The RYR1 g.1843C>T mutation is associated with the effect of the IGF2 intron3-g.3072G>A mutation on muscle hypertrophy

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Summary

Muscle growth is a complex phenomenon regulated by many factors, whereby net growth results from the combined action of synthesis and turnover. In pigs, two quantitative trait nucleotides (QTN) are known to have an important influence on muscle growth and fat deposition: one QTN is located in the ryanodine receptor 1 (RYR1) gene (RYR1 g.1843C>T) and the other, a paternally expressed QTN, is in the insulin-like growth factor 2 (IGF2) gene (IGF2 intron 3-g.3072G>A). The mutation in IGF2 abrogates in vitro interaction with a repressor, which leads to a threefold increase of IGF2 expression in post-natal muscle. The family of the calpains, a family of Ca\(^{2+}\)-sensitive muscle endopeptidases, and their specific inhibitor calpastatin play an important role in post-natal protein degradation, also influencing muscle and carcass traits. This study investigated the possible interactions between the genotypes of the RYR1 and IGF2 QTN on IGF2 expression. Samples were taken from several muscles and from pigs at several ages, and messenger RNA expression levels were measured using a real-time quantification assay. IGF2 expression in m. longissimus dorsi of animals with mutations in both IGF2 and RYR1 was significantly lower than in animals that inherited the IGF2 mutation but were homozygous wildtype for RYR1.

Keywords insulin-like growth factor 2, muscle, pig, quantitative trait locus, ryanodine receptor 1.

Introduction

Insulin-like growth factor 2 is a peptide hormone that stimulates responses in skeletal muscle, including effects on carbohydrate and fat metabolism, protein turnover, growth and differentiation in an autocrine/paracrine way (Florini et al. 1991; Oksbjerg et al. 2004). A paternally expressed quantitative trait locus (QTL) with major effect on muscle mass has been mapped to the IGF2 locus at the distal end of pig chromosome 2p (Jeon et al. 1999; Nezer et al. 1999). This QTL explained more than 30% of the variance for percentage lean meat in a cross between the European Wild Boar and Large White and more than 25% of the variance for average back-fat depth. A G-to-A transition at nucleotide 3072 in intron 3 (IGF2 intron3-g.3072G>A) is the causal mutation underlying this QTL (Jeon et al. 1999; Nezer et al. 1999). The mutation abrogates in vitro interaction with a nuclear factor, probably a repressor, and pigs inheriting the mutation from the sire have a threefold increase in IGF2 messenger RNA (mRNA) in post-natal muscle. The mutated A\(^{\text{Mut}}\) allele is associated with higher muscle growth rates, lower fat deposition rates and a bigger heart. The transition has no influence on IGF2 expression in pre-natal muscles (Van Laere et al. 2003). In pigs, the adult muscle fibre number is fixed at birth and post-natal growth is characterised by an extensive hypertrophic growth of the existing muscle fibres. Therefore, it has been hypothesised that IGF2 has a role in muscle hypertrophy (Novakofski & McCusker 1993).

Insulin-like growth factor 2 has been shown to mediate its effect in a primary human parathyroid cell culture model predominantly through ryanodine receptor 1 (RYR1)-mediated intracellular calcium stores. Ryanodine receptors are the calcium-dependent calcium release channels in the sarcoplasmic reticulum of skeletal muscle. Fujii et al. (1991) reported the identification of a mutation in porcine skeletal muscle RYR1 (g.1843C>T) associated with high lean meat percentage. This mutation also causes malignant hyper-
 thermia, with homozygous stress negative (NN), homozygous stress positive (nn) and heterozygous (Nn) being the three genotypes. The effects of the RYR1 QTL on muscle mass and fat deposition are major and of the same magnitude as those reported for the IGF2 locus. The two loci jointly explained 50% of the difference between Piétrain and Large White animals for muscularity and leanness in the Nezer et al. (1999) study.

In our study, we investigated post-natal IGF2 function by comparing IGF2 expression in muscles between the IGF2 intron3-g.3072G>A alleles. Because this mutation is also positively correlated with the lean meat percentage, we included the possible interaction between RYR1 and IGF2 in our study.

**Materials and methods**

**Animals and samples**

Animals originating from two damlines of Rattlerow-Segers (Lebbeke, Belgium), were used in experiment 1. Line A is a Landrace line and line B is a pure Large White line; both lines are homozygous wildtype (CC) for the RYR1 g.1843C>T mutation. Intact male piglets of the two dam lines were genotyped for the IGF2 intron3-g.3072G>A mutation. Because IGF2 is a paternally expressed gene in pigs (Nezer et al. 1999), piglets were selected based on their paternal allele: G\textsuperscript{pat}, the wildtype paternal allele, and A\textsuperscript{pat}, the mutant paternal allele.

