



Original article

Myokine interleukin-15 expression profile is different in suckling and weaning piglets



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ABSTRACT

Interleukin-15 (IL-15) is a cytokine highly expressed in skeletal muscle. The objective of the present study was to investigate the development of muscle IL-15 expression in suckling piglets and in early weaning piglets (day 14) at each level, that is, mRNA, protein, and secretion. Eight litters (eight piglets per litter) of newborn healthy piglets (Large × White × Landrace) with a similar initial weight (1618.0 ± 140.1 g) were chosen and divided into two groups. Group one used suckling piglets that were killed, respectively, at days 1, 7, 14, 21, and group two used early (day 14) weaning piglets that were killed respectively, at days 15, 17, 19, 21. In group one, IL-15 gene expression levels increased significantly ($P < 0.05$) along with increased body weight over time. IL-15 protein expression levels in piglets at day 21 of age were higher ($P < 0.05$) than those in piglets at other ages, and there was no difference ($P > 0.05$) among piglets at other ages. These findings indicated that increased IL-15 mRNA expression did not result in a corresponding increase of its protein expression. In group two, which used early weaning piglets from days 15–19, IL-15 mRNA and protein expression levels increased constantly ($P < 0.05$) and were higher ($P < 0.05$) than those in suckling piglets. Moreover, there was no gain of body weight ($P > 0.05$) compared with suckling piglets at day 14 of age. However, IL-15 protein expression levels in early weaning piglets at day 21 of age dropped significantly ($P < 0.05$) to the levels as suckling piglets at day 21 of age, while body weight increased ($P < 0.05$) markedly to the levels as suckling piglets at day 21 of age. In both groups, the serum IL-15 levels of piglets decreased significantly ($P < 0.01$) over time. Taken together, our results indicate that IL-15 expression differs in suckling piglets and in weaning piglets. It is speculated that IL-15 may play an important role in counteracting the effects of early weaning stress.

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1. Introduction

In general, weaning in piglets is a gradual process representing the shift from the piglets dependent on sow's milk to other food

sources (Moeser et al., 2007). In many modern swine production systems, however, weaning is an abrupt process occurring early in life at around 21 days of age. Piglets must adjust themselves to rapidly adapt to dramatic changes in dietary and environmental factors. Such changes may lead to weaning stress in piglets, and affect their health and welfare through a decline in feed intake and an increase in susceptibility to disease (De Jonge et al., 1996; Jones et al., 2001). Furthermore, weaning upregulates the expression of inflammatory cytokines in piglets (Pie et al., 2004).

Interleukin-15 (IL-15) is a cytokine first identified in the supernatant of monkey epithelial cell line CV-1/EBNA as a T-cell stimulatory factor that enhances the antitumor response (Grabstein et al., 1994; Carbo et al., 2001). However, the IL-15 gene is expressed not

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in activated T-cells but in placenta, skeletal muscle, kidney, lung and heart (Carbo et al., 2001). IL-15 possesses numerous physiological functions, including regulating T-cell growth and development, muscle protein accretion, and lipid metabolism (Ajuwon and Spurlock, 2004). The role of IL-15 in regulating T-cell growth and development is discussed extensively by other researchers (Carbo et al., 2001). IL-15 is highly expressed in skeletal muscle and is proposed to play a crucial role in skeletal muscle (Quinn et al., 1995; Fehniger and Caligiuri, 2001). Indeed, IL-15 can accumulate increased amounts of contractile proteins and muscle-specific myosin heavy chain by stimulating differentiated myocytes and muscle fibers. IL-15 can stimulate mouse skeletal myoblast differentiation in certain conditions (Quinn et al., 1997; Carbo et al., 2001). Apart from promoting muscle fiber growth and anabolism during normal and pathological conditions, IL-15 can also antagonize muscle wasting in some disease models (Carbo et al., 2000; Sugiura et al., 2002). In regard to the regulation of lipid metabolism, IL-15 acts directly on the adipocyte to regulate lipogenesis or lipolysis (Alvarez et al., 2002; Ajuwon and Spurlock, 2004). Cell culture studies, short-term *in vivo* studies, and human genotype association studies all support the model that muscle-derived IL-15 can reduce fat deposition and adipocyte metabolism through a muscle-to-fat endocrine pathway (Quinn, 2008). Although the mechanism of IL-15 decreasing fat mass in animals remains largely unknown, it is confirmed that factors such as leptin and food intake can be ruled out (Alvarez et al., 2002). Additionally, low-level IL-15 expression is strongly upregulated in response to the stimulation of inflammatory mediators, such as interferon- α (IFN- α) (Carroll et al., 2003; Ajuwon et al., 2004). Collectively, IL-15 secreted from skeletal muscle, also named myokine, may function as a powerful homeo-rhetic factor that mobilizes and directs energy away from the adipocyte to other cells during the acute phase of the inflammatory response (Ajuwon and Spurlock, 2004; Pedersen and Fischer, 2007).

