Alteration of inflammatory cytokines, energy metabolic regulators, and muscle fiber type in the skeletal muscle of postweaning piglets

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Alteration of inflammatory cytokines, energy metabolic regulators, and muscle fiber type in the skeletal muscle of postweaning piglets[1]

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ABSTRACT: This study was conducted to determine the alterations of inflammatory cytokines, energy metabolic regulators, and muscle fiber type in the LM of the piglets postweaning. Crossbred piglets (Landrace × Large White) weaned at 14 d age were randomly selected from 8 litters and slaughtered at 0 (W0), 1 (W1), 3 (W3), 5 (W5), or 7 (W7) days postweaning. The glycogen content, free glucose concentration, and enzyme activities, including ATPase (Na^+/K^+, Ca^{2+}/Mg^{2+}), creatine kinase, and lactic dehydrogenase (LDH), were detected in the skeletal muscle tissue. Concentrations of proinflammatory cytokines, including IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), and anti-inflammatory cytokines, including IL-10 and transforming growth factor-β1 (TGF-β1), were measured in serum. The mRNA abundance of the above cytokines, energy metabolic regulators, and muscle fiber type related genes were determined via real-time quantitative PCR analysis. The adenosine monophosphate–activated protein kinase α (AMPKα) signaling was measured by Western blot analysis. Our results showed ATPase activities were lower on W7 d but LDH activity was higher on W3 d after weaning ($P < 0.05$). Serum TNF-α concentration was markedly increased on W1 d, then returned to the value of preweaning ($P < 0.05$), and almost all the values of inflammatory cytokines were reduced to a low point on W5 d after weaning. Additionally, the IL-6 mRNA abundance was upregulated during W3 to W7 d, but cytokine TNF-α was upregulated just on W7 d ($P < 0.05$). The mRNA abundance of AMPKα and uncoupling protein (UCP) 3 were both higher on W1 and W3 d, and UCP2 was higher on W7 d postweaning ($P < 0.05$). Myosin heavy chain (MyHC) I and MyHC IIx–type fibers were enhanced on W1 d, then returned to the value of preweaning, and the MyHC IIb–type fiber was significantly increased on W5 and W7 d ($P < 0.05$). Meanwhile, the value of P-AMPKα/T-AMPKα increased on W3 d postweaning ($P < 0.05$) compared with that on W0 d. These results indicate that weaning stress induced inflammation in skeletal muscle tissue during at least 7 d postweaning. It upregulated the expression of proinflammatory cytokines, which then stimulated the AMPKα and UCP involved in energy metabolism events, accompanied by significant alterations in muscle fiber type.

Key words: early weaning, energy metabolism, inflammation, piglet, skeletal muscle

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INTRODUCTION

Regular weaning for piglets is an average of 21 to 28 d at most pig farms; early weaning occurring at 14 to 21 d of age is 1 of the most stressful events in a pig's life (Davis et al., 2006; Yin et al.,2014a,b; 2015a,b). Postweaning piglets grow up in a competitive environment and face many challenges called stressors, and finally, weaning stress syndrome occurs (Moeser

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et al., 2007). A great deal of experimental data demonstrates that weaning results in impairment in intestinal morphology and barrier function of piglets especially in the first 2 wk after weaning (Wijtten et al., 2011; Hu et al., 2013; Wang et al., 2015). It reduces the efficiency of nutrient utilization in the intestine and peripheral tissue, such as skeletal muscle, then contributes to the decreased growth performance of pigs. Skeletal muscle is also 1 of the organs that appears to be most affected by early disturbances (Warner and Ozanne, 2010).

Proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (**TNF-α**) and anti-inflammatory cytokines such as IL-10 and transforming growth factor-β1 (**TGF-β1**) regulate skeletal muscle development (Urso, 2013; Späte and Schulze, 2004). However, few studies have investigated the gene expression levels of inflammatory cytokines in the skeletal muscle of early weaning piglets. Adenosine monophosphate–activated protein kinase α (**AMPKα**) and silent information regulator 1 (**SIRT1**) regulating each other could affect sensing energy status (Cantó and Auwerx, 2009; Price et al., 2012), and uncoupling proteins (**UCP**) 2 and 3 regulate whole-body metabolism (Schrauwen and Hesselink, 2002). They are all involved in modulating skeletal muscle fiber type (Hesselink et al., 2001; Chalkiadaki et al., 2014; Scheffler et al., 2014).

However, there are few available data regarding the biological changes in the skeletal muscle tissue of piglets after weaning. Therefore, the objective of this experiment was to identify alterations in the inflammation, energy metabolic regulators, and muscle fiber type in the skeletal muscle of piglets weaned early and slaughtered on varying days postweaning.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures outlined in this experiment were approved by the committee on animal care of the Institute of Subtropical Agriculture of the Chinese Academy of Sciences (Yin et al., 2010c).

