OVINE DOUBLE-MUSCLING: MUSCLE STRUCTURE & PROTEIN PATTERN

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Introduction

To improve carcass muscularity, breeds with hypertrophied muscle can be used. Belgian strains of Texel sheep harbor a QTL (Quantitative Trait Locus) with considerable effect on muscle development[(1,2)]. This effect does not seem to be associated with sensory qualities degradation. Improvement of muscularity is often accompanied, in many species, by a decrease in meat sensory quality in relation to changes in muscle contractile and metabolic properties and to structural modifications as fibre area[(3)].

Proteins are the main constituents of the muscular cell. They determine structural and functional characteristics of the cell and to a certain extent the sensorial quality of meat. 2-D electrophoresis (2-DE) is of a great interest since it allows the concomitant separation of hundred or even thousand of proteins. Thus, 2-DE is a powerful tool for studying protein expression in relation with different factors.

Until now the study of sarcoplasmic proteins, constituting approximately 30% of total proteins was little developed. However this fraction contains the majority of metabolism proteins and of signal transduction pathways.

The current study aims at determining the effect of the Belgian Texel QTL on structural traits and protein composition of two leg muscles.

The comparative study of two muscle types, hyper- vs normo-developed, originating from double-muscled vs not double-muscled haplotype gives us the ability to identify sarcoplasmic proteins implicated in muscular development.

Objectives

The current study aims at determining the effect of the Belgian Texel QTL on structural traits and protein composition of two leg muscles.

Methodology

Animals and muscular samples

We used crossbred lambs originating from F2 crossing between Romanov ewes and double-muscled Belgian Texel rams. Animals were genotyped on "Texel" Locus for the QTL associated to the double-muscled phenotype(2). 15 homozygote Texel (TT) and 17 homozygote Romanov (RR) lambs were selected for analysis. Animals were slaughtered at fixed weight of 33 kg for females and 39 kg for males. After slaughter, carcasses were dressed according to commercial practices and were weighted. Carcasses were split down the vertebral column in the sagittal plane and through the pubic symphysis. Hind legs were split down transversally between the last lumbar vertebra. The left hind leg was weighted. Semimembranosus (Sm) and Vastus medialis (Vm) muscles were removed and weighted. Sm was cut transversally to fibre direction in midbelly muscle region. The transversal area of Sm was recorded. The two muscles were sampled in the middle part for histochemical and 2D-electrophoresis analyses. Muscle samples were taken from Sm and Vm within 30 min after slaughter, frozen in liquid nitrogen. Then samples for electrophoresis were reduced to a fine powder under liquid nitrogen using a mortar and a mechanical pestle and stored at –80°C until protein extraction was performed. Samples for histology were kept at –80°C.

Histochemistry

Transverse cryo-sections (10 µm) from Sm (15 TT and 17 RR) and Vm (4 TT and 9 RR) muscle samples were incubated with amylase and stained with PAS[4,5]. According to this method, basal lamina were stained, showing muscle fibre and capillaries contours. Height light microscope images per section, corresponding to the total area of 0.5 mm^2, were analysed. Numbers of fibres and capillaries were recorded. The transversal section of fibres was measured by computer image analysis. In Sm muscle, an extrapolated total number of capillaries and fibres was calculated using the muscle transversal section area. GLM procedure was applied for mean comparison between the two haplotypes (TT, RR). Means were adjusted to carcass weight. The sex effect was removed.

Sarcoplasmic protein extraction

2-DE gels were performed on 5 TT and 5 RR Sm muscles and 4 TT and 4 RR Vm muscles. Gels were produced in triplicate. The sarcoplasmic fraction was obtained using a method inspired from the subcellular fractionation of Pietrzak[6]. The extraction buffer consisted of 50mM KCl, 4mM $MgCl_2$, 20mM Tris, 2mM EDTA, 1% (w/v) DTT and 5mM Pefabloc at pH7. 150mg of muscle were added to 1.5mL of extraction buffer in an Eppendorf containing a glass bead. Homogenisation was performed in Retsch MM2 agitator (Retsch, Haan, Germany) for 1h at 4°C. Extracts were centrifuged at 10,000g for 15min at 6°C and the supernatant was collected. Samples were frozen in liquid nitrogen and stored at –80°C.