In spite of these interesting observations concerning IL-15 effects on T-cells, muscle and adipocyte, few studies has been devoted to investigating the development of muscle IL-15 expression at each level, that is, mRNA, protein, and secretion. Most of previous studies measured only IL-15 mRNA, it is unclear if such changes were accompanied by changes in IL-15 protein production or secretion from muscle tissue (Pattison et al., 2003; Nieman et al., 2004; Pistilli et al., 2007). In addition, IL-15 expression may be controlled at multiple levels (such as translation and secretion) besides transcription (Tagaya et al., 1996). To our knowledge, the models previous studies used mainly focused on human and rat (Yang et al., 2013; Molanouri Shamsi et al., 2014; Rinnov et al., 2014), and not a single study has considered the dynamic changes of IL-15 expression at each level in suckling piglets and in weaning piglets. Moreover, previous studies usually consider the intestinal and immune systems rather than muscle tissues of piglets when it comes to the adverse effects of weaning stress (Pie et al., 2004; Wang et al., 2008; Jiang et al., 2009; Campbell et al., 2013). However, we need a greater understanding of the biological impact of stress to improve strategies to overcome weaning stress. IL-15 plays an important role in preserving muscle weight and protein content (Carbo et al., 2000). Moreover, early weaning may result in inflammation in piglets, and IL-15 expression is associated with inflammation (Stegall and Krolick, 2000; Sugiura et al., 2002; Pie et al., 2004; Quinn, 2008). Based on these facts, we hypothesized that myokine IL-15 expression may be different in suckling and weaning piglets, and IL-15 may play an important role in counteracting the effects of early weaning stress. To demonstrate this hypothesis, we conducted this study to examine the development of muscle IL-15 expression in suckling piglets and in weaning piglets at each level, that is, mRNA (via real-time PCR), protein (via

immunohistochemical evaluation), and secretion (via ELISA method).

2. Materials and methods

2.1. Animals and diets

All procedures followed in the present experiment were approved by the Committee of Animal Care of the Institute of Subtropical Agriculture, the Chinese Academy of Sciences.

Eight litters (eight piglets per litter) of newborn healthy piglets (Large \times White \times Landrace) with a similar initial weight (1618.0 ± 140.1 g) were chosen and divided into two groups. Group one used suckling piglets that were killed, respectively, at days 1, 7, 14, 21 of age, and group two used early (day 14) weaning piglets that were killed, respectively, at days 15, 17, 19, 21 of age. After weaning, they were fed commercial diets (Anyou Group, Jiangsu, China). All the piglets had *ad libitum* access to water, diets and milk from sows throughout the whole experimental period.

2.2. Sample collection

Body weights of piglets were recorded after an overnight fast. Before slaughter, plasma was collected into 10 mL tubes from the jugular vein for IL-15 determination. Serum was separated by centrifugation at 827 rcf (Beckman Coulter, Allegra X-22R Centrifuge, made in USA) for 15 min at 4 °C and then stored at -80 °C until analysis. The piglets were electrically stunned, exsanguinated and eviscerated. Immediately, samples (about 5 g) of the *longissimus lumborum* muscle dissected from the carcasses were placed in 10% neutral buffered formalin, or in liquid N₂ and then stored at -80 °C, respectively, until further analyses.

2.3. Analysis of secreted adipokines in serum by ELISA

The serum concentration of IL-15 was quantified using ELISA kits (Sigma, St. Louis, MO, USA) according to the manufacturers' instructions. All the samples were measured in six replicates.

2.4. Real-time PCR

Total RNA was extracted from the muscle tissues using the TRIzol reagent (Invitrogen) as described in our previous study (Duan et al., 2014). Primers for the selected genes were designed using the Oligo 6.0 software (Table 1). RT was performed using the AMV Reverse Transcriptase Kit (Promega). The relative expression levels of the target genes were determined using quantitative real-time PCR, performed with an ABI 7900 PCR system (ABI Biotechnology). The final volume of the reaction mixtures (20 μ L) contained diluted complementary DNA and SYBR Green I (Molecular Probes) as a PCR core reagent. Beta-actin was used as a

Table 1
Primers used for real-time PCR.

