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Expression of *IGFBP-3* and *IGFBP-5* genes in muscles of pigs representing five different breeds*

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ABSTRACT

Insuline like growth factors binding proteins (IGFBP 1-6) modulate action of insuline like growth factors (IGFs) - hormones engaged in the regulation of muscle growth of mammalian organism. The aim of our study was to analyse changes in the expression level of *IGFBP-3* and *IGFBP-5* genes in muscles during development of pigs (between 60 and 210 days of age) and to evaluate if there are differences in the expression level of these genes between groups of pigs representing different breeds. We did not observe a significant changes in *IGFBP-5* mRNA level during postnatal development of pigs. However, *IGFBP-3* was shown overexpressed at the early developmental stages, when compared to late developmental stages.

The intra-breed analyses revealed that *IGFBP-5* mRNA level was significantly lower in Duroc pigs and tract of *IGFBP-3* was significantly higher in Pietrain pigs than in the other investigated breeds: Puławska, Polish Large White and Polish Landrace.

KEY WORDS: pigs, *IGFBP-3, IGFBP-5*, somatothropic axis, expression, Real Time PCR

INTRODUCTION

Somatothrophic axis is a system of hormones, receptors and binding proteins, which control growth and development. The key element in mammals is growth

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hormone (GH) - well known stimulator of anabolic processes. GH acts also through insuline growth factors (IGFs). There are two IGFs - IGF1 and IGF2, and both of them may act in the endocrine and auto/paracrine manner. It was shown that IGFs can stimulate differentiation and proliferation of myogenic cells (Florini et al., 1986). What is more, a causative mutation in a non-coding fragment of *IGF2* gene (IGF2 SNP G3072A), affecting gene expression, weight of muscle and fat deposition, has been identified in pigs (Van Laere et al., 2003). The role of insulin like growth factors binding proteins (IGFBPs) is to modulate IGFs action through prolonging IGFs half-life, transporting IGFs to specific cells and tissues and regulating their access to IGF1 receptor. There are six IGFBPs. The predominant *IGFBP* in muscles is *IGFBP-5* (James et al., 1993), whereas in blood plasma - *IGFBP-3*. Recently, full-length cDNA, DNA sequence, polymorphisms and expression profile of another member of IGF axis - insulin-like growth factor-binding protein acid-labile subunit (*IGFALS*) - has been characterized (Li et al., 2007). Nevertheless, many studies indicate that IGFBPs may act in IGFs independent manner (Cobb et al., 2004). Kamanga-Sollo (2005) has shown that *IGFBP-3* and *IGFBP-5* mediate TGF-beta and myostatin-induced suppression of proliferation in porcine embryonic myogenic cell cultures. Transgenic mice overexpressing IGFBP-3 demonstrated significantly reduced birth weight and postnatal growth (Modric et al., 2001). In IGFBP-5-overexpressing mice increased neonatal mortality, reduced female fertility, whole-body growth and muscle development inhibition were observed (Salih et al., 2004). Moreover, Tilley (2006) revealed that expression of *IGFBP-3* and *IGFBP-5* is altered in growth retarded foetal pigs. These data suggest that *IGFBP-3* and *IGFBP-5* may potentially affect meatiness of farm animals in IGF-dependent or independent manner.

Several studies demonstrated associations between IGFBP-3 plasma concentration and growth, voluntary feed intake, and gain: feed ratio in pigs (Owens et al., 1999; Saleri et al., 2001). Recently, Wang (2009) has described effect of polymorphism in intron 2 (the combined mutations of G897T-G903A-C911T) of *IGFBP-3* on backfat thickness and meat colour in Chinese population of pigs. Although, the knowledge of function of IGFBPs in mammalian organism is increasing, effects of IGFBPs on muscle of farm animals is unknown. Therefore, the aim of the present study was to analyse expression level of *IGFBP-3* and *IGFBP-5* in muscles during development of pigs (between 60 and 210 days) and to evaluate if there are differences in the expression level of these genes between groups of pigs representing five breeds, selected for different traits. Comparison of gene expression in muscle tissue of pigs differing in muscularity has been performed previously (Stachowiak et al., 2010). Authors compared expression of *ADIPOR1* gene in the same Polish pig breeds as it was used in our experiment, expecting differences in expression level between breeds displaying different muscularity.

