeldt et al., 1989). IGF-1 is generated by the liver under the control of growth hormone; it has a fundamental role in prenatal and postnatal development. Multiple signaling pathways correlated with IGF-1 are activated by binding to the IGF-1R, and IGF1 effects are modulated by multiple IGFBPs. Both IGF-1 and IGF-2 have a crucial effect on muscle building, they promote muscle differentiation *in vitro* and enhance muscle maintenance and repair *in vivo* (Samantha Gardner et al., 2010). The activation of IGF1 and IGF2 are mediated by IGF1R, although two types of IGF receptors exist, IGF1R and IGF2R (Ryo et al., 2002). IGF1R is co-activated many signaling factors, such as bone morphogenetic proteins, ligands of the epidermal growth factor receptor etc. (Alexander, 2009), but its most famous signaling pathways are the mitogen-activated protein kinase (MAP K) cascade and the phosphoinositide 3-kinase (PI-3 kinase) /Akt pathway (Arnulfo Quesada et al., 2008). Both MAPK and PI3K/Akt pathways play a key role in multiple cellular processes, such as cell proliferation, apoptosis, transcription and cell migration (Song et al., 2005; Serge et al., 2010). IGF2 main function involves in gestation stage. It is a growth promoting hormone during gestation, as well promotes progesterone secretion during the luteal phase (Wendy and Patricia, 2008). Therefore, it did not change approximately with arginine supplementation in weaning piglets. IGFBP5 function was being identified with increasing *in vitro* studies. It can inhibit IGF-1 induced skeletal muscle and breast cancer cell proliferation (Rozen et al., 1997; Ewton et al., 1998).

Many studies have been investigated that some stimulition impacted on the expression of many genes in IGF signaling pathway. Dietary Zn supplementation increased IGF1 and IGF1R expression in the small-intestinal mucosa and exerts its beneficial effects on the intestinal growth of weanling piglets (Li et al., 2006). We have also shown IUGR condition could lower the expression level of IGF1 in muscle of piglets (Chen Rongjun et al., 2011). Recently, increasing efforts were made to study the role of

arginine in the growth and development of young mammals. It is an essential amino acid for maximal growth of neonatal pigs. Yao et al., (2008) had investigated that dietary arginine supplementation can increases mTOR signaling activity in skeletal muscle of milk-fed piglets (Yao et al., 2008). Dietary arginine supplementation can promote muscle over fat gain and improve the metabolic profile as well as reducing body white fat in diet-induced obese rats (Jobgen et al., 2006). We asked whether arginine supplementation might act via the IGF signaling pathway. *In vitro*, IGFBP-5, IGF1 and IGF2 expression are able to stimulate the PI3K/AKT/mTOR pathway when translated and secreted from the cell leading to a positive feedback loop resulting in the synergistically enhanced expression of IGF1, IGFBP-5.2 and IGFBP4 (Bower and Johnston, 2010). *In vivo*, our results also demonstrated that dietary arginine supplementation can incite the IGF1, IGF2 and IGFBP5 expression in piglets. Bower and Johnston (2010) had discussed the relationship of amino acids and IGF-1 in primary cell cultures from fast myotomal muscle of Atlantic salmon (Salmo salar). Both amino acids and IGF-1 had a synergistic effect on IGF-1 expression in cells.

Two of the main metabolic functions of arginine convert to nitric oxide (NO) via nitric oxide synthase and stimulating growth hormone (Scott et al., 2011; Li et al., 2007). Arginine promotes GH releasing hormone (GHRH), as well as increase GH release by suppressing endogenous growth hormone inhibiting hormone (Vincenzo Rochira et al., 2008). Growth hormone is released into the blood stream, and then stimulates the liver to produce IGF-1 (Scarth, 2006). But IGF-2 is not influenced by GH, which mediated the embryonic growth in mammals (Walock et al., 2011). Consequently, we can see IGF2 expression is not changed too much in liver of these two arginine and control groups (Figure 2). Therefore, we deduced that arginine may play an initial switch role in the GH/IGFs axis. It also has been noticed that arginine can stimulate protein synthesis via facilitating mitogen-activated protein kinase (MAPK) activation and consequently cytokine production. MAPK is key activators in cell cycle control and cell differentiation (Leise and Michael, 2000; Dhanasekaran and Johnson, 2007). As we mentioned in IGF1R function, it also can trigger the MAPK pathway in organism. For this reason, arginine may activate IGFs and then affect the downstream pathways. In our investigation, IGF1 expression in arginine group was increased in three tissues by the stimulation of arginine, showing the main function of IGF1 in IGF mediated signal pathway. The IGF2 mRNA expression of arginine group in muscle was much higher than in liver and kidney. This may be for the reason that IGF2 was known to be more potent in stimulating myogenesis than IGF-1. While *IGF2* gene is highly active during fetal development, it is much less active in the adult body (Beverly et al., 2001). IGF1R expression was much higher in liver in arginine group than control group, indicating that IGF1R play a important role in activating IGFs, for it can bind IGF1 and IGF2. IGF2R target IGF2 for degradation via another way; the lysosomal pathway, it may trigger a signaling cascade leading to cardiac muscle cell hypertrophy, as well as clear IGF2 from the cell surface to attenuate signaling (Boker et al., 1997; Chu et al., 2008). Therefore, we can see IGF2R barely altered in these three tissues. IGFBP family members have different functions in modulating IGF-1 bioactivity at the tissue level (Jones and Clemmons, 1995). In myoblast cell line, IGFBP5 regulates the cyto-differentiation by binding to IGF2 (Ren et al., 2008). In muscle tissue, arginine activated IGF2 expression and IGFBP5 mRNA abundance also increased corresponding to this, but in liver, IGFBP5 was enriched whereas IGF2 did not alter.

These findings suggest that arginine may modulate the expression of IGF signaling factors, and IGF system involved in the improved growth performance of weaning piglets. Our results also have important implications for improving swine endocrine homeostasis. The more co-relationship for IGFs and arginine in the growing of weaning piglets need to undergo further studies.

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