Genes	Primers	Sequences (5'–3')	Size, bp
IL-15	Forward	GCATCCAGTGCTACTTGTGTR	118
	Reverse	TGCCAGTTGCTTCTGTITT	
IL-6	Forward	CCTCTCCGGACAAAACCTGAAR	117
	Reverse	TCTGCCAGTACCTCCTTGCT	
TNF- α	Forward	CCACGCTCTTCTGCTACTGCR	168
	Reverse	GCTGTCCCTCCGGCTTTGAC	
β -actin	Forward	TGCGGGACATCAAGGAGAAAG	216
	Reverse	AGTTGAAGGTGCTCTCTGG	

housekeeping gene or an 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 (C_t). The comparative C_t value method using the formula $2^{-\Delta\Delta C_t}$ was employed to quantify the expression levels of IL-15, tumor necrosis factor- α (TNF- α) and IL-6 relative to those of β -actin using the following formula:

$$2^{-\Delta\Delta C_t} (\Delta\Delta C_t) = \left(C_{t \text{ gene of interest}} - C_{t \beta\text{-actin}} \right)_{\text{treat}} - \left(C_{t \text{ gene of interest}} - C_{t \beta\text{-actin}} \right)_{\text{control}}$$

2.5. Examination of IL-15 protein by immunohistochemistry

An immunohistochemical streptavidin-peroxidase (SP) method was used as described by one previous study (Xiang et al., 2012).

2.6. Statistical analyses

All the results are expressed as means with their standard errors. Statistical analyses were carried out using one-way ANOVA, SAS 8.2 (SAS institute, Inc.), followed by a Tukey's test of multiple comparisons. In case of a P value < 0.05 , differences were considered to be statistically different.

3. Results

3.1. The change of body weights in piglets

The final body weights of all piglets are listed in Table 2. In group one, the body weights of suckling piglets increased significantly ($P < 0.05$) over time. In group two, the body weights of early weaning piglets at day 21 of age were significantly higher ($P < 0.05$) than those of piglets at other ages. There was no difference in body weights between suckling piglets and early weaning piglets at day 21 of age.

3.2. Serum IL-15 activity and its development in the suckling and weaning piglets

The development of serum IL-15 levels in the suckling and weaning piglets are shown in Table 3. In both groups, the serum IL-15 levels of piglets decreased significantly ($P < 0.01$) over time. Moreover, serum IL-15 levels in early weaning piglets at day 15 of age were higher ($P < 0.01$) than those in suckling piglets at day 14 of age. However, in early weaning piglets at day 17 of age, serum IL-15 levels were equal to those in suckling piglets at day 14 of age.

3.3. Development of gene expression

The expression levels of genes (IL-15, IL-6, and TNF- α) in the muscle tissue of piglets are shown in Table 4. In group one, the expression levels of IL-15 in suckling piglets from day 1 to day 21 of age increased significantly ($P < 0.05$). However, there was no

difference ($P > 0.05$) between piglets at day 14 of age and piglets at day 21 of age. In group two, IL-15 expression levels in early weaning piglets at day 19 of age were higher ($P < 0.05$) than those in piglets at other ages. However, there was no difference ($P > 0.05$) among piglets at other ages. Furthermore, IL-15 expression levels in early weaning piglets were higher ($P < 0.05$) than those in suckling piglets. In group one, IL-6 expression levels increased significantly ($P < 0.05$) in suckling piglets from day 1 to day 14 of age, and dropped significantly ($P < 0.05$) at day 21 of age, whereas in group two, they increased significantly ($P < 0.05$) in early weaning piglets from day 13 to day 15 of age and dropped significantly ($P < 0.05$) at day 21 of age. The trend in IL-6 expression in suckling piglets was similar to that in early weaning piglets. The expression levels of TNF- α were higher ($P < 0.05$) in early weaning piglets at day 21 than other early weaning piglets, and there was no difference ($P > 0.05$) among other piglets in two groups.