MATERIAL AND METHODS

The expression of *IGFBP-3* and *IGFBP-5* genes was analysed in two muscles: *M. longissimus dorsi* and *M. semimembranosus* of 177 sows from 5 breeds: Duroc, Pietrain, Puławska, Polish Large White (PLW) and Polish Landrace (PL). Animals were kept in Pilot Plant of the National Research Institute of Animal Production in Pawłowice under the same housing and feeding conditions. Animals of each breed were divided into 6 age groups (5-6 sows per group), according to the day of slaughter, 60-, 90-, 120-, 150-, 180- and 210-days old pigs. Animals were related - all sows within the breed had the same father (except the Pulawska breed - 3 fathers), and their mothers were sisters. Tissue fragments were collected immediately after slaughter and kept in liquid nitrogen during transportation. Animals of all breeds were stress resistant (RYR1 C/C) except Pietrain pigs, were C/T heteozygotes occured.

The total RNA was extracted once, using TRI-Reagent (Sigma) and Silent Crusher S homogenizer (Heidolph), according to the method described by Chomczynski (1993). The quantity of extracted RNA was estimated by BioPhotometer (Eppendorf), and its quality was evaluated by gel electrophoresis.

The RNA (1 µg) was reverse transcribed into cDNA at 37ºC using High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems), according to manufacturer's protocol.

Primers and probes for *IGFBP-3* and *GAPDH* genes were described previously (Johnson et al., 2002; Van Laere et al., 2003), whereas primers for *IGFBP-5* were designed with Primer Express software (Applied Biosystems) (Table 1).

Relative Quantification of the expression was performed on 7500 Real-Time PCR System using labeled TaqMan® Tamra probes and TaqMan® Universal PCR Master Mix with UNG AmpErase (Applied Biosystems). Reactions, in a total volume of 50 µl, were performed in duplicate and according to the TaqMan® Universal PCR Master Mix protocol. The protocol included two initial steps: 50ºC for 2 min (UNG incubation) and 95ºC for 10 min (AmpliTaq Gold activation) and 40 cycles of 95ºC for 15 s (denaturation) and 1 min at 60ºC (annealing/extending). *GAPDH* was used as an endogenous control. The results were analysed using Sequence Detection System software v. 2.0 (Applied Biosysytems).

For statistical analysis results from two muscles were averaged. Preliminary statistical analysis included comparison of mRNA level between five different breeds without distinguishing developmental stages and comparison of mRNA level between six different developmental stages (60-210 days of age) without distinguishing breeds. Further, we compared mRNA level between two developmental stages - early (60-120 days of age) and late (150-210 days of

Table 1. Primers and probes used in the study

Gene	Primers		TaqMan TAMRA Probes		References
GAPDH	F	ACCAGGGCTGCTTTTAACTCTG	FAM	ACCTCCACTACATGGTCTACATGTTCCAGTATGATT	Van Laere et al., 2003
	R	TGACAAGCTTCCCGTTCTCC			
IGFBP3	F	AGCACGGACACCCAGAACTT	FAM	TCCTCTGAGTCCAAGCGCGAGA	Johnson et al., 2002
	R	CGGCAAGGCCCGTATTC			
IGFBP5	F	AAAGAAGCTGACCCAGTCCAAGT	FAM	TGAGAACACCGCCCACCCTCGA	designed by authors
	R	CTCATCTCAGGCGCCAAGAT			

age) in each breed separately. We also compared mRNA expression between three breed groups (high muscularity group - H Pietrain breed, intermediate muscularity - I Large White, Landrace and Duroc and low muscularity - L Puławska). The comparison has been made in the early developmental stage and late developmental stage separately. All statistical analysis was performed using One Way Anova (Tukey Test; SAS Institute) .

RESULTS AND DISCUSSION

Preliminary statistical analysis showed that expression of *IGFBP-5* mRNA was approximately 5-7 fold lower in Duroc than in other breeds ($P<0.001$). The highest expression of *IGFBP-5* mRNA was observed in Large White. On the other hand, *IGFBP-3* mRNA was the most abundant in Pietrain breeds (almost 2-fold higher than in Duroc, $P<0.05$) (Figure 1). Comparison of breed groups (H, I L) revealed no statistical differences in *IGFBP-5* gene expression, while *IGFBP-3* mRNA was statistically more abundant in high muscularity group at the early developmental stages ($P<0.001$) (Figure 2).

