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Original Article

Evaluation of *PFKM*, *TFDP2*, and *HIP2* Gene Expression and Muscle Growth in Sheep

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Abstract

Genes involved in metabolic pathways contributing to hormone-induced muscle hypertrophy are possible markers that can be used to select for increased muscle growth. *Phosphofructokinase muscle-type*, *transcription factor dp-two*, and *huntingtin-interacting protein* are three candidate genes which are known to be involved in metabolic pathways controlling energy metabolism and the cell cycle. The objective of this study was to analyze the effect of testosterone on the mRNA abundance from these three genes in splenius and semitendinosus muscles in rams and wethers at four different age points. Analysis of gene expression showed no differences in mRNA abundance between semitendinosus and splenius muscles in both rams and wethers at four different age groups for the three genes analyzed. Further expression analysis is needed to determine the contribution of other candidate genes to controlling hypertrophy in sexually dimorphic muscles.

Key words: Gene expression, muscle growth, sheep, testosterone

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Introduction

Selection for increased muscle growth rate is a primary breeding objective of the lamb meat industry. It has been well established that intact males exhibit superior growth rate and muscling compared to females due to the presence of testosterone (Arnold et al. 1997), but the molecular mechanisms contributing to this physiological response are not fully understood. Only certain muscles display increased growth rate in response to testosterone, and these are referred to as sexually dimorphic (Sauerwein and Meyer 1989). The onset of puberty and subsequent testosterone release, which occurs around 100 d of age in sheep, coincides with an increase in protein accretion rate and hypertrophy in sexually dimorphic muscles. Identifying genes that are differentially expressed in response to testosterone and growth state could lead to the identification of molecular markers contributing variation to muscle anabolism. In pigs, it was demonstrated that several genes known to be involved in muscle growth are down regulated in the absence of testosterone (Yao et al. 2009). Studies in sheep have also identified differential expression in several key genes related to muscle anabolism, including *insulin-like growth factor one* (*IGF-1*) and *androgen receptor* (*AR*) (Mateescu and Thonney 2005). However, muscle growth rate is a quantitative trait that is controlled by many genes and the environment. Additional genes contributing variation to muscle growth rate need to be identified to fully understand the physiological response to testosterone.

For this study, candidate genes were selected based on previously known involvement in energy metabolism. The genes selected were *Phosphofructokinase muscle-type* (*PFKM*), *transcription factor dp-two* (*TFDP2*), and *huntingtin-interacting protein* (*HIP2*). These three genes were previously shown to be differentially expressed in the longissimus dorsi of callipyge lambs (Fleming-Waddell et al. 2007) and are also known to be involved in the regulation of cell growth, cell division, and energy production. In this study, we analyzed the expression of *PFKM*, *TFDP2*, and *HIP2* in both sexually dimorphic and non-sexually dimorphic muscles, in the presence or absence of testosterone, across different age points.

Materials and Methods

The population for this project consisted of eighteen sets of twin lambs, with one individual from each set castrated at birth. Each set of twins was randomly assigned to one of four slaughter groups corresponding to 77, 102, 135, and 160 d of age. Slaughter ages were selected to give two time points before and after puberty, which occurs around 100 d in sheep. Semitendinosus (non-sexually dimorphic) and splenius (sexually dimorphic) muscles were previously collected and weighed (Mateescu and Thonney 2005) and samples were snap-frozen in liquid nitrogen at slaughter and stored at -80°C for RNA extraction.

Total RNA was isolated from muscle samples using a ToTALLY RNA extraction kit from Ambion Inc. (Austin, TX). A 100-mg sample of frozen muscle tissue was homogenized in 1 ml of denaturation solution. RNA was subsequently isolated using a two-step extraction with phenol:cholorform:IAA, followed by acid-phenol:chloroform. RNA was precipitated with isopropanol and washed with 70% ethanol following collection by centrifugation. Total RNA was rehydrated in resuspension solution at 65°C. RNA samples were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies) and were stored at -80°C.

First-strand complementary DNA (cDNA) was synthesized using a Quantitect Reverse Transcription kit from Qiagen (Germantown, MD). Purified RNA was briefly incubated at 42°C in gDNA wipeout buffer to remove genomic DNA contamination. Reverse transcription was carried out using a master mix prepared with Quantitect Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The reaction was incubated at 42°C for 20 min followed by inactivation at 95°C. First-strand cDNA was stored at -20°C.

