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Microstructure of porcine meat with the Rendement Napole gene*

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The Rendement Napole gene (*RN*) consists of a mutation at the γ3 subunit of AMP-activated protein kinase (AMPK) in pigs. AMPK works as an energy level sensor and controls numerous cellular pathways. The study aimed at identifying the differences in microstructure occurring between the normal porcine meat and porcine meat encoded by the RN gene. Using molecular techniques 194 commercial pigs were sampled to determine their genotypes. Meat from the *semimembranosus* muscle was examined using optic (OM) and transmission electron microscopy (TEM). The genotypes rn^I/rn^I, rn^V/rn^V, rn^V/rn^I, RN/rn^V and RN/rn^I were identified. OM revealed broadening of the sarcoplasmic space in *RN* animals, while TEM showed disordering of muscle fibres and alteration of the sarcomeric structure. Mitochondrial degradation was found in both *RN* and normal animals, however, the morphological changes observed seemed to be caused by the mutated AMPK.

KEY WORDS: AMPK / microstructure / muscle / pork / PRKAG3 / *RN* gene

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First observed in France by Monin and Sellier [1985], a dominant *RN* gene has an undesirable effect on the Napole yield (*Rendement Napole*) of pork. It also affects negatively an economic effect of meat processing due to poor yields in the production of cured and cooked hams resulting from a lowered final pH that directly leads to decreased water-holding capacity [Lundström *et al.* 1996, Hamilton *et al.* 2000, Moeller *et al.* 2003]. A primary issue observed in RN meat is a marked increase in glycolytic potential, due to glycogen accumulation, which affects the *post-mortem* processes, with the meat developing PSE (pale, soft exudative) features [Lundström *et al.* 1998, Josell *et al.* 2000]. In 2000, a dominant mutation of the codon 200 of the PRKAG3 gene was identified [Milan *et al.* 2000], which codifies the regulatory γ subunit of a protein-kinase AMP activated (AMPK). This mutation causes a substitution of arginine by glutamine corresponding to the *RN* gene [Milan *et al.* 2000]. Another polymorphism, substitution of isoleucine at position 199 by valine, along with the mutation on site 200, has been found implicated in glycogen accumulation [Ciobanu *et al.* 2001].

Incidence of the *RN* gene has been reported in Europe and North America [Fernandez and Monin 1994; Meadus *et al.* 1998], but no reports exist from the other geographic regions. Lara *et al.* (2003, unpublished) found a 10.13% incidence of the *RN* gene in a local abattoir in Chihuahua, Mexico.

Since it was discovered, many studies have focused on the *RN* gene, but only two dealt with the microstructure of affected muscle. Estrade *et al.* [1993a] based upon biopsies of pigs affected by the *RN* gene reported a dramatic alteration in the sarcoplasmic reticulum appearance, with glycogen accumulation easily observed in that compartment. In another study by Estrade *et al.* [1993b], a change of mitochondrial morphology related to glycolytic fibres was observed, which suggested a modification of energy metabolism. Besides the observations derived from these two studies, nothing is known about the microstructure of *RN*-affected porcine meat. To address this deficit, our objective was to investigate the changes in microstructure of fresh, RN-affected porcine meat compared with normal meat in order to elucidate the mechanism of its effect.

Material and methods

One hundred and ninety-four randomly selected, commercial animals were sampled in a federally inspected local abattoir in Chihuahua, Mexico. The sampling process lasted two months and was conducted on nine occasions. Animals were mainly imported from the state of Sonora and from different parts of Chihuahua state. Blood samples for genotyping were obtained from the slaughter line in vacuum tubes containing EDTA. Meat samples were collected from the *semimembranosus* muscle 22 h *post-mortem* with a knife and were transported to the laboratory at 4°C.