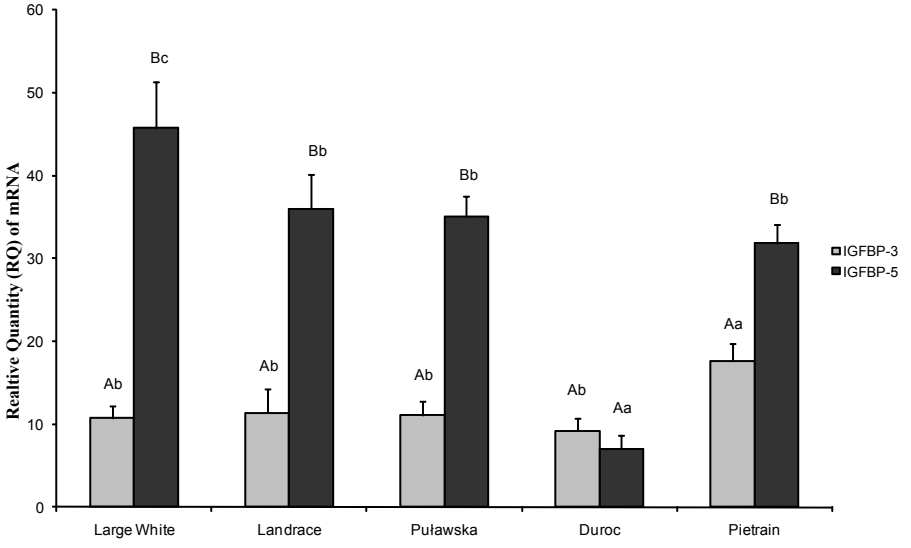

Figure 1. Relative quantity (RQ) of *IGFBP-3* and *IGFBP-5* transcripts in muscles of different breeds of pigs without distinguishing developmental stages. Different letters indicates significant differences; small letters - $P<0.05$, capital letters - $P<0.001$

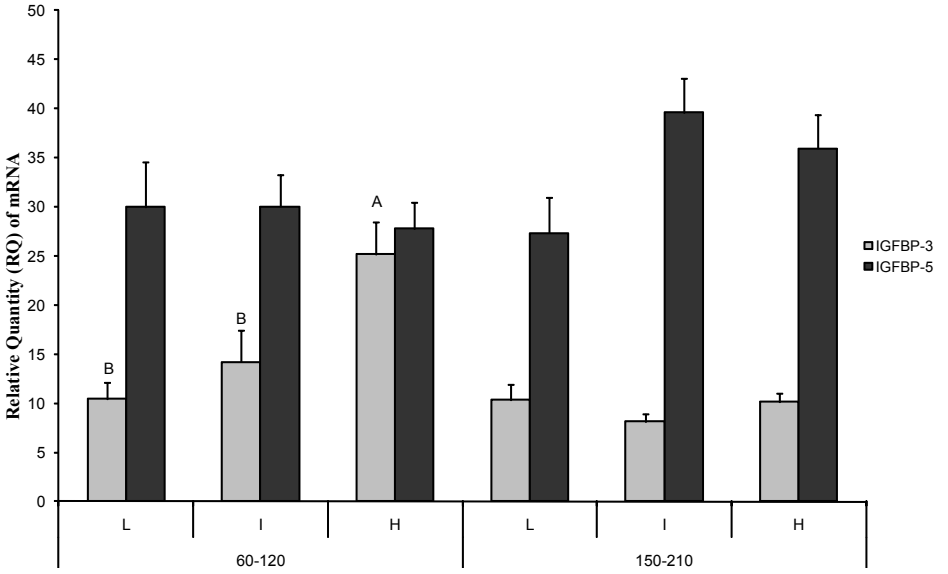

Figure 2. Relative quantity (RQ) of *IGFBP-3* and *IGFBP-5* transcripts in muscles of three breed groups (high muscularity group - H Pietrain breed, intermediate muscularity - I Large White, Landrace and Duroc and low muscularity - L Puławska) at the early developmental stage (60-120 days of age) and late developmental stage (150-210 days of age). Different letters indicates significant differences; small letters - $P<0.05$, capital letters - $P<0.001$