2-dimensional electrophoresis

Immobilised pH gradient (IPG) isoelectric focusing (IEF) was carried out in a Protean IEF cell (Bio-Rad), using Bio-Rad ReadyStrip, 17cm, pH5–8. 110 or 300µg of protein were loaded onto the strips for analytical or preparative gels, respectively. Proteins were loaded by inclusion of an adequate volume of extract in a buffer consisting of 7M urea, 2M thiourea, 2% (w/v) CHAPS, 5mM Pefabloc, 0.2% (w/v) DTT and 0.2% carrier ampholytes. Strips were rehydrated overnight. For the subsequent IEF, voltage was increased gradually to 8,000V until a total of 80,000Vh. Strips were immediately frozen and stored at -20°C until further use. Prior to SDS–PAGE, strips were equilibrated for 15min followed by 20min in a solution of 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 50mM Tris, supplemented successively with 1% (w/v) DTT or 2.5% (w/v) iodoacetamide and bromophenol blue as a dye. SDS–PAGE was performed in a protean IIxi cell (Bio- Rad) on 12% polyacrylamide gels at 15mA per gel, until the dye track reached the end of the gels. Analytical and preparative gels were silver stained following the protocol of Yan *et al.*[(7)].

Image analysis

Gels images were acquired through a GS-800 densitometer and analysed using the PDQuest software (Bio-Rad). After automated detection and matching, highly saturated or ill-defined spots were manually removed and matching across gels was inspected and corrected when necessary. Intensity was expressed in ppm and data were analysed using the software SASx8.1 and a One-Way ANOVA test was used to study genotype effect on protein expression.

Protein isolation and identification by MS

Spots were excised from preparative gels using pipette tips. Gel pieces were placed into a 1.5mL Eppendorf and destained for 10min with a solution containing 30mM KFe and 100mM Sodium thiosulfate, then the gel pieces were washed three times in milliQ Water for 10min. The trypsic digestion and the desalting were done according to manufacturer's protocol (Montage In Gel DigestZP Kit, Millipore).
Resulting peptides mixtures were loaded directly onto the MALDI target. The matrix solution (5mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid) was added immediately and allowed to dry at room temperature. A Voyager DE-Pro model of MALDI-ToF mass spectrometer (Perseptive BioSystems, Farmingham, MA, USA) was used in positive-ion reflector mode for peptide mass fingerprinting. External calibration was performed with a standard peptide solution (Proteomix, LaserBio Labs, Sophia-Antipolis, France). Internal calibration was performed using peptides resulting from auto-digestion of porcine trypsin. Monoisotopic peptide masses were assigned and used from NCBI database searches with the "Mascot" and "Profound" softwares (http://www.matrixscience.com and http://prowl. rockefeller.edu).

Results & Discussion

Muscle structure

Results of dissection and histochemistry are presented in table 1. The Sm muscle was heavier and thicker in TT haplotype than in RR. Conversely, the Vm weight was not different between the two haplotypes. These observations showed that hypertrophy was differentially expressed according to the muscle. This is in accordance with previous research on double-muscled cattle[8]. In both muscles, fibre area was not different between haplotypes. The extrapolated total number of fibres of Sm was higher in TT than in RR. It can be concluded that the Sm hypertrophy is not due to fibre hypertrophy but to hyperplasia. These characteristics are comparable to those described in double-muscled cattle presenting a mutation on the gene coding for myostatin[8,9]. The Belgian Texel QTL for muscle hypertrophy has also been identified in the chromosomal region of the myostatin encoding gene[1]. However, sequencing of the gene did not reveal mutation. The ratio of capillaries to fibre number was lower in Sm muscles of TT lambs. This ratio was not different among genotypes in Vm. These results mean that the Sm hypertrophy is not associated to a proportional increase of blood supply.

2-DE and mass spectrometry

On over 800 proteic spots detected on gel, we have determined 102 spots which varied statistically significantly between the 2 genotypes on Sm. Among these 102 spots, 87 were over expressed in TT genotype. 35 of the 102 spots were picked off and analysed for identification by MALDI-ToF mass spectrometry, and 19 were identified (Table 2). In the Vm 74 spots were defined as varying with genotype. Among them, 29 were over expressed in TT genotype. 16 of the 74 spots were analysed by mass spectrometry and we successed to identify 5 proteins (Table 2). Three of the identified proteins were common to both muscles.