DNA was extracted from blood samples according to Rudbeck and Dissing [1998]. PCR amplification of a segment containing the 199 and 200 sites of the PRKAG3

gene was performed using NAP-F 5′-GGAACGATTCACCCTCAACT-3′ and NAP-R 5′-AGCTCTGCTTCTTGCTGTCC-3′ primers (INVITROGEN) on an Amplitron II thermocycler (THERMOLYNE). RFLPs of the PCR products were done separately using *Bsa*HI and *Bsr*BI to detect the mutation on the site 199 and 200, respectively (both enzymes from BIOLABS Inc.). Final diagnosis was done combining both results; an animal that carried one allele with the 200Q substitution was considered an *RN* carrier without considering the 199 site.

Two hours after collection, the muscle was carefully cut with a scalpel into blocks of 2-3 mm^3 without damaging meat internal structure. Blocks were fixed in a 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer of pH 7.2, rinsed with cacodylate buffer and stored at 4°C until the sampling process ended. Samples were classified according to their genotype. Equal numbers of samples of each genotype were selected, postfixed in 2% osmium tetroxide and embedded in Epon 812 resin (EMS).

Semi-thin sections (0.6 µm) were obtained in a MT-7000 ultramicrotome (RMC), stained with Multiple Stain solution (POLYSCIENCES Inc.) and assessed with an Axioskop 2 plus microscope (CARL ZEISS Inc.)

Thin sections (12 nm) were contrasted with 1% uranyl acetate and 2% lead citrate, and assessed with a JEM-1010 transmission electron microscope at an accelerating voltage of 60 kV.

Results and discussion

Out of six possible genotypes five were obtained. The number of non-*RN* carriers amounted to 115 animals (59.3%) and corresponded to the R200/199V (rn^I/rn^I) genotype, four animals (2.1%) were of the R200/I199 (rn^V/rn^V) genotype, and 66 (34.0%) were determined as R200/I199,199V (rn^V/rn^I). The number of *RN* carriers amounted to seven animals (3.6%) with the R200,200Q/I199,199V (RN/rn^V) while two (1%) occurred to be of the R200,200Q/199V (RN/rn^I) genotype. No homozygous *RN* carriers were found. The *RN* gene incidence from the studied pig population was 4.6%.

The incidence of the *RN* gene observed in this study is considered low, and does not represent a threat to the pork industry in Chihuahua. Although an overview of the incidence of the *RN* gene in Mexico cannot be extrapolated from this study; the presence of the *RN* gene was confirmed. The low incidence resulted in not all genotypes being represented. This, however, was not considered important because of the dominance of the *RN* gene.

Two samples of each genotype were randomly selected. Pictures were viewed and taken. The most representative pictures obtained of each genotype are presented in Figure 1. It is easy to appreciate differences between non-carriers (Fig. 1 – A, B, C) and carriers (Fig.1 – E, F) of the *RN* gene. Non-carriers show organized fibres arranged in large groups, compared to carriers, which have less fibre organization, with fibres arranged in smaller groups; the most evident difference is the enlargement of the sarcoplasmic compartment.