Eight lactating sows (Landrace × Large White) with similar initial BW of new born piglets (BW = 1,618.0 ± 165.1 g) were selected. The piglets were weaned at 14 d of age (BW = 3,981.2 ± 195.3 g), and 1 piglet per litter was randomly selected (n = 8 per time) and slaughtered at 0 (**W0**), 1 (**W1**), 3 (**W3**), 5 (**W5**), or 7 (**W7**) days postweaning. The BW of the piglets slaughtered at W7 was significantly higher than that of the other groups (Duan et al., 2015). Care from birth to weaning was performed according to routine procedures on the source farm. After weaning, all the piglets were fed with creep feed (Artificial milk 101, Anyou Feed, Taicang China). The formulated composition of the diet provided for the first 7 d postweaning is shown in Table 1, and the diet was formulated to meet the requirements suggested by NRC (1998). Piglets were fed ad libitum and had unlimited access to clean drinking water throughout the experiment.

Table 1. The formulated composition of initial nursery diet provided for the first 7 d postweaning

Ingredient, %	Minimum
Cp	20.00
Lysine	1.40
Crude fat	2.98
Crude fiber, maximum	4.00
Crude ash, maximum	8.00
Calcium	0.50
Phosphorous	0.40
Salt	0.30

Sample Collection

The litter was split evenly across slaughter days. Before slaughter, the piglets were fasted overnight, and a blood sample was collected by vein puncture, then centrifuged at 3,000 × g for 15 min (4°C; Li et al.,2011a), and the separated serum was stored at −20°C until analysis (Yin et al.,2010a). After slaughter using electrical stunning, the LM samples (1 × 1 × 1 cm) were rapidly excised from the left side of the carcass and placed in liquid nitrogen (−196°C) and then stored at −80°C for further analysis, including muscle glycogen, glucose, enzyme activity, and gene or protein expression level (Yin et al.,2010b).

Measurement of Muscle Glycogen, Glucose, and Enzyme Activities

Glycogen content, free glucose concentration, and ATPase (Na^+/K^+, Ca^{2+}/Mg^{2+}) activity determination were conducted according to the manufacturer's instructions for Glycogen Assay, Glucose Assay, ATPase (Na^+/K^+, Ca^{2+}/Mg^{2+}) Activities Assay Kits (Nanjing Jiangcheng Biotechnology Inst., Nanjing, China) and Creatine Kinase (**CK**) Assay and Lactic Dehydrogenase (**LDH**) Assay Kits (Cusabio Biotechnology Inst., Wuhan, China). For glycogen content assay, muscle samples (0.5 g) were homogenized in 2.5 mL of perchloric acid (0.6 M) using an Ultraturrax homogenizer (Turratec, TE-102, Tecnal, Brazil). Homogenate was then centrifuged at 3,000 × g (4°C) for 15 min, and the supernatant was filtered through Whatman No. 54 filter paper (GE Healthcare, PA). The filtered supernatant was adjusted to spectrophotometrically determine the glycogen content as described previously (Amdi et al., 2013). Total glycogen and free glucose were ex-

Table 2. Primers used for real-time PCR analysis

Gene[1]	Primer sequences (5′–3′)	Size, bp	TA,[2] °C
IL-1β	F: CAAGGAGATGACAGCGATGA R: TCTTCGGGTAACTTTTGGGG	136	60
IL-6	F: CCTCTCCGGACAAAACTGAA R: TCTGCCAGTACCTCCTTGCT	117	58
TNF-α	F: CCACGCTCTTCTGCCTACTGC R: GCTGTCCCTCGGCTTTGAC	168	62
IL-10	F: AAGGAGGAGAAGGGGTAGGT R: GCTCGGAATGGAAAGTTTGG	136	56
TGF-β1	F: TTTCGCCTCAGTGCCCA R: GCCAGAATTGAACCCGTTAA	493	62
AMPKα	F: CAGACAGCCCTAAAGCAAGA R: CTCCAGCACCTCATCATCAA	311	60
SIRT1	F: CTACTGGTCTTACTTTGAGGG R: CAAGGGATGGTATTTATGCT	522	58
UCP2	F: CTTCTGCGGTTCCTCTGTGT R: CATAGGTCACCAGCTCAGCA	641	60
UCP3	F:GAGATGGTGACCTATGATGT R: CGCAAAAAGGAAGGTGTGAA	260	62
MyHC I	F: GGCCCCTTCCAGCTTGA R: TGGCTGCGCCTTGGTTT	63	60
MyHC IIa	F: TTAAAAAGCTCCAAGAACTGTTTCA R: CCATTTCCTGGTCGGAACTC	100	58
MyHC IIb	F: CACTTTAAGTAGTTGTCTGCCTTGAG R:GGCAGCAGGGCACTAGATGT	83	56
MyHC IIx	F: AGCTTCAAGTTCTGCCCCACT R: GGCTGCGGGTTATTGATGG	76	62
β-actin	F: TGCGGGACATCAAGGAGAAG R: AGTTGAAGGTGGTCTCGTGG	216	64

[1]TNF-α = tumor necrosis factor-α, TGF-β1 = transforming growth factor-β1, AMPKα = adenosine monophosphate–activated protein kinase α, SIRT1 = silent information regulator 1, UCP = uncoupling protein, MyHC = myosin heavy chain.