For the second experiment, 13 sows were mated with two males. The sows were heterozygous for the RYR1 locus (CT) and homozygous for the IGF2 mutation (AA). Sire 1 was heterozygous for the IGF2 locus and homozygous wildtype (CC) for the RYR1 locus. Sire 2 was heterozygous for both mutations. Piglets were genotyped at birth for IGF2 (A\textsuperscript{pat} or G\textsuperscript{pat}) and for RYR1 (CC, CT or TT). Pigs with different genotypes of IGF2 and RYR1 were selected to obtain six groups of animals: G\textsuperscript{pat}/CC, G\textsuperscript{pat}/CT, G\textsuperscript{pat}/TT, A\textsuperscript{pat}/CC, A\textsuperscript{pat}/CT and A\textsuperscript{pat}/TT.

For both experiments, blood samples were taken at 2–4 weeks of age by puncture of the jugular vein. The piglets were weaned at 4 weeks of age and kept in the rearing unit until 8 weeks. They were then fattened in the finishing unit at a different site until 26 weeks (experiment 1) or approximately 109 kg (experiment 2). In the first experiment, pigs were slaughtered at four ages: 4 (weaning), 8, 16 and 26 weeks (slaughter weight) and samples were taken from m. masseter (M), m. triceps brachii (TB), m. psoas major (PM), m. longissimus dorsi (LD) and heart (H). These skeletal muscles were selected because of large differences in oxi-do-glycolytic metabolism. In experiment 2, pigs were slaughtered at an average live weight of 109 kg and samples were taken of TB, LD and H. In both experiments, the animals were electrically stunned and slaughtered in compliance with the current ethical guidelines for animal welfare. Carcasses were chilled at 4 °C. The entire muscle was removed from the carcass after which the epimysium was carefully dissected. Muscle samples were taken within an hour after slaughtering. A sample of about 2 g was taken and snap-frozen in liquid nitrogen. The samples were stored at −80°C until further analysis.

**Extraction of DNA and total RNA**

DNA was extracted from whole blood and tissue according to Sambrook & Russell (2001). Total RNA was extracted from muscle samples using TRI Reagent™ (Sigma-Aldrich). The amount of extracted DNA or RNA was quantified by measuring the absorbance at 260 nm with a UV-spectrophotometer.

**Genotyping**

An allelic discrimination assay was performed for the IGF2 intron3-g.3072G>A mutation using the ABI Prism 7700 sequence detection system (Applied Biosystems). For analysis of the g.1843C>T substitution in RYR1 found by Fuji et al. (1991), a 427-bp sequence was amplified using forward primer 5’-CGCTTTCAACCCTTCTCTCA-3’ and reverse primer 5’-ACAAGCTGGGACAAGCAGA-3’. Digestion of the resulting PCR product with HhaI was carried out using standard conditions.

**Measurement of IGF2 expression using quantitative real-time PCR**

A standard protocol for the reverse transcription step was followed (Sambrook & Russell 2001). PCR primers and Taqman® probes were according to Van Laere et al. (2003). Real-time PCR was performed using the ABI Prism 7700 sequence detection system. The expression levels of each group of animals were normalised to GAPDH levels and displayed as the fold change relative to the experimental group with the lowest expression of IGF2.

**Statistical analyses**

Insulin-like growth factor 2 expression data were analysed using the comparative Ct method (Livak & Schmittgen 2001). In experiment 1, data were analysed with a univariate general linear model that included IGF2 paternal allele, muscle and age group as fixed factors and their two-way interactions. In experiment 2, data were analysed with a univariate general linear model with IGF2 paternal allele, RYR1 genotype, muscle and age group as fixed factors and their two-way interactions. When RYR1 genotype, IGF2 paternal allele, muscle or age group was significant, means were compared with the Tukey’s comparison of mean test. For significant two-way interactions, means for one factor were compared within the other factor. P-values < 0.05
were considered significant. The statistical analyses were done using **SAS** 8.0 for Windows.

**Results**

Relative **IGF2** expression at four ages (4, 8, 16 and 26 weeks) and three muscle types (H, M, LD) were determined using real-time PCR (Fig. 1). Numbers of animals per group varied from 6 to 14. The univariate general linear model that included **IGF2** paternal allele, muscle and age group as fixed factors and their two-way interactions explained 84% of the variation ($P < 0.0001$). The two-way interaction between age group and muscle was significant ($P < 0.0001$). In all three muscle types and across all ages, there was significantly higher **IGF2** expression in animals that inherited the mutant allele from their father ($A^{pat}$) compared with those animals that received the wildtype allele from their sire ($G^{pat}$). **Insulin-like growth factor 2** expression was higher in animals at 4 weeks of age compared with animals at 26 weeks in both genotypes and for all muscle types, except for the **IGF2** expression in the heart of animals that inherited the wildtype allele from their father. However, there was a significant decrease with age in all muscles. Finally, in both genotypes and across all ages, the **IGF2** expression in M (red muscle) was higher than the expression of **IGF2** in LD (white muscle) except in animals with the wildtype allele at 4 weeks, where there was no difference.