3.4. IL-15 examination by immunohistochemistry

The expression levels of IL-15 protein in muscle tissues tested by the immunohistochemical SP method are shown in Fig. 1. In group one, IL-15 expression levels in piglets at day 21 of age were higher ($P < 0.05$) than other suckling piglets, and there was no difference ($P > 0.05$) among other suckling piglets in the expression levels of IL-15. In group two, the expression levels of IL-15 started to rise ($P < 0.05$) from day 15 to day 17 of age, and then to decrease ($P < 0.05$) from day 17 to day 21 of age. There was no difference in IL-15 expression between suckling piglets and early weaning piglets at day 21 of age.

4. Discussion

IL-15 is indicated to have limited ability to induce muscle growth in healthy animals. However, IL-15 may stabilize skeletal muscle protein in pathological conditions characterized by myonuclear apoptosis and muscle protein breakdown (Frost and Lang, 2003; Costelli et al., 2006). For example, in healthy, growing rats, administration of IL-15 resulted in only more than 3-fold decreases in the rate of muscle protein degradation, accompanied by a slight depression in muscle protein synthetic rates and small increases in muscle weight and protein accretion. However, in tumor-bearing rats, administration of IL-15 induced close to a 10-fold decrease in muscle proteolysis rates, associated with significant preservation of muscle weight and protein content (Carbo et al., 2000). Intriguingly, IL-15 also has anti-inflammatory and anti-apoptotic activity in various tissues and disease states, besides the important physiological functions in skeletal muscle (McInnes et al., 1997; Vainer et al., 2000; Shinozaki et al., 2002; Umemura et al., 2002; Hiromatsu et al., 2003; Obermeier et al., 2006; Budagian et al., 2006; Quinn, 2008). In the present study, IL-15 mRNA expression increased over time in suckling piglets to meet the needs of the body development. While in early weaning piglets, IL-15 mRNA was significantly higher than that in suckling piglets over time. In addition, expression levels of IL-6 and TNF- α increased significantly in early weaning piglets at day 19 and 21 of age, respectively. IL-6 and TNF- α , inflammatory cytokines, have direct effects on skeletal

Table 2
The body weights of the suckling and weaning piglets.

Item	Group one (suckling piglets)				Group two (early weaning piglets)				SEM	P-value
	1 d	7 d	14 d	21 d	15 d	17 d	19 d	21 d		
Final body weight, kg	1618.0 ^d	2934.8 ^c	3981.2 ^b	4639.8 ^a	3884.8 ^b	4112.6 ^b	4166.0 ^b	4438.6 ^a	217.5	<0.05

^{a,b,c,d} Values with unlike letters within a row were significantly different ($P < 0.05$).

Table 3

Serum concentration of IL-15 in the suckling and weaning piglets.

Item	Group one (suckling piglets)				Group two (early weaning piglets)				SEM	P-value
	1 d	7 d	14 d	21 d	15 d	17 d	19 d	21 d		
IL-15, ng/mL	1.07 ^a	0.55 ^c	0.51 ^{dc}	0.27 ^e	0.73 ^b	0.51 ^{dc}	0.59 ^{bc}	0.39 ^{de}	0.14	<0.001

^{a,b,c,d,e} Values with unlike letters within a row were significantly different ($P < 0.05$).

Table 4

Relative mRNA expression levels of selected genes in suckling and weaning piglets.

Items	Group one (suckling piglets)				Group two (early weaning piglets)				SEM	P-value
	1 d	7 d	14 d	21 d	15 d	17 d	19 d	21 d		
IL-15	0.22 ^d	0.55 ^{cd}	0.97 ^{bc}	0.99 ^{bc}	1.27 ^{ab}	1.42 ^{ab}	1.80 ^a	1.37 ^{ab}	0.41	<0.001
IL-6	0.19 ^c	0.68 ^{ab}	0.71 ^{bc}	0.47 ^b	0.23 ^c	0.60 ^{ab}	0.70 ^a	0.63 ^{ab}	0.20	<0.01
TNF- α	0.14 ^b	0.13 ^b	0.13 ^b	0.18 ^b	0.14 ^b	0.18 ^b	0.15 ^b	0.53 ^a	0.10	<0.001

^{a,b,c,d} Values with unlike letters within a row were significantly different ($P < 0.05$).

muscle mass and physiologic activities, and thus are involved in muscle wasting (Reid and Li, 2001; Dyck et al., 2006; Dirks and Leeuwenburgh, 2006). These findings indicate that early weaning stress of the piglets may cause inflammation and strongly elevate IL-15 mRNA expression. Therefore, IL-15 expression levels were relatively low in healthy, growing animals, compared with in inflammation-bearing animals. To some extent, our results are in agreement with previous studies (Stegall and Krolick, 2000; Sugiura et al., 2002; Frost and Lang, 2003; Pie et al., 2004;

Costelli et al., 2006). Moreover, the observations of Carbo et al. (Carbo et al., 2000) can be explained by our results.