A quantitative polymerase chain reaction (qPCR) was carried out to quantify the amount of *PFKM*, *TFDP2*, and *HIP2* mRNA transcript in the muscle samples. SYBR Green PCR master mix from Fermentas (Glen Burnie, MD) and 5 µM

primers were used to amplify each q-PCR product. Standard curves and primer efficiencies for each gene were generated using a 10-fold dilution series (1000 - 0.001 ng cDNA each) and threshold cycles (Ct) were plotted for regression analysis using the MY IQ software. Threshold cycle correlation coefficients above 0.99 across the standard curve dilutions were considered acceptable for RT-PCR analysis. Efficiency values were ≥ 95% for each of the four primers. The 50s ribosomal subunit L1 (RPLA) was used as a control gene for this experiment. Gene expression was quantified using the comparative threshold cycle (Ct) method. Delta Ct values for each muscle were defined as the Ct difference between the gene of interest and the internal control *RPLA*. Delta Ct values were analyzed for differences using the General Linear Model procedure of SAS (Cary, NC). The model statement included muscle (splenius and semitendinosus), age class (77, 105, 133, and 161 d), sex (ram and wether), and their interaction as fixed effects. Comparisons between class statements were made using the results of the PDIFF option. For all tests, a *P*-value of <0.05 was considered to be statistically significant.

Table 1: Delta Ct values for each muscle calculated across four time points (77, 102, 135, 160 d) in rams and wethers. Delta Ct values were calculated by taking the difference between the Ct value for each gene and the Ct value for the control (*RPLA*) in each muscle sample. There was no significant differential expression for the effect of age, muscle type, or their interaction

			Gene		
Sex	**Age, d**	**Muscle**	***PFKM*[1]**	***TFDP2*[2]**	***HIP2*[3]**
Ram			ΔCt (adjusted to *RPLA*)		
	77	*Splenius*	-1.43	-1.91	0.90
		Semitendinosus	0.72	0.87	0.90
	102	*Splenius*	-1.39	-0.33	-0.63
		Semitendinosus	-2.35	-1.50	-3.18
	135	*Splenius*	-2.86	-0.46	-0.23
		Semitendinosus	-2.69	-0.63	-2.65
	160	*Splenius*	-1.26	-1.08	-0.25
		Semitendinosus	-2.29	-1.49	-1.09
Wether					
	77	*Splenius*	0.16	-0.50	-0.57
		Semitendinosus	-1.05	-0.23	-1.77
	102	*Splenius*	-1.64	-2.33	-2.23
		Semitendinosus	-2.13	-1.34	0.26
	135	*Splenius*	-0.98	-0.19	0.18
		Semitendinosus	-1.57	-1.49	-0.66
	160	*Splenius*	-1.63	-1.55	-0.86
		Semitendinosus	-2.39	-1.80	-0.19
P-value (interaction)			0.21	0.07	0.08

[1]PFKM=phospofructokinase muscle-type
[2]TFDP2=Transcription factor dp-two
[3]HIP2A=huntingtin interacting protein

Results and Discussion

No statistically significant differences in gene expression were found for *TFDP2*, *PFKM*, or *HIP2* due to age, muscle, or sex, and the age x muscle x sex interactions (Table 1). All three genes were differentially expressed in relation to muscle hypertrophy (Fleming-Waddell et al. 2007) in a microarray analysis of gene expression between normal and callipyge lambs, but expression analysis in the present study did not validate these findings. The lack of differential expression indicates that these genes are not likely significant factors in testosterone mediated muscle hypertrophy. The lack of differential expression between sexually dimorphic muscles in both the presence and absence of testosterone provides evidence that testosterone is unlikely to regulate the expression of these genes. Alternative candidate genes identified in the microarray analysis by Flemming-Waddel et al. (2007) are likely regulating this metabolic process. Further investigation of the metabolic pathways involved in testosterone-mediated muscle hypertrophy is necessary to identify the genes which could be used as markers for increased muscle anabolism in sheep.

Conclusion

The three genes *Phosphofructokinase muscle-type* (*PFKM*), *transcription factor dp-two* (*TFDP2*), and *huntingtin-interacting protein* (*HIP2*) do not appear to be differentially regulated in response to testosterone during muscle hypertrophy in sheep. The lack of differential expression of these three genes indicates there is a need to investigate alternative genes involved in muscle metabolism. The identification of differentially expressed genes involved in muscle metabolism is important for the development of marker assisted selection programs to integrate genomic information into modern sheep production systems. Further analysis of the gene networks involved in muscle growth is needed to identify regulators of muscle hypertrophy in sheep to increase the efficiency of selection and ultimately production.

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