Using these same animals, the relative **IGF2** expression was compared at two ages (4 and 16 weeks) in four muscle types (LD, PM, TB and M) (Fig. 2). The univariate general linear model, with **IGF2** paternal allele, muscle and age group as fixed factors and their two-way interactions, explained 87% of the variation ($P < 0.0001$). Two-way interactions were not significant. Again, higher **IGF2** expression was found in animals that inherited the **IGF2** mutant allele from their father ($A^{pat}$) compared with the animals that inherited the paternal wildtype allele ($G^{pat}$). Expression in M was highest, **IGF2** expression in LD was intermediate, and the lowest expression was found in PM and TB, in both ages and for both **IGF2** paternal alleles.

Expression of **IGF2** was measured in H, LD and TB muscles in six pigs each of five genotypes: $G^{pat}$/CC, $G^{pat}$/CT, $G^{pat}$/TT, $A^{pat}$/CC, $A^{pat}$/CT and $A^{pat}$/TT. The univariate general linear model with **IGF2** paternal allele, **RYR1** genotype, muscle and age group as fixed factors and their two-way interactions explained 74% of the variation ($P < 0.0001$). Two-way interactions were not significant. The data confirmed that the **IGF2** paternal allele influenced **IGF2** expression in all muscles ($P < 0.0001$). The **RYR1** genotype only affected **IGF2** expression in LD ($P = 0.0096$). When LD of $A^{pat}$/CC and $A^{pat}$/TT animals were compared, a

**Figure 1**: Analysis of insulin-like growth factor 2 (**IGF2**) expression at 4 weeks (4), 8 weeks (8), 16 weeks (16) and 26 weeks (26) in heart (H), m. longissimus dorsi (L) and m. masseter (M). $G^{pat}$: piglets inherited the wildtype **IGF2** allele from their father. $A^{pat}$: piglets inherited the mutant **IGF2** allele from their father. Letters indicate significant differences within a genotype (A-I: $G^{pat}$, S-Z: $A^{pat}$), ages and muscle types. Asterisks indicate significant differences between genotypes at the same age in the same muscle; *: $0.05 < P < 0.01$, **: $0.01 < P < 0.005$.

**Figure 2**: Analysis of insulin-like growth factor 2 (**IGF2**) expression at 4 weeks (4) and 16 weeks (16) in m. longissimus dorsi (L), m. psoas major (PM), m. triceps brachii (TB) and m. masseter (M). $G^{pat}$: piglets inherited the wildtype **IGF2** allele from their father. $A^{pat}$: piglets inherited the mutant **IGF2** allele from their father. Letters indicate significant differences within a genotype (A-D: $G^{pat}$, V-Z: $A^{pat}$), ages and muscle types. Asterisks indicate significant differences between genotypes at the same age in the same muscle; *: $0.05 < P < 0.01$, **: $0.01 < P < 0.005$. ns: not significant.
significantly higher expression was found \((P = 0.02)\) in the stress resistant (CC) group \((2^{-ΔΔCt} \text{ value of 7.13})\) compared with the \(A^{pat}/TT\) group \((2^{-ΔΔCt} \text{ value of 2.30})\). No effect of genotype on \(IGF2\) expression was found in H and TB tissues (data not shown).

**Discussion**

The \(IGF2\) mutation causes an increase in heart weight and carcass lean meat content at the expense of fat, but has no effect on birth weight and growth rate (Jeon et al. 1999; Nezer et al. 1999; Van Laere et al. 2003). In a separate study, we demonstrated that an increased weight of the LD relative to the carcass weight at 26 weeks occurred in pigs that inherited the mutant \(IGF2\) allele from the sire (Van den Maagdenberg et al. 2006a). To examine whether the \(IGF2\) effect was muscle- or development-dependent, \(IGF2\) expression of animals in two genotypes \((G^{pat} \text{ and } A^{pat})\) at four ages (4 weeks or weaning, 8, 16 and 26 weeks or slaughter weight) was monitored in three muscles (H, LD and M). The \(IGF2\) expression in piglets that inherited the mutant \(IGF2\) allele from the sire was higher than in those who inherited the wildtype \(IGF2\) allele. From 4 to 26 weeks of age, \(IGF2\) expression significantly decreased, irrespective of muscle or \(IGF2\) paternal allele. Our findings are in concordance with the findings of Lee et al. (1993), who used Northern blot assays to show that \(IGF2\) mRNA abundance was high in porcine foetal muscle tissue and declined during the perinatal period and through 42 days post-natal. Also, Gerrard et al. (1998) reported a gradual decrease in \(IGF2\) gene expression from 59 days prenatally to adult age. Our results on \(IGF2\) expression declining after birth also correspond to the general knowledge that relative muscle growth decreases with age (Sara & Hall 1990; Novakofski & McCusker 1993).