In the current study, the trend of change in IL-15 mRNA expression was almost consistent with that of IL-15 protein and final body weights in early weaning piglets. Moreover, their IL-15 protein expression increased significantly from day 15 to day 17 of age, and dropped significantly to the levels as suckling piglets at day 21 of age. However, their body weights did not increase from day 15 to day 17 of age and were as many as those of suckling

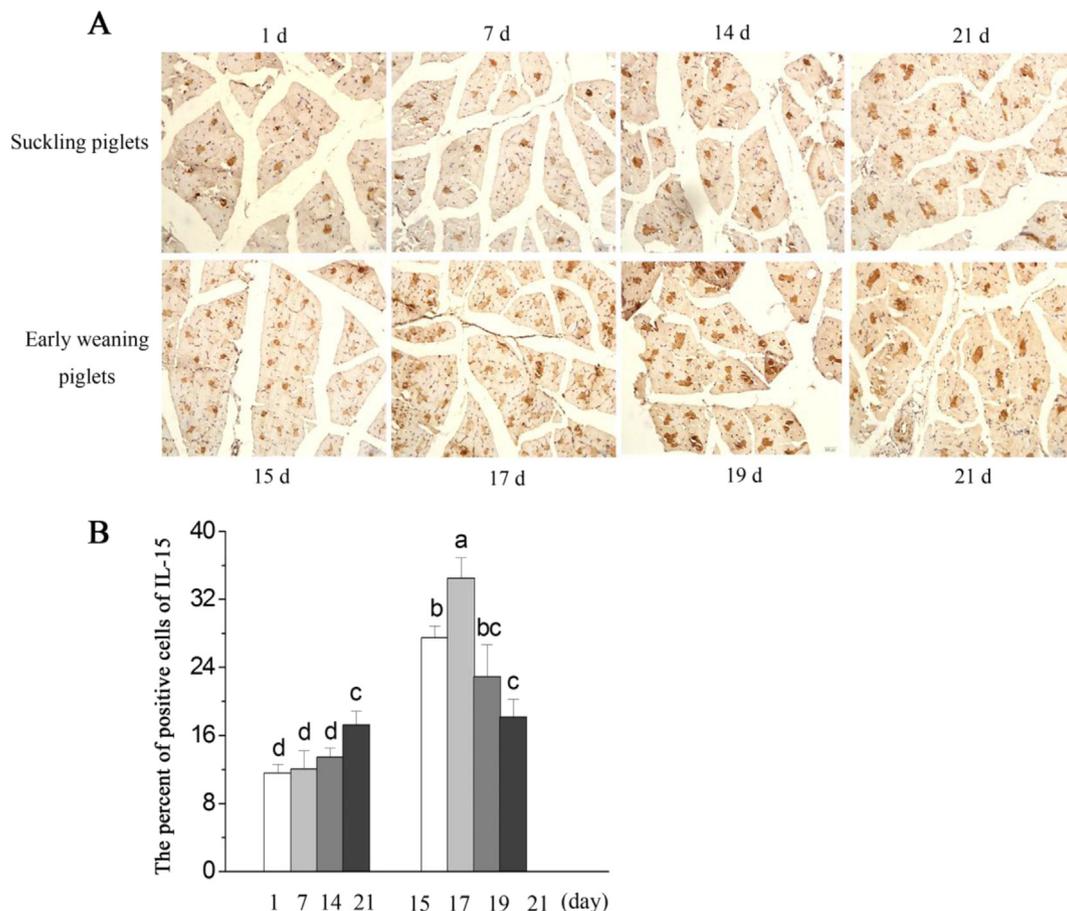


Fig. 1. Expression of IL-15 protein in suckling and weaning piglets. (A) IL-15 was evaluated by an immunohistochemical streptavidin-peroxidase (SP) method. (B) The percent of positive cells of IL-15 protein in muscle tissues tested by the immunohistochemical SP method are shown ($\times 400$). Data are expressed as means \pm SE ($n = 8$).

piglets at day 14 of age. Surprisingly, their body weights at day 21 of age increased significantly and were as many as those of suckling piglets at day 21 of age. These results suggest that inflammation in muscle may be caused by early weaning stress, resulting in no weight gain. The increased expression of IL-15 at mRNA and protein levels may be a mechanism whereby the piglets were able to counteract this weaning stress. However, at day 7 after weaning, early weaning stress was markedly suppressed with a marked increase in body weight and a significant decrease in expression levels of IL-15 mRNA and protein. However, the present study had some limitations, because feed intake of early weaning piglets can also influence their final body weights. Despite IL-15 mRNA and protein expression tended to increase in early weaning piglets, there was no such trend in suckling piglets.