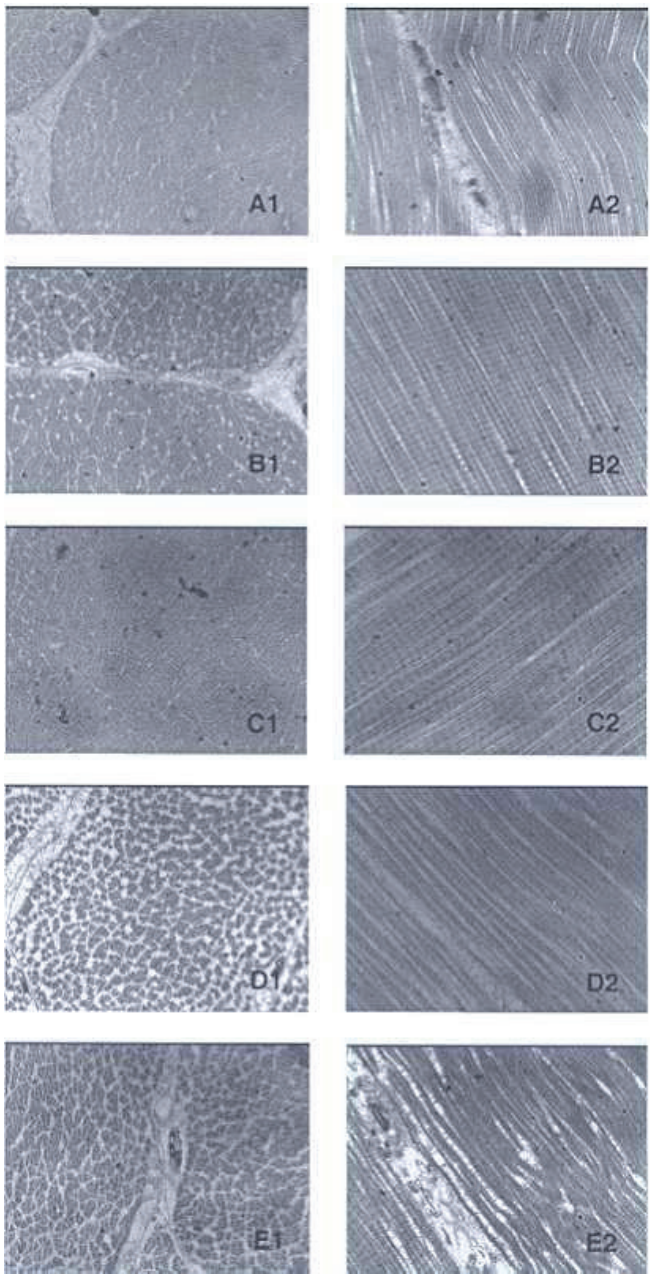

Fig. 1. Light micrographs of transverse (1) and longitudinal (2) sections of porcine *semimembranosus* muscle according to *RN* genotype, rn^I/rn^I (A); rn^V/rn^V (B); rn^V/rn^I (C); RN/rn^V (D) and RN/rn^I (E) (× 100).

Trends in research into the *RN* gene have focused on glycogen accumulation issues, disregarding structural issues observed by Estrade *et al.* [1993ab]. Comparing our micrographs with those presented by Estrade *et al.* [1993ab] shows that the modification visible in their biopsies was present in the 24 h *post-mortem* muscle examined in our study. This contradicts the data of Deng *et al.* [2002], who concluded that the change taking place in *RN* meat is a result of an altered maturation process. If the modification involved the maturation process, differences in the microstructural appearance would have been expected between the two publications of Estrade *et al.* [1993ab] which were published based on muscle biopsies made before killing, and our study that was conducted using meat samples taken 24 h *post-mortem*.

Microscopy showed an enlarged sarcoplasmic space that probably could be filled with water and solutes derived from *post-mortem* metabolism [Pearson and Young 1989]. It can be assumed that the space has been depleted of glycogen granules abnormally stored by means of an altered glycolytic metabolism, as mentioned by Estrade *et al.* [1994].

Because of the uniformity observed between RN carriers and non-carriers when OM was used, only two genotypes were selected for TEM examination. Selected genotypes were chosen according to their having more striking differences, with the rn^V/rn^V genotype chosen as a non-carrier and the RN/rn^V genotype as a carrier of the *RN* gene.