We found a significant difference between \(IGF2\) expression in m. masseter, a slow oxidative, red muscle and m. longissimus dorsi, a fast glycolytic, predominantly white muscle, across \(IGF2\) paternal allele and ages. This suggests that \(IGF2\) expression might be related to the muscle fibre metabolic type, which we investigated using two intermediate muscles, PM and TB. We found that the \(IGF2\) expression in these muscles was significantly lower than the expression in LD and M. The relative weights of the individual muscles in a newborn animal are dependent on the animal’s immediate needs which are different from those of the adult animal. Therefore, a transition must take place in the relative weights of the muscles as related to their overall increase in weight in the growing animal. To survive, the new-born piglet must be able to suck. To carry out this task it must have well-developed muscles in its jaws (Berg & Butterfield 1976). Because M is indispensable for suckling and the mastication of feed, it must develop early in post-natal life. The LD helps to flex the vertebral column and move the neck as well as respiratory movements, also immediate needs of a new-born animal. Therefore, \(IGF2\) expression was significantly higher in early developing muscles (M and LD) than in late developing muscles (PM and TB).

In pigs, the adult muscle fibre number is fixed at birth and is determined prenatally by the number of myoblasts formed and by the timing of myoblast differentiation into muscles. Post-natal muscle growth is characterised by an extensive hypertrophic growth of the existing muscle fibres (Novakofski & McCusker 1993). As Van Laere et al. (2003) showed, the \(g.3072G>A\) transition in \(IGF2\) has no influence on \(IGF2\) expression in pre-natal muscles, but has an effect on the expression of \(IGF2\) postnatally. Also, \(IGF2\) has an effect on muscle growth (Sara & Hall 1990; Novakofski & McCusker 1993). Results of other studies (Ewton et al. 1987; Florini et al. 1991; Oksbjerg et al. 2004) suggest that \(IGF2\) has a role in muscle hypertrophy. In a previous study (Van den Maagdenberg et al. 2006a), we demonstrated that CAST was significantly higher \((P < 0.005)\) and CAPN2/CAST was significantly lower \((P < 0.05)\) in animals that inherited the mutant \(IGF2\) allele from their father. This suggests that the influence of \(IGF2\) on muscle hypertrophy is twofold: on the one hand it has an effect on muscle growth by satellite cell activity (Florini et al. 1991) and on the other hand it suppresses muscle protein degradation through CAST (Ewton et al. 1987).

In skeletal muscle, \(RYR1\) is the predominant intracellular calcium-dependent calcium channel (Sutko & Airey 1996; Dulhunty & Pouliquin 2003). A significant effect of the \(RYR1\) genotype on \(IGF2\) expression was found in LD. Animals with the double-mutant genotype \((A^{pat}/TT)\) had significantly lower \(IGF2\) expression compared with the animals that inherited the mutant \(IGF2\) allele from the sire but were homozygous wildtype for the \(RYR1\) genotype \((A^{pat}/CC)\). The \(IGF2\) expression in \(A^{pat}/CT\) animals was intermediate. A similar trend, although not significant, was found in LD in animals that received the wildtype \(IGF2\) allele from the sire. A possible explanation for this could be that there was altered \(Ca^{2+}\) dependence of the mutant \(RYR1\) receptor. Despite the fact that the \(RYR1\) mutation increases lean meat percentage, \(IGF2\) expression was lower in TT animals compared with CC animals. A direct effect of the \(RYR1\) mutation on lean meat percentage, independent of \(IGF2\), was found in a larger dataset (Van den Maagdenberg et al. 2006b). Also, the influence of \(IGF2\) on \(RYR1\) was less in malignant hyperthermia susceptible (MHS) pigs than in normal pigs.

No significant effect of \(RYR1\) genotype within the same \(IGF2\) paternal allele was found in heart. These data support Ervasti et al. (1991) who found no differences in the ryanodine binding properties of cardiac sarcoplasm reticulum (SR) isolated from MHS and normal pigs, in contrast to differences in skeletal muscle SR. The cardiac SR calcium-release mechanism is normal in MHS individuals probably because the predominant \(RYR1\) protein expressed in cardiac

muscle is RYR2 instead of RYR1 (Ervasti et al. 1991; Zissimopoulos & Lai 2005). The effect of IGF2 on muscle growth might partially be mediated by the calpain/calpastatin system and that this is dependent on RYR1-mediated Ca\(^{2+}\) transport.

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**References**