The neonatal period of piglets is characterized by rapid gain in skeletal muscle mass, accompanied by a marked elevation of protein synthesis and lipid accumulation (Davis et al., 2000). As mentioned earlier, IL-15 released from skeletal muscle may directly influence adipose tissue metabolism (such as inhibiting lipid accumulation) through a muscle-to-fat endocrine pathway (Quinn, 2008; Li et al., 2014). Based on these observations, we hypothesized that serum IL-15 levels in neonatal period may be on the decrease over time, whereas IL-15 levels in skeletal muscle may be on the increase. To test the hypothesis, we assessed the development of serum IL-15 levels in suckling piglets and in weaning piglets. As expected, serum IL-15 levels in neonatal period significantly decreased over time in suckling piglets. Moreover, its trend of change was opposite to that of IL-15 mRNA in muscle. Our results are in agreement with other findings (Davis et al., 2000; Li et al., 2014), indicating that IL-15 released from skeletal muscle gradually diminishes to a point where it cannot meet the development requirements of muscle protein synthesis. In early weaning piglets, serum IL-15 levels were also on the decrease over time. It should be pointed out that serum IL-15 levels in early weaning piglets at day 15 of age were higher than those in suckling piglets at day 14 of age. So were IL-15 gene and protein expression levels. These results indicate that early weaning stress significantly increases IL-15 expression at mRNA, protein and secretion levels. However, in early weaning piglets at day 17 of age, serum IL-15 levels dropped significantly to the levels as in suckling piglets at day 14 of age. Additionally, their trend of change in serum IL-15 levels was opposite to that of IL-15 mRNA and protein expression levels. Collectively, these results suggest that early weaning stress markedly affects circulating IL-15 concentrations, and IL-15 was mainly expressed in skeletal muscle instead of being secreted into blood in order to constantly promote body growth.

As discussed above, IL-15 mRNA and protein is mainly expressed in skeletal muscle, and IL-15 expression levels may be related to inflammatory states resulting from early weaning stress. However, little information exists on the modulation of IL-15 expression and secretion in muscle tissue of suckling and weaning piglets. Previous studies have almost exclusively used human and rat models. They show that muscle IL-15 expression, at least at the mRNA level, is mediated by muscle activity and advanced age (Ostrowski et al., 1998; Pattison et al., 2003; Nieman et al., 2004; Riechman et al., 2004; Rinnov et al., 2014; Pistilli et al., 2007). Several studies also show that hormonal (Lambert et al., 2004), nutritional (Sun and Zemel, 2007) and inflammatory factors may influence muscle IL-15 mRNA transcription and circulating IL-15 protein levels. For example, stimulation of several inflammatory mediators (such as TNF- α) upregulates intracellular and secreted IL-15 levels (Stegall and Krolick, 2000; Sugiura et al., 2002). Our results show that early weaning may influence myokine IL-15 expression at each level, that is, mRNA, protein, and secretion.

5. Conclusion

Results from our study indicate that in response to early weaning stress, IL-15 mRNA and protein expression increases in early weaning piglets initially. It drops soon after with a concomitant increase in body weight gain and a decrease in IL-15 expression. Additionally, the trend of change in serum IL-15 levels in both groups of piglets was opposite to that of IL-15 mRNA and protein expression levels. Our findings suggest that early weaning stress significantly increases IL-15 expression at mRNA, protein and secretion levels. Furthermore, IL-15 is mainly expressed in skeletal muscle rather than being secreted into blood in order to constantly promote body growth. Unexpectedly, in suckling piglets, increased expression levels of IL-15 mRNA did not yield corresponding increase of its protein expression levels. Therefore, we suspect that IL-15 may play an important role in counteracting the effects of early weaning stress. Clearly, further research is required to test whether administration of IL-15 to early weaning piglets may counteract weaning stress of piglets.

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