CHARACTERIZATION OF THE PORCINE *RN*$^-$ MUTATION IN *PRKAG3* BY PROTEOME ANALYSIS

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INTRODUCTION

The porcine dominant mutation (denoted *RN* $^-$) resulting in a ~ 70 % increase in muscle glycogen content with large effects on meat quality and processing yields was recently found to located in the *PRKAG3* gene encoding a muscle specific isoform of the regulatory γ3 subunit of adenosine monophosphate activated protein kinase (AMPK) (Milan *et al*., 2000). The *RN* $^-$ genotype was found to possess a single nucleotide substitution from CGA to CAA in codon 200 resulting in a nonconservative amino acid substitution from an arginine to a glutamine (Milan *et al*., 2000).AMPK is a heterotrimer consisting of one catalytic (α) and two regulatory subunits (β and γ). Two isoforms of the catalytic subunit (α1 and α2) have been described while the regulatory subunits are present in three isoforms (β1-3 and γ1-3). The exact physiological relevance of these isoforms has not been completely described, but the γ3 isoform have been found to be musclespecific (Cheung *et al*., 2000) consistent with *RN* $^-$ animals showing high glycogen content in muscle but not in liver (Estrade *et al*., 1993). AMPK acts as a metabolic sensor that monitors cellular energy levels and when activated, AMPK inhibits ATP consuming pathways and stimulates ATP regenerating pathways (Hardie and Carling, 1997). AMPK is allosterically activated by an increase in the AMP:ATP and cratine:phosphocreatine ratios which promotes its phosphorylation by an upstream kinase (AMPKK) and inhibits its dephosphorylation (Hardie *et al*., 1998 ; Kemp *et al*., 1999). AMPK has been shown to inhibit enzymes involved in glycogen, fatty acid and cholesterol synthesis and to stimulate fatty acid oxidation, glycolysis and glucose uptake (Holmes *et al*., 1999 ; Kemp *et al*., 1999 ; Winder and Hardie, 1999 ; Ojuka *et al*., 2000).

In the work of Estrade *et al*. (1994) selected enzymes from the glycogen anabolism were found to have higher activity in *RN* $^-$ carriers, while no difference in activity were detected in enzymes from the glycogen catabolism. Furthermore, slightly higher activities of citrate synthase and mitochondrial respiration were found in *RN* $^-$ animals. These results were confirmed by Lebret *et al*. (1999) who reported a more oxidative metabolism of glycolytic muscle in *RN* $^-$ carriers, as shown by increased activity of β-hydroxy-acylcoenzyme A dehydrogenase and citrate synthase, while the activity of lactate dehydrogenase was lower.

In order to study the effects of the *RN* $^-$ mutation on the protein expression in skeletal muscle tissues, we have performed comparative proteome analysis on samples from 23 animals (11 *rn*+/*rn*+ and 12 *RN* $^-$/*rn*+).

MATERIAL AND METHODS

Animals and tissue samples. All animals in this experiment were from sets of siblings originating from crosses between a Danish Landrace/Yorkshire sow and a Hampshire boar. The pigs were raised on a commercial farm, and slaughtered at the experimental slaughter plant at the Danish Institute of Agricultural Sciences according to Danish governmental regulations on treatment of livestock. Tissue samples from *Longissimus dorsii* were taken at the position of the last pair of ribs immediately after bleeding and were frozen instantly in liquid N_2, and kept frozen at - 80 °C until use. Fractions of muscle proteins were obtained by collection of the muscle fluid emerging from the muscle during post mortem storage (Honikel, 1987).

