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EFFECT OF MC4R POLYMORPHISM ON PHYSIOLOGICAL STRESS RESPONSE IN PIGS

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SUMMARY

Melanocortin-4 receptor (MC4R) is a G-protein coupled receptor predominantly expressed in hypothalamic regions which are known for their roles in feeding behavior, energy homeostasis and HPA axis regulation. In this study, we analyzed the effect of a missense mutation (Asp298Asn) in the porcine MC4R gene on physiological stress response and carcass composition in pigs of two crosses: A (♀Duroc x ♂Swedish Landrace) x ♂Pietrain (n=25) and B (♀Swedish Landrace x ♂Large White) x ♂Pietrain (n=21). All pigs included in this study were heterozygous (Nn) for the stress syndrome gen. Blood samples were collected before loading and at exsanguinations to measure cortisol, lactate, glucose, serum enzymes activity and some haematological parameters. Because only one pig with AA genotype was observed, there was no indicated effect of this genotype on investigated parameters. The heterozygous (AG) pigs showed a lower increase (P<0.05) in CK and AST activity after exsanguinations as well as trend towards lower increase (P<0.10) in cortisol and lactate levels and higher increase (P<0.10) in RBC and haemoglobin content. Higher increase (P<0.05) in LDH activity was observed in GG homozygous pigs from group B, but not in pigs from group A. In addition, the heterozygous (AG) pigs had a higher backfat thickness and lower estimated lean (P<0.05) than homozygous (GG) pigs. These results may support a possible role of the MC4R Asp298Asn polymorphism in the genetic basis of stress response and economically important traits in pigs.

Key- words: MC4R, pig, stress response, carcass composition

INTRODUCTION

The control of stress in modern pig production is an important component of farm and pre-slaughter management with effect on production efficiency, meat quality and animal welfare. The most common practice includes adjustment of the environment to animal needs. An alternative approach to increase adaptive abilities of livestock and well-being is selection of genetically superior animals in relation to stress response (Newman, 1994). Significant gene effects on biological measures of stress response were detected on several porcine chromosomes (Desautes et al., 2002) and involvement of genetic influence on variability of neuroendocrine stress response was suggested. A melanokcortin-4 receptor (MC4R) gene codes a protein, melanocortin-4 receptor, one of the five identified melanocortin receptors. MC4R is a G-protein coupled receptor widely expressed in the central nervous system (CNS), including a number of areas that are known to regulate feed intake and energy homeostasis (Kishi et al., 2003; Mountjoy et al., 1994). A presence of MC4R in both the parvocellural and magnocellular division of the paraventricular nuclei of the hypothalamus, suggest their role in regulation of activity of the hypothalamus-pituitary-adrenal axis (HPA) via vasopressinergic and corticotropic neurons (Mountjoy et al., 1994). This hypothesis is supported by the finding that MTII, a preferable MC4R agonist, increase plasma corticosterone level and that this increase is attenuated by the selective MC4R antagonist HS014 (Lu et al., 2003). In pigs, MC4R locus was mapped on porcine chromosome 1q22-27 (Kim et al., 2000a). A missense mutation which results in replacement of aspartic acid (Asp) with asparagine (Asn) at position 298 of the protein sequence in a highly conserved region of the seventh transmembrane domain, was shown to be associated with growth rate, feed

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intake and backfat thickness (Kim et al., 2000b; Hernandez-Sanchez et al., 2003; Houston et al., 2004; Jokubka et al., 2006). A functional analysis of this mutation showed a defective signaling of the mutate MC4R protein (Asn298) to adenilate cyclase in the membrane of neural cells that results in lowered intracellular levels of cAMP after correct ligand binding to receptor (Kim et al., 2004). In this investigation we premised that presence of Asn298 protein variant of MC4R has a similar effect on physiological stress response used as selective MC4R antagonist. In line of this, lower stress response could be expected in pigs with Asp298Asn mutation than in pigs with none mutate MC4R receptor protein. Therefore, the aim of this study was to investigate the effect of MC4R polymorphism on physiological stress response caused by pre-slaughter handling of pigs and some carcass traits.