[2]TA = annealing temperature.

pressed as milligrams of glycogen per 1 g wet muscle tissue, and the enzyme activity was expressed as unit per liter. In the assays, the intra-assay CV was <5%, and the interassay CV was <8%.

Measurement of the Cytokine Levels in the Serum

The serum concentrations of IL-1β, IL-6, TNF-α, IL-10, and TGF-β1 were determined using a radio immunoassay method with kits for porcine that are commercially available from Chemclin Biotech Co., Ltd. (Beijing, China; Li et al., 2011b).

Quantitative Real-Time PCR Analysis

Total RNA was isolated from the muscle tissue using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of RNA were determined by ultraviolet spectroscopy using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE). Thereafter, about 1 μg of total RNA was incubated with DNase I (Fermentas, Thermo Fisher Scientific, MA) followed by reverse transcription using a First-Strand cDNA Synthesis Kit (Promega, Madison, WI) according to the manufacturer's protocol. The cDNA was synthesized with Oligo dT and superscript II reverse transcriptase, and the cDNA was stored at −80°C before further processing. The primer sequences for selected genes are listed in Table 2. The relative expressions of the target genes were determined by real-time PCR performed using an ABI7900HT PCR system (Applied Systems, Forrest City, CA). Real-time PCR was duplicated for each cDNA sample using SYBR Green I as the PCR core reagents in a final volume of 20 μL (Liu et al.,2012; Wu et al.,; Zhang et al.,2013). The PCR conditions were as follows: incubation for 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing, and extension for 60 s at (56°C to 64°C). Target genes' mRNA expression levels (in arbitrary units) were acquired from the value of the threshold cycle ($\mathbf{C_t}$) of the real-time PCR as related to that of β-actin using the comparative C_t method through the formula $2^{-\Delta\Delta Ct}$ ($\Delta\Delta C_t = [C_{t\ \text{gene of interest}} - C_{t\ \beta\text{-actin}}]_{\text{treat}} - [C_{t\ \text{gene of interest}} - C_{t\ \beta\text{-actin}}]_{\text{untreat}}$). Beta-actin, as a housekeeping gene whose mRNA expression level was stable in different tissues, was used as an internal control to normalize the expression of target genes.

Western Blot Analysis

An about 80- to 100-mg LM sample was used to extract total protein using a RIPA (Radio-Immunoprecipitation Assay) lysis buffer (50 m*M* Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 m*M* sodium chloride, 1 m*M* EDTA) containing a protease inhibitor cocktail and a phosphatase mixture (Roche, Nutley, NJ). The protein concentration was measured by BCA Protein Assay Reagents (Thermo Fisher, Waltham, MA) and normalized with Mili-Q water. About 30 μg of total protein was separated by a reducing SDS-PAGE. Western blots were incubated with primary antibodies of rabbit anti-phospho (**P**)-AMPKα (Thr172) and anti-total (**T**)-AMPKα at a dilution of 1:1,000 (Cell Signaling Technology, Beverly, MA) after blocking with 5% nonfat milk. The membranes were then rinsed in TBST (Tris-buffered saline, 0.1% Tween 20.) and incubated with a second antibody, peroxidase-conjugated anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA), for 1 h at a dilution of 1:5,000. Mouse anti-β-actin (Santa Cruz Biotechnology) diluted at 1:1,000 was used as the internal control (Tan et al., 2011; 2015). The bands of the protein were visualized using a chemiluminescent reagent (Pierce, Rockford, IL) with a digital luminescent image analyzer LAS-1000 (Fujifilm, Japan). We quantified the

Table 3. The glycogen content, glucose concentration, and enzyme activities in LM of piglets postweaning