The pattern observed using TEM was very similar to that observed with OM. In transverse sections, *RN* carriers showed disorganized myofibrils, and an enlarged sarcoplasmic space compared to non-carriers. A change in myofibril shape was observed (Fig. 2): non-carriers showed polygonal myofibrils with sharp corners (Fig. 2 – A1) whereas *RN*-carrier myofibrils had rounded corners which in some cases, were oval (Fig. 2 – B1). Longitudinal sections of *RN* carriers (Fig. 2 – B2) showed an alteration in the sarcomere structure, which appeared more relaxed than of sarcomeres from non-carriers (Fig. 2 – A2). Furthermore, sarcomere was by about 56% longer in *RN* carriers (Fig. 2 – B2) and the A band by 77% longer than in non-carriers (Fig. 2 – A2). Altered was also the width of *RN* carrier sarcomere, which occurred by 20% narrower, while the M line was 5% narrower than in non-carriers (Fig. 2 – A2). This can be interpreted as thickness shrinkage of myofibrils of *RN* carriers. Mitochondrial degradation was observed in pigs of all genotypes (Fig. 3), with the extent of degradation variable in the same fields of the same sample.

Change of the sarcomere dimensions observed with TEM could be related to misbalanced osmolarity, as suggested by Estrade *et al.* [1993a], probably caused by the abnormal accumulation of excess lactic acid. Mitochondrial degradation can be considered normal because of cytochrome C-activated apoptosis; respiratory chain proteins are vulnerable to changes in the mitochondrial membrane potential by action of ions, like lactate [Wang *et al.* 2001, Yuan *et al.* 2002].

It is well known that an alteration in glycogen metabolism exists in RN animals [Monin and Sellier 1985, Estrade *et al.* 1994, Milan *et al.* 2000], and many efforts

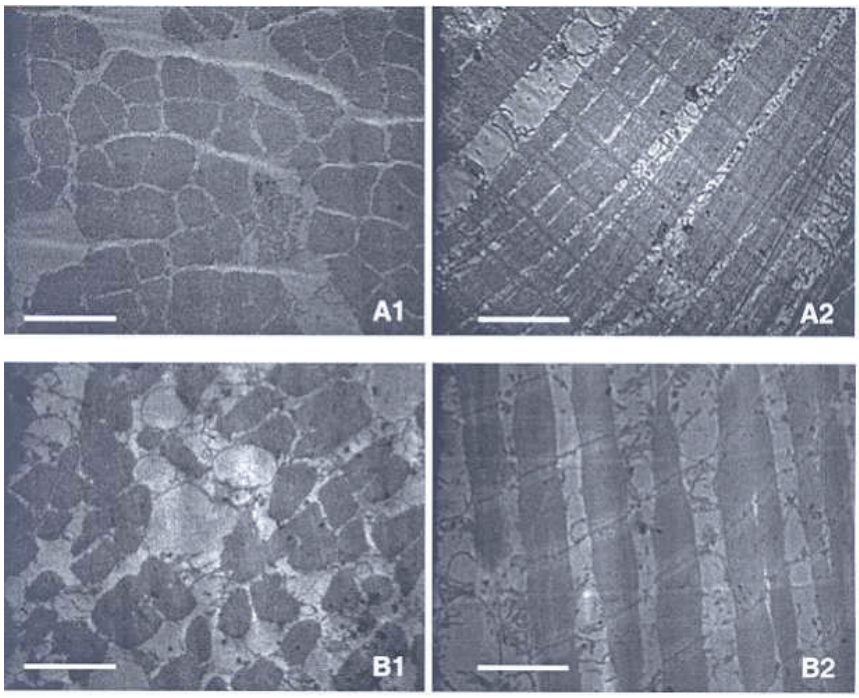

Fig. 2. Transmission electron micrographs of transverse (1) and longitudinal (2) section of porcine *semimembranosus* muscle according to *RN* genotype: non-*RN* carrier (A); *RN* carrier (B), (× 100). Bar equals 1 μm.