Two-dimensional electrophoresis (2DE). Two-dimensional electrophoresis with immobilized pH gradients (IPG-Dalt) was carried out essentially as described by Görg *et al*. (2000). Tissue samples (50 mg) were homogenized in 1 mL thiourea lysis buffer. A crude fractionation of muscle tissues were in some cases obtained by centrifugation of muscle samples for 15 min at 1000 x g. The resulting muscle fluids were enriched in cytoplasmic proteins, and free from contractile proteins that otherwise dominates the 2DE images. The crude extracts were vigorously vortexed for 2 hours at RT, followed by centrifugation in 30 min at 10.000 x *g*. Samples of muscle fluid (fractionated muscle) were prepared by five times dilution in thiourea lysis buffer followed by vortexing for 1 hour and centrifugation in 30 min at 10.000 x *g*. The individual IPG dry strips (pH 4 – 9) were rehydrated overnight with sample (50 µg per strip) in reswelling solution. The isoelectric focusing was performed using a Pharmacia Muliphor Instrument at 20 °C under a layer of silicon oil, using a maximum current of 1 mA and a stepwise increasing voltage starting at 50 V increasing over time to 3500 volt, reaching a total of 700.000 volthours. The focused IPG strips were equilibrated for 2 x 15 min with gentle shaking in 5 mL equilibration buffer. DTT (1 % w/v) was added to the first, and iodacetamid (5 % w/v) to the second equilibration step. The strips were mounted on top of the SDS gel and sealed with melted 0.5 % w/v agarose in 1 x running buffer containing a trace of bromophenol blue. The proteins were separated according to size in 10 % SDS-PAGE (25 cm X 30 cm) with a continuous buffer system (Laemmli, 1970). The gels were casted and electrophorized using the Investigator^{TM} 2-D Electrophoresis System (Genomic Solutions) allowing the simultanous handling of 10 gels. The gels were electrophorized at 50 mA for 3 hours followed by 110 mA until the dye front reached the bottom of the gels.

Visualization of proteins. The 2DE gels were silver stained by the silver-diamin method essentially as described by Hochstrasser *et al*. (1988). Briefly, gels were fixed overnight (40 % v/v ethanol, 10 % v/v acetic acid) followed by sensitizing (1 % v/v glutardialdehyde, 0.5 M sodium-acatate) for 30 min. Staining was carried out in 260 mM $AgNO_3$, 1.2 % v/v ammonia and 90 mM NaOH for 20 min and the gels were developed in 0.12 % v/v formaldehyde (37 %), 0.76 mM citric acid. After developing, the gels were stored in destaining solution (7 % v/v acetic acid, 20 % v/v ethanol, 1 % v/v glycerol) for 1 hour followed by storage in 5 % v/v glycerol until drying.

Image Analysis. The dried 2D gels were scanned and analyzed using the Bioimage 2D analyzer software (Genomic Solutions). Spots were detected and the gels were matched and manually corrected before a match-ratio integrated quantification was performed. Spots differing significantly in integrated intensity dependent on genotype were detected by a two-tailed t-test assuming equal variance.

RESULTS AND DISCUSSION

The aim of this work was to characterize the porcine *RN*$^-$ genotype by comparative proteome analyses. Two-dimensional gel electrophoresis and digital image analyses were employed on samples originating from animals characterized as carriers or non-carriers of the *RN*$^-$ mutation. More than thousand protein spots were detectable on each gel, but a majority were rejected from analyses due to uncertain spot boundaries or too high or low intensities. In 2DE gels of muscle and fractionated muscle (figure 1) app. 600 and 300 spots, respectively, were considered for further analyses.