Item	Day postweaning[1]					*P*- value
	W0	W1	W3	W5	W7	
Muscle glycogen, mg/g	9.89 ± 0.33	9.85 ± 0.18	9.69 ± 0.23	9.84 ± 0.43	9.85 ± 0.28	0.71
Muscle free glucose, mg/g	0.21 ± 0.03	0.25 ± 0.09	0.31 ± 0.11	0.29 ± 0.08	0.23 ± 0.05	0.29
Na^+/K^+-ATPase, U/L	36.70 ± 0.68^a	33.35 ± 0.36^{a,b}	35.56 ± 0.38^a	36.64 ± 0.68^a	31.24 ± 0.60^b	0.02
Ca^{2+}/Mg^{2+}-ATPase, U/L	0.62 ± 0.03^a	0.55 ± 0.02^{a,b}	0.60 ± 0.02^a	0.63 ± 0.03^a	0.50 ± 0.06^b	0.01
Creatine kinase, U/L	1.15 ± 0.11	1.12 ± 0.07	1.06 ± 0.06	1.16 ± 0.08	0.99 ± 0.20	0.18
Lactic dehydrogenase, U/L	1.85 ± 0.17^{a,b}	2.12 ± 0.09^a	1.74 ± 0.11^b	1.84 ± 0.13^{a,b}	2.01 ± 0.19^{a,b}	0.09

^{a,b}Means with different superscripts are significantly different ($P < 0.05$), $n = 8$.

[1]All the piglets were randomly slaughtered at 0 (W0), 1 (W1), 3 (W3), 5 (W5), or 7 (W7) days postweaning.

resultant signals using Alpha Imager 2200 software (Alpha Innotech Corp., San Leandro, CA).

Statistical Analysis

The data obtained were analyzed using 1-way ANOVA with the aid of the SAS 8.2 software package (SAS Inst. Inc., Cary, NC). Differences between significant mean values were separated using Duncan's multiple range tests and were considered statistically significant at $P < 0.05$.

RESULTS

Glycogen Content, Glucose Concentration, and Key Enzyme Activities

The glycogen content, free glucose concentration, and the enzyme activities of ATPase (Na^+/K^+, Ca^{2+}/Mg^{2+}), CK, and LDH in the LM are shown in Table 3. No significant difference was observed in the muscle glycogen, free glucose, and CK kinase activity ($P > 0.05$). The enzyme activities of ATPase (Na^+/K^+, Ca^{2+}/Mg^{2+}) decreased by about 14.88% and 19.35% on W7 d postweaning ($P < 0.05$) compared to the value from W0, respectively, and the values of other groups were not significantly different. Moreover, the activity of LDH increased about 14.59% to a peak on W1 d ($P < 0.05$), then decreased and returned to the value of preweaning (W0 d; $P < 0.05$).

Serum Concentration of the Cytokines and Their mRNA Expression Levels in Skeletal Muscle

We detected the proinflammatory and anti-inflammatory factors' concentrations in serum and their mRNA expression levels in the LM tissue postweaning. As shown in Fig. 1A and 1B, the serum level of proinflammatory factor TNF-α increased to a significant peak on W1 d ($P < 0.05$), and the levels of the cytokine IL-1β, IL-6, and TNF-α decreased to a marked low point on W5 d ($P < 0.05$); for the serum anti-inflammatory factor level, IL-10 and TGF-β1 also decreased to a significant low point on W5 d ($P < 0.05$), and then the values began to increase. The values of IL-1β, TNF-α, and TGF-β1 were restored d to those of preweaning on W7 d, but not those for the cytokines of IL-6 and IL-10 ($P < 0.05$). As shown in Fig. 2, the relative mRNA expression levels of IL-1β, IL-10, and TGF-β1 were not significantly different among all the groups ($P > 0.05$). However, we observed that the mRNA expression level of IL-6 was downregulated on W1 d, then upregulated on W3 d ($P > 0.05$), and the values between W3 and W7 d were not different ($P > 0.05$). The mRNA expression level of TNF-α markedly increased on W7 d ($P < 0.05$), but the value was stable from W0 to W5 d.

Transcripts of the Energy Metabolic Regulators and Muscle Fiber Type Related Genes

The relative mRNA expression levels of the related key genes are shown in Fig. 3. The mRNA expression level of AMPKα was enhanced on W1 and W3 d compared with W0 d preweaning ($P < 0.05$). However, the mRNA expression level of SIRT1 significantly decreased after weaning compared with the value from W0 d ($P < 0.05$). Moreover, the mRNA expression level of UCP2 increased to its highest point on W7 d ($P < 0.05$), which was not significantly different from the value on W3 d. Meanwhile, the mRNA expression level of UCP3 was stimulated on W1 d but then inhibited on W5 and W7 d relative to the value from preweaning (Fig. 3A; $P < 0.05$).