In Sm, some of the identified spots corresponded to same proteins. Among those redundant proteins, Creatine kinase (CK) was observed at different molecular weights (Mw), only one spot corresponded to the theoretical Mw. The five others CK spots were probably fragments of the whole protein. According to their observed Mw, the Glycogen myophosphorylase (GP) spots also corresponded to fragments. Due to its high Mw and basic pI, the whole protein can not be seperated on our 2-D gels. Moreover there is an exposed serine in the 3D structure of ovine GP that could explain a specific clivage creating a 35 kDa fragment. Concerning Pyruvate Kinase (PK), two spots were also evidenced at a lower Mw than their theoretical weight.

In a previous study, protein fragments were also observed very early after slaughter[10]. We may suppose that the presence of fragments was due to early proteolysis. In this case, we hypothesize that the fragments expression is more a reflect of initial rate of entire protein than a mark of more acute proteolysis in hypertrophied Sm.

The two other spots of PK were positioned at their theoretical Mw. They probably corresponded to the entire protein, but they varied by their pI. It is also the case for the enolase proteins. These different pI locations of a same protein onto the gel map could correspond to different isoforms due to post translational modifications.

All of these proteins as well as the lactate dehydrogenase were over expressed in the Sm of TT and are enzymes related to glycolytic metabolism. Enolase and PK are enzymes of distal glycolysis, as for CK it is an enzyme essential to the refilling of cellular ATP stock, in fast glycolytic muscles it is essential to ensure fast spatio-temporal energy buffering. Moreover, it is noticeable that CK Knock Out mice exhibit a marked decrease in skeletal muscle weights[11] which support the link between CK over expression and Sm hypertrophy in TT.

This over expression was in accordance with a previous study showing a higher level of the fast isoform, associated to glycolytic metabolism, of the myosin heavy chain in Sm muscle of the TT genotype[2]. It also can be related to the lower level of capillary supply. These particularities suggested an evolution of this hypertrophied muscle metabolism to a more glycolytic pattern.

Two spots were chaperone proteins: the small heat shock protein 27kDa (HSP-27) and the Glutathione S-transferase Pi (GST-Pi). They were over expressed in Sm of TT genotype. HSP-27 has been reported to play a central role in the structural and functional organization of the three-dimensional intermediate filament and the actin microfilament system[12]. Moreover, HSP-27 has been found to be over expressed in hypertrophied cultured cells[13]. HSP-27 exist in many isoforms[14] and Kim[15] reports that one isoform is more expressed in glycolytic muscle which agrees with the switch in fiber's type towards a more glycolytic one in TT animals muscles[2]. Thompson proposed that HSP-27 could be involved in long term skeletal muscle adaptation to hypertrophy[16].

The GST-Pi is involved in the mechanisms of cellular detoxification and cellular resistance to oxidative stress[17]; a lot of oxidative and cytotoxic molecules are produced during muscle metabolism so GST-Pi over expression agrees with higher metabolism in Sm of TT.

The transferrin was also over expressed in TT genotype. It is a protein usually synthesised in liver which binds iron and delivers it to cells. It has been suggested that it could be synthesised by growing cells when vasculature is insufficient, and that transferrin enhances action of neural tissue on growth and development of cultured skeletal myoblasts. The Sm from TT genotype has a less developed vasculature than Sm from RR genotype (Table 1) and given its increased metabolism, its iron needs are elevated so local expression of transferrin could be augmented to supply this need.

As for the α1-antitrypsin, it is a member of the serine protease inhibitor protein family (serpins) and is the principal serum inhibitor of proteolytic enzymes such as elastase, serpins are also able to inhibit cystein proteinase like cathepsin K, L and S in muscle[18]. Here we show that α1-antitrypsin protein was under expressed in both muscles of the TT genotype. So in this study α1-antitrypsin seems to be a marker of TT genotype.

Most of varying proteins in the Vm were under expressed in TT genotype (Table 2), where hypertrophy seemed to be repressed. Markers of glycolytic metabolism were under expressed like the triose phosphate isomerase and GP.

The only identified over expressed spot was the Antiquitin, a member of aldehyde dehydrogenase superfamily, which is supposed to be involved in osmotic stress regulation[19].