Hoeflich et al. (2004) investigated expression of insulin-like growth system genes in muscles of mice selected for lean body mass, protein amount and body weight. They demonstrated that the level of *IGFBP-3* mRNA is higher in muscles of mice with lower lean body mass, which is contradictory to our results - we observed the highest level of *IGFBP-3* mRNA in Pietrain breeds in the preliminary statistical analysis, when all developmental stages were combined. Moreover, when breed groups differing in muscularity were compared, *IGFBP-3* mRNA was the most abundant in the H group at 60 and 90 days of age, which points on function of *IGFBP-3* in the development of muscle mass. The reason for the discrepancy between our results and obtained by Hoeflich (2004) may be inter-species differences. Another possible explanation is that in Pietrain animals a few C/T heterozygotes in *RYR1* locus occured. Recently, a common molecular mechanism for *IGF2* and *RYR1* in regulation of muscle development has been proposed (Stinckens et al., 2007). If this is the case, a relationship between *RYR1* and another member of IGF axis cannot be excluded.

In the study performed by Hoeflich et al. (2004), lines of mice selected for lean body mass displayed significantly lower expression of *IGFBP-5* than lines selected for protein amount. There were no differences between mice of high lean body mass and low lean body mass as well as between mice of high protein amount and low protein amount. In our study, we observed significantly lower expression of *IGFBP-5* in Duroc breed than in all other breeds. However, analysis of breed groups at different developmental stages revealed no differences in *IGFBP-5* mRNA level between groups with different muscularity. This may suggest that *IGFBP-5* expression is not associated with meatiness, but with another phenotypic feature typical for Duroc breed. Recently, it has been proposed that the *IGFBP-5* gene was associated with the variation in meat quality, especially in pH value (Wang et al., 2010). In order to verify this hypothesis further studies on associations between *IGFBP-5* polymorphism, expression and meat quality traits are necessary.

Comparison of *IGFBP-5* mRNA level between two developmental stages - early (60-120 days of age) and late (150-210 days of age) in each breed separately revealed no statistically significant differences. Moreover, we did not observed any significant changes during development when all breeds were combined (Figures 3 and 4).

On the other hand, expression of *IGFBP-3* was upregulated at 120 days of age, when all breeds were combined ($P<0.05$). What is more, in Pietrain breed expression of *IGFBP-3* mRNA was 2.5-fold higher at early developmental stages, when compared to late developmental stages ($P<0.001$). Similar trend was observed in all other breeds except Duroc (Figures 3 and 4).

To date, ontogeny of *IGFBPs* transcript levels in pigs muscles *in vivo* was studied by Northern Blot analysis only (Peng et al., 1996; Gerrard et al., 1999). In adipose tissue developmental expression patterns of these genes has been evaluated by Real-Time PCR and microarray technology but no significant changes in expression of *IGFBP-5* mRNA between 90 and 210 days of age has been identified (Hausman et al., 2008).

The study by Gerrard (1999), performed on foetal (between 30-109 days post-coitum), neonatal (21 days) and adult (6 months) pigs showed that *IGFBP-5* mRNA abundance decreased prenatally. Authors noted the lowest expression in neonatal pigs, whereas in adult pigs mRNA increased but not significantly (Gerard et al., 1999). We also did not observe significant changes in *IGFBP-5* mRNA expression.

Expression of *IGFBP-3* was analysed in 90 and 110 days of gestation and at 1day, 3 week, 3 and 6 month of age in the skeletal muscle of pigs (Peng et al., 1996). Significant decrease of mRNA levels was observed with the advancing