For the muscle fiber alteration, no significant difference was observed in the myosin heavy chain (**MyHC**) IIa mRNA expression level among all groups ($P > 0.05$). The mRNA expression level of MyHC I was higher on W1 d compared with W0 d ($P < 0.05$), then settled at a value that was not significantly different from that on W0 or W1 d. The mRNA expression level of MyHC IIb did not change until W5 d, when it increased to a peak and then decreased to a value that was still higher than that on W0 d ($P < 0.05$). In addition, the expression level of MyHC IIx mRNA also increase on W1 d, then

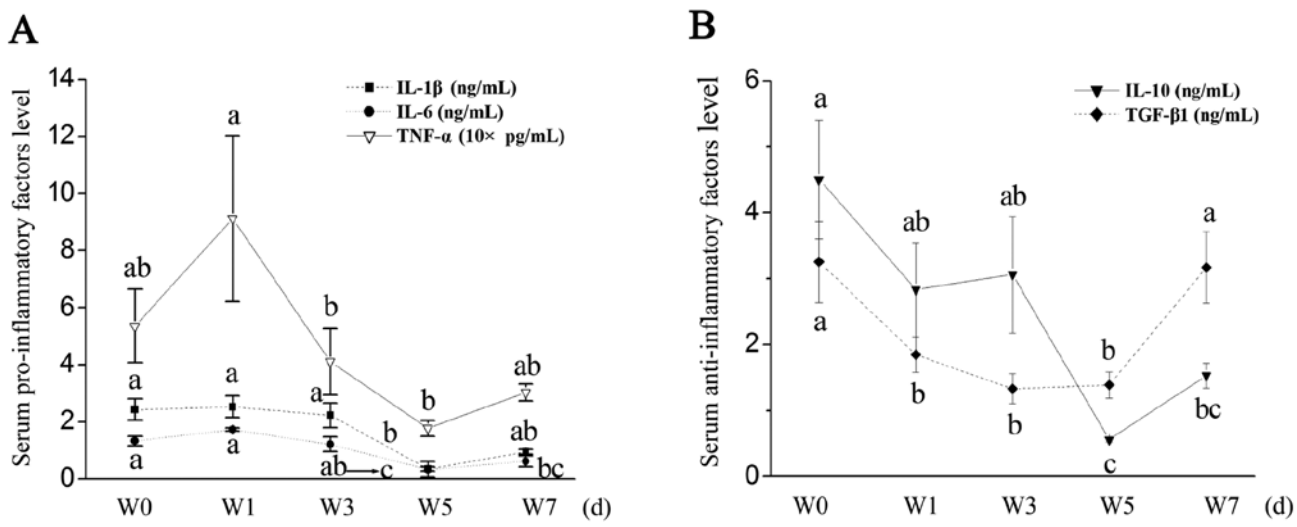

Figure 1. Serum concentrations of (A) proinflammatory cytokines IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) and (B) anti-inflammatory cytokines IL-10 and transforming growth factor-β1 (TGF-β1) of the piglets postweaning. All the piglets were randomly slaughtered at 0 (W0), 1 (W1), 3 (W3), 5 (W5), or 7 (W7) days after early weaning. Data are represented as mean ± SE ($n = 8$). [a–c]Values with different letters are considered significantly different ($P < 0.05$).

decreased, but the values from the other days were not significantly different ($P > 0.05$; Fig. 3B).

AMP-Activated Protein Kinase α Signal Pathway

The changes in AMPKα signaling were analyzed by Western blot for the phosphorylated form and total content of the protein (P-AMPKα/T-AMPKα) to calculate the relative expression level that reflects AMPK activation (Fig. 4). The AMPKα signaling was activated on W3 d and then returned to the value preweaning on W5 and W7 d postweaning ($P < 0.05$).

DISCUSSION

Skeletal muscle comprises almost 40% to 50% of total body mass of pigs and represents major metabolic activity (Matsakas and Patel, 2009). Thus, maintenance of skeletal muscle's normal function is important for muscle protein deposition and composition of livestock animals. It would be of interest to investigate whether there are some critical changes in muscle characteristics of piglets postweaning.

Early life stress alters behavior (Dybkjær, 1992; O'Mahony et al., 2009), and early weaning stress alters immunity (Kick et al., 2012) and induces oxidative stress (Yin et al., 2014a). All those events require energy expenditure. The present study showed that the ATPase activities including Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase in the LM were both reduced 7 d after weaning of the piglets. Additionally, the enzyme activity of LDH in the muscle tissue increased 1 d postweaning and then significantly decreased 3 d postweaning. Na^+/K^+-ATPase is an integral membrane protein and generates a Na^+ gradient that is used as a physiological driving force for the cotransport of AA and sugars (Wolitzky and Fambrough, 1986). Ca^{2+}/Mg^{2+}-ATPase can act as a pathway for rapid Ca^{2+} release from sarcoplasmic reticulum (Gould et al., 1987), and Ca^{2+} is also the second messenger of the whole-body metabolism. The decrease of Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase in skeletal muscle reduces muscle fundamental function in excitability and contractility (see the review by Clausen, 2013) and results in growth suppression of piglets. Moreover, LDH is a muscle injury biomarker (Bouzid et al., 2014). These results suggest that weaning stress could nega-