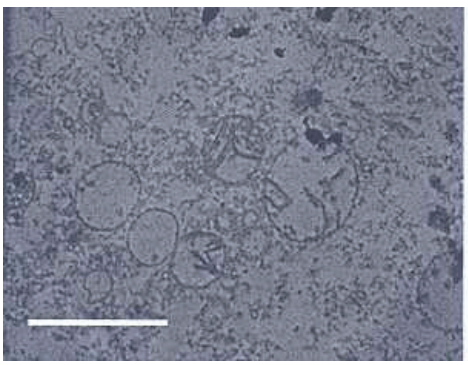

Fig. 3. Transmission electron micrograph of a group of mitochondria of pork meat presenting different stages of degradation (× 15,000). Bar equals 1 μm.

have been made to elucidate the relationship between the modifications caused by a simple mutation (R200Q) in AMPK and the RN phenotype. AMPK is an energy sensor activated by ATP-depleting stresses. When it is activated catabolic pathways are turned on, and anabolic pathways are turned off [Hardie 2004, Scott *et al.* 2004]. Andersson [2003] showed that, after physical stress, animals that carry the *RN* allele show normal glycogen degradation, but have an enhanced capacity to replenish it. This suggests that anabolic capacity is altered in *RN* carriers and the effect of the *RN* gene transpire as a diminution in AMPK activation.

A possibility that has not been considered so far resides in a variation of structural proteins due to modified activation of AMPK, which may be responsible for the dramatic differences observed in microstructure. Mu *et al.* [2003] have demonstrated in mice that inactivation of AMPK causes a change in the transcription of a large number of genes enrolled in multiple cellular functions, including those of structural proteins. UDP-glucose pyrophosphorylase, a key enzyme in glycogen synthesis, is overexpressed in *RN* carriers. This finding explains glycogen accumulation. With this evidence, we suggest that the microstructural alteration observed in RN meat could be related to an alteration in the transcription of genes encoding structural proteins, derived from the diminished activation of the AMPK.

The RN phenotype can be related to a muscular dystrophy, as in most cases dystrophies manifest as devastating pathologies. In humans, Duchenne muscular dystrophy (DMD), a result of deletion of the gene coding for dystrophin [Roberts 2001], causes disability in patients. However, in an animal model of DMD (*mdx* mice) no such disability occurs, mice have a better developed alternate pathway that counteracts the lack of the dystrophin gene [Barton *et al.* 2002, Harper *et al.* 2002]. RN pigs do not show disability, so structural integrity may not be so extensively affected as DMD. Furthermore, a similar compensatory pathway could also be present in pigs.

It can be concluded that the morphological changes observed seem to be caused by the 200Q mutation corresponding to the *RN* gene. However, more research is still needed to fully understand the effect of this mutation on the structure of muscle.

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Mikrostruktura mięśni świń z genem kwaśnego mięsa (RN)

S t r e s z c z e n i e

Gen kwaśnego mięsa (*Rendement Napole* – RN) jest wynikiem mutacji w podjednostce γ3 kinazy białkowej aktywowanej przez AMP (AMPK) świń. AMPK działa jako czujnik poziomu energii i kontroluje szereg ścieżek metabolizmu komórki. Badanie miało na celu identyfikację różnic mikrostrukturalnych w budowie mięśniówki normalnych i noszących gen RN. Wykorzystując techniki molekularne zgenotypowano 194 świnie z fermy produkcyjnej. Próbki mięśnia półbłoniastego (*musculus semimembranosus*) badano za pomocą mikroskopu optycznego (OM) i elektronowego mikroskopu transmisyjnego (TEM). Zidentyfikowano następujące genotypy: rn^{I}/n^{I}, rn^{V}/rn^{V}, rn^{V}/rn^{I}, RN/rn^{V} i RN/rn^{I}. Badanie OM ujawniło poszerzenie przestrzeni sarkoplazmatycznej u zwierząt z RN, podczas gdy badanie TEM wykazało dezorganizację włókien mięśniowych i zmianę struktury sarkomerycznej. Degradację (zwyrodnienie) mitochondrialną stwierdzono niezależnie od genotypu RN jednak obserwowane zmiany morfologiczne wydają się spowodowane zmutowaną AMPK.