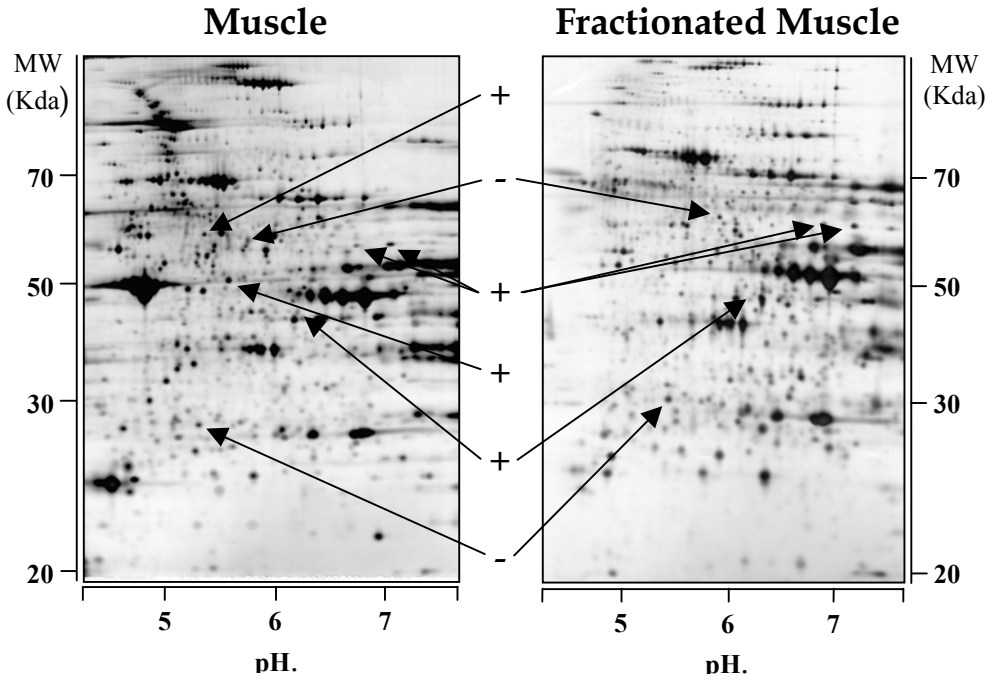

Figure 1. Silver stained 2DE gels of muscle (left) and fractionated muscle (right) from *RN*-carriers. The positions of the differentially expressed proteins are indicated. A (+) indicates that the protein is found in a relatively higher abundance due to the *RN*$^-$ genotype

The obtained gels were analyzed in three parallel comparisons as the 13 muscle samples were analyzed in two sets dependent on sex, while the 10 fractionated muscle samples were

analyzed in a third set. Within the three parallel comparisons, several proteins were found to differ in intensity dependent on genotype. A comparison of the protein patterns on muscle sample 2DE gels with that of 2DE gels of fractionated muscle sample revealed that out of 400 spot positions 200 were found in both types of sample 150 were found in muscle only while the remaining 50 spots were specific for the fractionated muscle sample. The 50 spots specific for the fractionated muscle are likely to represent protein fragments arised by degradation during the post-mortem processes in meat.
Seven spots differing significantly in integrated intensity dependent on genotype were revealed by the analysis. Two of the spots could only be found on the muscle 2DE gels while the remaining five spots were detectable on both muscle and fractionated muscle 2DE gels.

CONCLUSION

Two-dimensional gel electrophoresis and digital image analyses has been employed on samples originating from animals characterized as carriers or non-carriers of the *RN* $^{-}$ mutation. Spots differing significantly in integrated intensity dependent on genotype have been found and are to be identified in forthcoming work.

REFERENCES

Cheung, P. C., Salt, I. P., Davies, S. P., Hardie, D. G. and Carling, D. (2000) *Biochem. J.* **346** : 659-669.

Estrade, M., Ayoub, S., Talmant, A. and Monin, G. (1994) *Comp. Biochem. Physiol. Biochem. Mol. Biol.* **108** : 295-301.

Estrade, M., Vignon, X., Rock, E. and Monin, G. (1993) *Comp. Biochem. Physiol.* **104** : 321-326.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R. and Weiss, W. (2000) *Electrophoresis* **21** : 1037-1053.

Hardie, D. G. and Carling, D. (1997) *Eur. J. Biochem.* **246** : 259-273.

Hardie, D. G., Carling, D. and Carlson, M. (1998) *Annu. Rev. Biochem.* **67** : 821-855.

Hochstrasser, D. F., Harrington, M. C., Hochstrasser, A. C., Miller, M. J. and Merril, C. R. (1988) *Anal. Biochem.* **27** : 424-435.

Holmes, B. F., Kurth-Kraczek, E. J. and Winder, W. W. (1999) *J. Appl. Physiol.* **87** : 1990-1995.

Honikel, K. O. (1987) In "Evaluation and Control of Meat Qality in Pigs", p.129-142. Editors P. V. Tarrant, G. Eikelenboom and G. Monin. Dordrecht: Martinus Niijhoff

Kemp, B. E., Mitchelhill, K. I., Stapleton, D., Michell, B. J., Chen, Z. P. and Witters, L. A. (1999) *Trends. Biochem. Sci.* **24** : 22-25.

Laemmli, U. K. (1970) *Nature* **227** : 680-685.

Lebret, B., Le Roy, P., Monin, G., Lefaucheur, L., Caritez, J. C., Talmant, A., Elsen, J. M. and Sellier, P. (1999) *J. Anim. Sci.* **77** : 1482-1489.

Milan, D., Jeon, J. T., Looft, C., Amarger, V., Robic, A., Thelander, M., Rogel-Gaillard, C., Paul, S., Iannuccelli, N., Rask, L., Ronne, H., Lundstrom, K., Reinsch, N., Gellin, J., Kalm, E., Roy, P. L., Chardon, P. and Andersson, L. (2000) *Science* **288** : 1248-1251.

Ojuka, E. O., Nolte, L. A. and Holloszy, J. O. (2000) *J. Appl. Physiol.* **88** : 1072-1075.

Winder, W. W. and Hardie, D. G. (1999) *Am. J. Physiol.* **277** : E1-10.