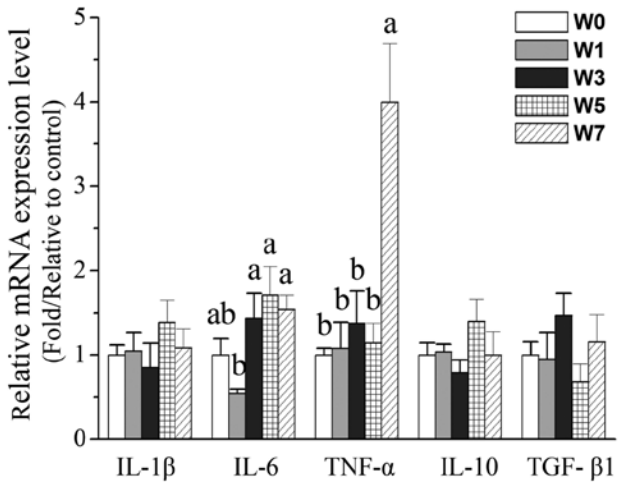

Figure 2. The relative mRNA expression levels of proinflammatory cytokines IL-1β, IL-6, tumor necrosis factor-α (TNF-α) and anti-inflammatory cytokines IL-10 and transforming growth factor-β1 (TGF-β1) in LM muscle of the piglets postweaning. All the piglets were randomly slaughtered at 0 (W0), 1 (W1), 3 (W3), 5 (W5), or 7 (W7) days after early weaning. Values are shown as fold relative to control. Data are represented as mean ± SE ($n = 8$). [a,b]Values with different letters are considered significantly different ($P < 0.05$).

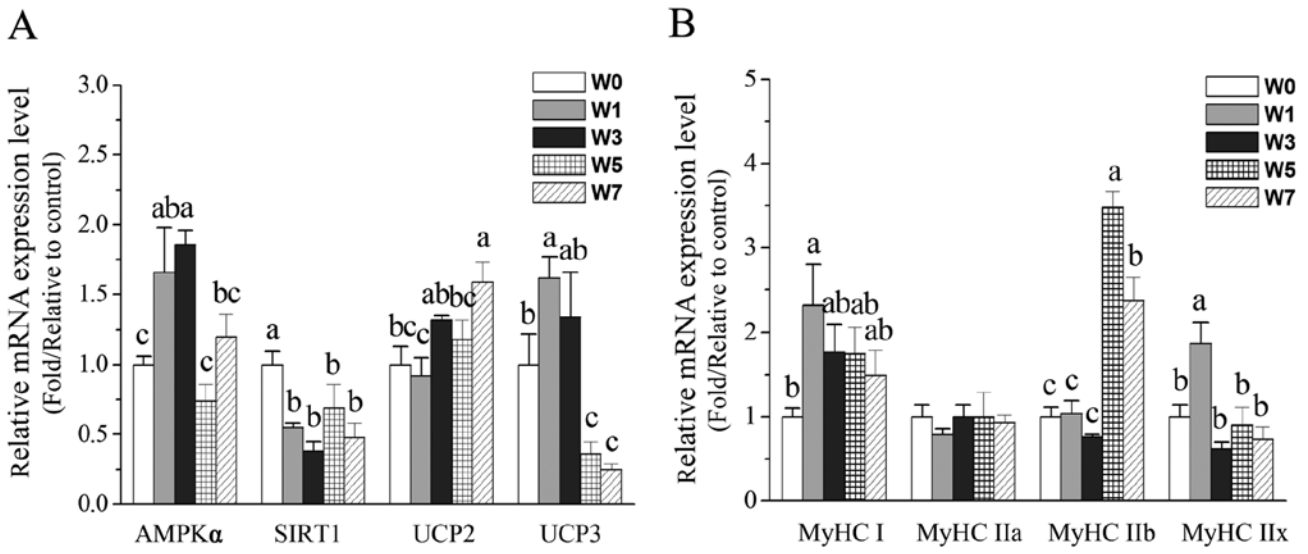

Figure 3. The relative mRNA expression levels of genes associated with (A) energy metabolic regulators and (B) muscle fiber type in LM of the piglets postweaning. All the piglets were randomly slaughtered at 0 (W0), 1 (W1), 3 (W3), 5 (W5), or 7 (W7) days after early weaning. Values are shown as fold relative to control. Data are represented as mean ± SE ($n = 8$). [a–c]Values with different letters are considered significantly different ($P < 0.05$). AMPKα = adenosine monophosphate–activated protein kinase α, SIRT1 = silent information regulator 1, UCP = uncoupling protein, MyHC = myosin heavy chain.

tively influence the enzyme activity of ATPase and LDH of skeletal muscle tissue of piglets postweaning.