As for GST-Pi and GP, conversely to their expressions in Sm, they were under expressed in Vm of TT genotype. So we found them more expressed in the TT genotype

in the most developped muscle (Sm). Moreover GST-Pi over expression is associated to cancerous proliferation[20], i.e. an uncontrolled hyperplasia. So GST-Pi could play a role in the maintenance of the hyperplasia in Sm muscle of TT animals.

Conclusions

To conclude we have shown that some proteins can be linked to a TT genotype, but it is impossible to generalize results from one muscle to others. With regard to the GP and GST-Pi, their expressions seem to be linked to hypertrophy.
Additional studies are needed for a better comprehension of muscular development mechanisms and the maintenance of muscularity.

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Tables and Figures

Table 1. Means of dissection and histochemical measurements and genetic effects

Measurements	Mean	SD	QTL effect	
			Test[a]	TT – RR[b]
Leg weight. g	2312.5	115.6	*	89.64
Muscle weight. g				
Sm	230.4	23.7	***	43.67
Vm	39.8	6.8	NS	-1.3
Transversal area of Sm. cm^2	25.4	4.7	***	2.93
Fiber transversal area. μm^2				
Sm	1756	507.1	NS	-232.95
Vm	3123	649.8	NS	-31.2
Capillary/Fiber Number				
Sm	0.81	0.17	*	-0.17
Vm	1.06	0.12	NS	-0.035
Fiber total number in Sm	1 593 664	54 9318	*	525 334
Capillary total number in Sm	1 235 259	360 499	NS	186290

[a]NS = P > 0.10 ; *P < 0.05 ; ***P < 0.001

[b]A positive value for TT – RR corresponds to an additive increase due to the Belgian Texel QTL. T = Texel. R = Romanov.

Table 2. Mass spectrometry identified proteins and their expression levels in TT genotype vs RR genotype.

Semimembranosus			
Acc N°	Protein ID	Expression TT/RR	p
gi|6013379	**Glutathione S-transferase Pi** (*Capra hircus*)	2.5	<0.001
gi|61553385	Heat Shock Protein 27 kDa (*Bos taurus*)	2.4	<0.01
gi|57163939	**Glycogen myophosphorylase** (*Ovis aries*) Fgt	3.9	<0.0001
gi|57163939	**Glycogen myophosphorylase** (*Ovis aries*) Fgt	1.9	<0.0001
gi|27806645	Enolase (*Bos taurus*)	2.5	<0.01
gi|27806645	Enolase (*Bos taurus*)	2.4	<0.01
gi|33286422	Pyruvate kinase (*Homo sapiens*)	2	<0.001
gi|33286422	Pyruvate kinase (*Homo sapiens*)	1.9	<0.01
gi|478822	Pyruvate kinase (*Homo sapiens*) Fgt	2.2	<0.001
gi|478822	Pyruvate kinase (*Homo sapiens*) Fgt	2	<0.001
gi|6729828	Creatine kinase (*Oryctolagus cuniculus*) Fgt	1.9	<0.01
gi|6729828	Creatine kinase (*Oryctolagus cuniculus*) Fgt	1.9	<0.01
gi|6729828	Creatine kinase (*Oryctolagus cuniculus*) Fgt	1.9	<0.01
gi|4838363	Creatine kinase (*Bos taurus*) Fgt	2.8	<0.05
gi|4838363	Creatine kinase (*Bos taurus*) Fgt	2.3	<0.05
gi|4838363	Creatine kinase (*Bos taurus*)	2	<0.001
gi|59858383	Lactate dehydrogenase (*Bos taurus*)	2	<0.01
gi|29135265	Transferrin (*Bos taurus*)	3.6	<0.001
gi|57526646	**α1-antitrypsin** (*Ovis aries*)	0.4	<0.0001
Vastus medialis			
Acc N°	Protein ID	Expression TT/RR	p
gi|25108887	Antiquitin (*Homo sapiens*)	2	<0.05
gi|59858493	Triose phosphate isomerase (*Bos taurus*)	0.3	<0.05
gi|57526646	**α1-antitrypsin** (*Ovis aries*)	0.5	<0.05
gi|57163939	**Glycogen myophosphorylase** (*Ovis aries*) Fgt	0.4	<0.05
gi|6013379	**Glutathione S-transferase Pi** (*Capra hircus*)	0.5	<0.05

Fgt: fragment of protein

In bold: proteins identified in Sm and also in Vm muscle