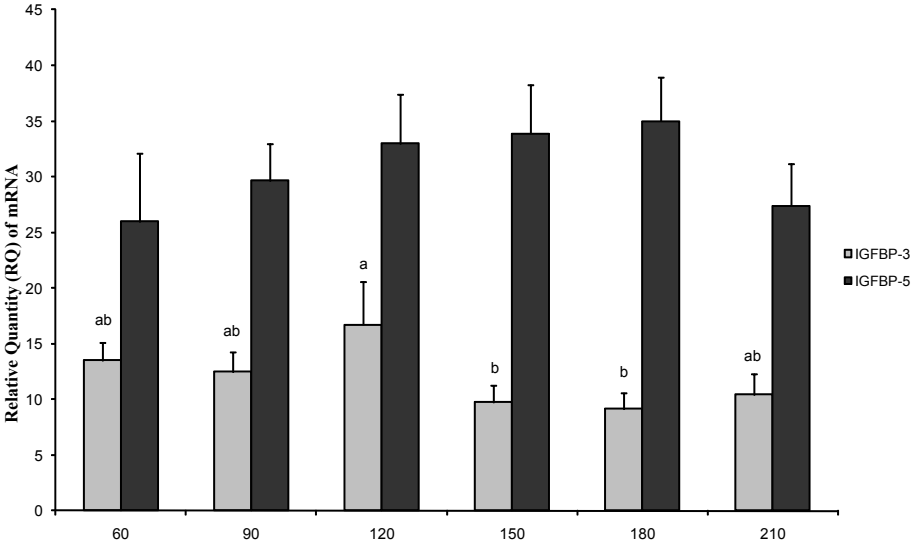

Figure 3. Relative quantity (RQ) of *IGFBP-3* and *IGFBP-5* transcripts in muscles during six different developmental stages without distinguishing breeds. Different letters indicates significant differences; small letters - P<0.05, capital letters - P<0.001

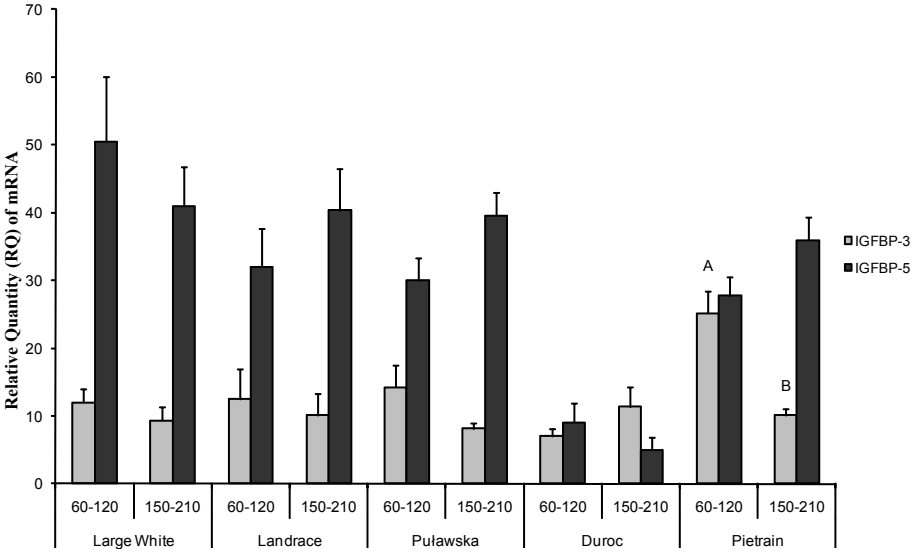

Figure 4. Relative quantity (RQ) of *IGFBP-3* and *IGFBP-5* transcripts in muscles of pigs at the two developmental stages - early (60-120 days of age) and late (150-210 days of age) in each breed separately. Different letters indicates significant differences; small letters - P<0.05, capital letters - P<0.001

age. Unfortunately, our results cannot be compared with the previous one as the studied developmental stages were different.

Developmental expression patterns of *IGFBP-3* mRNA in adipose tissue of two pig breeds were analysed by Guo (2008). Using Real-Time PCR, they established that *IGFBP-3* expression was the highest at 30 days, declined significantly at 60 days ($P<0.05$) and maintained steady, but a little fluctuant between ages in each breed to the age of 150 days. These findings generally correspond to our results for the muscle tissue.

Analysis of *IGFBP-5* mRNA expression in muscle tissue revealed no significant changes during development of pigs and *IGFBP-3* expression altered only slightly. These results show that expression of *IGFBP-3* and *5* mRNA is rather stable during postnatal development, which is in agreement with previous results for muscle and fat tissue.

On the other hand, expression of *IGFBP-5* was very clearly underexpressed in Duroc breed, when compared to other breeds and *IGFBP-3* was overexpressed in Pietrain breed at the early developmental stages. These may suggest that *IGFBP-3* and *5* genes function as regulators of muscle tissue formation. Further studies are needed in order to identify DNA polymorphism responsible for expression differences and to evaluate if the variation in *IGFBP-3* and *5* mRNA abundance in muscles has phenotypical consequences.

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