In this experiment, the mRNA expression level of the proinflammatory cytokines IL-6 and TNF-α in the LM were both markedly upregulated on 3 and 7 d after weaning of the piglets, but there were no differences in the mRNA abundance of IL-10 and TGF-β1 among all the groups. There are few studies on the inflammatory cytokine expression levels postweaning in skeletal muscle, but there are some for those in the intestine. Our results agree with an earlier report that no significant increase was observed in anti-inflammatory cytokines of IL-10 and TGF-β1 mRNA expression levels after early weaning (21 d of age), but the value of proinflammatory cytokines such as IL-6 and TNF-α on 3 d postweaning increased in the intestine (Hu et al., 2013). Another report also showed that mRNA abundance of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) was upregulated between d 0 and 2 after weaning and rapidly returned to preweaning values between d 2 and 8 in the intestine after weaning (Pié et al., 2004). However, the values of IL-6 and TNF-α did not return to their previous levels in the present experiment. The intestine is the organ that digests and absorbs nutrients and transports the nutrients (e.g., AA and fatty acids) to other tissues, including skeletal muscle. Maybe there are certain relationships in cytokine expression and secretion between the intestine and skeletal muscle. Both IL-6 and TNF-α are cytokines produced by skeletal muscle called myokines. Interleukin-6 works as an energy sensor and has beneficial effects on metabolism via the AMPK pathway. When muscle energy status is low, the level of IL-6 gene expression and protein release are enhanced (Keller et al., 2001); then increased glucose uptake and fatty acid oxidation result in decreased muscle protein accretion (Pedersen and Febbraio, 2008). Tumor necrosis factor-α directly impairs skeletal muscle oxidative metabolism by signaling through the inflammatory nuclear factor-κB pathway (Remels et al., 2010) and is associated with muscle wasting and weakness (Reid and Li, 2001). Meanwhile, we found that the serum concentration of the inflammatory cytokines showed a different trend; TNF-α significantly increased on d 1 after weaning, then returned to the preweaning value in the following days, and all the other cytokines were reduced almost to a low point on 5 d postweaning. We speculate that the gene expression level of the inflammatory cytokines in skeletal muscle is only 1 of the factors regulating the circulating concentration of the cytokines to retain the integrative metabolism balance of the body. Maybe piglets subjected to weaning stress could develop a self-recovery system via feedback regulation discussed in our recent research (Yin et al., 2014b). Another finding also demonstrates that circulating cytokine levels do not correctly reflect tissue levels and their potential activity as a result of unknown autocrine or paracrine effects. Tissue-specific expression levels further complicate the analysis of their exact action (Späte and Schulze, 2004).

In the present study, we found that AMPKα mRNA abundance was upregulated 1 d after weaning and then returned to the preweaning values on d 5. But the mRNA expression level of another energy sensor, SIRT1, was markedly reduced starting 1 d after weaning of the piglets. Adenosine monophosphate–activated protein kinase α functions as a sensor of cellular energy status (e.g., low ATP) and as a master regulator of metabolism. It is activated by metabolic stress and switches off anabolic

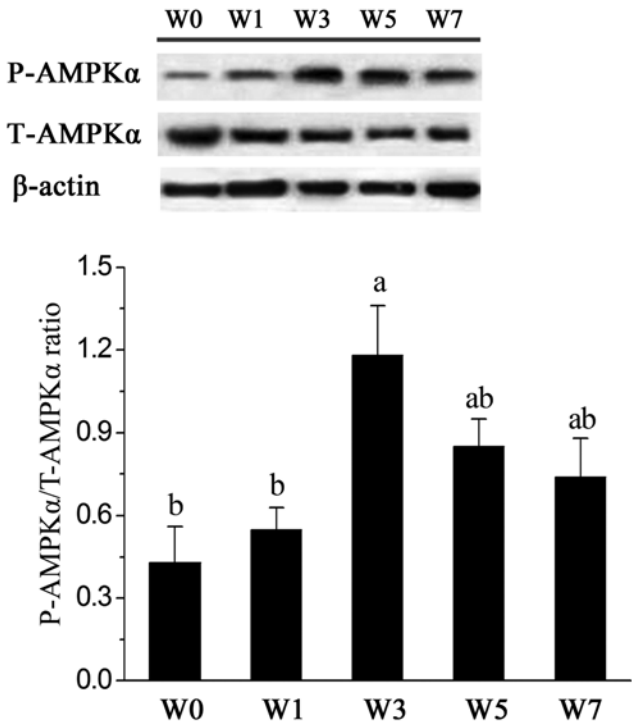

Figure 4. The relative protein expression level of adenosine monophosphate–activated protein kinase α (AMPKα) in LM of the piglets postweaning. All the piglets were randomly slaughtered at 0 (W0), 1 (W1), 3 (W3), 5 (W5), or 7 (W7) days after early weaning. Western blot analysis was employed; the expression level was the ratio of phosphor (P) form and total (T) content of AMPKα protein (P-AMPKα/T-AMPKα), and β-actin was used as an internal control. Data are represented as mean ± SE ($n = 8$). ^{a,b}Values with different letters are considered significantly different ($P < 0.05$).

pathways that consume ATP and reduced nicotinamide adenine dinucleotide phosphate while switching on catabolic pathways that generate ATP for energy homeostasis. In the study, the ratio of phosphorylated protein to total protein expression level of AMPKα (P-AMPKα/T-AMPKα) in the muscle tissue also increased 3 d after weaning. Adenosine monophosphate–activated protein kinase α might be a critical center of inflammation and energy metabolism under weaning status. Several studies highlighted that AMPKα signaling is activated following energy expenditure, resulting in the arrest of skeletal muscle protein synthesis and cell growth, with the stimulation of muscle proteolysis (Gwinn et al., 2008; Lantier et al., 2010). Stress induces inflammation, and the inflammatory process induces energy expenditure. A previous report indicates that proinflammatory cytokines could increase respiration and expression of genes linked to mitochondrial uncoupling and energy expenditure in culture muscle cells or muscle in vivo (Puigserver et al., 2001). In agreement with that report, the present study indicated that the mRNA expression levels of UCP2 and UCP3 were upregulated 3 d and 1 d after weaning, respectively, and the value of UCP3 returned to the preweaning value on d 5. It is suggested that mitochondrial function is involved in the inflammation that induced energy metabolism in the muscle tissue of piglets postweaning. Uncoupling protein 2 and UCP3 increase the proton conductance of the mitochondrial inner membrane. Uncoupling protein 2 not only regulates energy metabolism but also prevents the formation of reactive oxygen species and modulates the ATP:ADP ratio. Loss of UCP2 attenuates mitochondrial dysfunction (Kukat et al., 2014). Uncoupling protein 3 is involved in mitochondrial fatty acid metabolism to generate ATP (see the review by Schrauwen and Hesselink, 2002), and it seems to protect mitochondria against oxidative stress (Nabben and Hoeks, 2008), which enhances the expression of UCP3 mRNA in skeletal muscle (Flandin et al., 2005); overexpression of UCP3 neutralizes oxidative stress in mouse myotubes (Barreiro et al., 2009). The research of our lab also indicates that the UCP2 mRNA expression level in the jejunum is upregulated 5 d after weaning (Yin et al., 2014b). The UCP seem to be a feedback regulator on early weaning stress generating reactive oxygen species.

Skeletal muscle exhibits remarkable plasticity in responding to a variety of external stimulations. This was the first study to determine the changes in skeletal muscle fiber type of piglets postweaning. The present experiment showed that the expression of MyHC I and IIx increased 1 d after weaning and then returned to the preweaning value (0 d). For MyHC IIb, no significant difference was noted. However, the mRNA abundance of MyHC IIb increased to a peak 5 d after weaning. On the basis of MyHC protein expression form, muscle in rodents can be classified into 4 types, including MyHC I, IIa, IIb, and IIx. The MyHC I and IIa fibers are characterized by high endurance and mitochondria content. In contrast, MyHC IIb fiber generally has low endurance and lower mitochondria content. The MyHC IIx fiber is an intermediate type (Pette and Staron, 2000; Zierath and Hawley, 2004). In this experiment, an elevation in the muscle fiber type of MyHC IIb was observed in the piglets postweaning. The energy axis AMPK/SIRT1 sensing the cellular energy status can regulate muscle fiber type by affecting mitochondria (Parsons et al., 2003; Hardie et al., 2012; Chalkiadaki et al., 2014). Adenosine monophosphate–activated protein kinase activation evokes the slow and oxidative myogenic program in mouse skeletal muscle (Ljubicic et al., 2011). It appears likely that energy metabolism contributes to the skeletal muscle fiber type alterations in response to weaning status.

From a nutritional perspective, controlling early skeletal muscle inflammation and facilitating the muscle energy balance may have potential benefits in alleviating weaning stress and improving skeletal muscle growth and development for the following meat production. In

a follow-up study, metabonomics may be used to investigate the critical intermediates of AA and fatty acids in LM of piglets postweaning and other AMPKα-related effectors to further illustrate the underlying mechanism.

In summary, the primary findings might provide new insight into skeletal muscle metabolism of piglets subjected to early weaning stress. The status of stress decreased the ATPase activity but increased the LDH activity in the LM. It also altered the circulating and mRNA expression level of inflammatory cytokines and modulated the muscle fiber type with significant elevation of the MyHC IIb type. Additionally, AMPKα involved in the energy metabolism system was stimulated as a result of inflammation and contributed to the fiber type alteration. These factors may be the key points of weaning-associated skeletal muscle tissue injury of piglets and could become targets to modulate using nutritional approaches.

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