MATERIAL AND METHODS

A total of 46 pigs of two crosses: A (♀Duroc x ♂Swedish Landrace) x ♂Pietrain (n=25) and B (♀Swedish Landrace x ♂Large White) x ♂Pietrain (n=21), weighing between 100 and 110 kg were used in this experiment. All of the pigs were fattened at the same farm, in the 4 pens, held between 12 and 16 pigs of both sex, each. The transportation conditions and pre-slaughter handling procedures were similar for all the pigs. All pigs were transported together by truck, in the early morning. The fasting time preceding loading was about 12 h. The travelling distance from the farm to the abattoir was 80 km. After arrival at the abattoir pigs were rested in the same batch for 2 h without feed but with free access to fresh water. Blood samples were collected from each of animals before loading (basal level; sample 1) and at exsanguinations (sample 2). Samples were taken from the jugular vein using commercial vacutainer system and serum collection tube for biochemical analyses and tube with anticoagulant (K_3 EDTA) for DNA isolation, hematological analyses and plasma cortisol measure. Serum glucose concentration and CK, AST, ALT and LDH activity were measured using the automated biochemistry analyzer Olympus AU 400 (Olympus Diagnostica GMBH, Hamburg, Germany) and Olympus AU® Series Reagents. LDH isoenzymes were determined by an LDH isoenzyme electrophoresis kit (P/N 655940; Beckman Instruments; Fullerton, Calif) and expressed as a percentage of the total LDH activity. Plasma cortisol level was determined using an ELISA commercial kit (DRG Cortisol EIA-1887, DRG Diagnostics, Germany) and serum lactate concentration using enzymatic method (kit 256773; Boehringer Mannheim GmbH Diagnostica). Hematological parameters were determined in EDTA-treated blood using an automated hematological analyser (Cell-Dynn 3500, Abbott, IL, USA). Genomic DNA was extracted from frozen blood samples using a standard method. The Asp298Asn MC4R polymorphism was identified using a PCR–RFLP technique according to the protocol described by Stachowiak et al. (2005). The halothane genotype was determined using by the method by Fuji et al. (1991).
After slaughter hot carcass weight was recorded. Backfat thickness was measured at two different points, 7 cm off the midline of the split carcass on the last rib level (BF-1) and at the split of the carcass on the most thickness place above *M. gluteus medius* (BF-2). In addition, the diameter of *Longissimus dorsi* muscle (MLD) was measured and lean meat content was estimated by "two points" method according to Croatian Regulations (N.N. 119/1999). On the MLD the initial (pH_i) and the ultimate (pH_u) pH were taken by transferable pH-meter at 45 min and 24 h after slaughter, respectively. The water holding capacity (WHC) was measured on the same muscle at 24 h *post mortem* according to Honikel et al. (1998). The data were analysed statistically by SAS software (v. 8.1, 1999). The chi-square test was used to compare the observed genotype frequencies with Hardy-Weinberg equilibrium assumption. The blood parameters data were analysed estimating the ratio between the value obtained in sample 2 (at exsanguinations) and the baseline level before transport (sample 1). The effect of MC4R genotype, crossbreed and their interaction on increase of blood parameters were analysed using non-parametric permutation test (PROC MULTTEST). Carcass and meat quality parameters were analysed using the GLM procedure with MC4R genotype, crossbreed and their interaction as fixed effects and carcass weight as a covariate.

RESULTS AND DISCUSSION

The investigation was carried out on commercial fattened pigs from two different origin mostly used for fresh meat production. The crossbreed A are of Spain origin and the crossbreed B are the pigs produced according to national breeding programme in Croatia. All the pigs included in this study were heterozygous (Nn) for the stress syndrome gen. The allele and genotype frequencies for Asp298Asn polymorphism of MC4R are shown in table 1.

Table 1. Genotypes and allele frequencies for the Asp298Asn MC4R polymorphism

Crossbreed	n	Genotype frequency			Allele[1] frequency	
		GG	AG	AA	G	A
$A_{(DuSL)\ Pi}$	25	0.43	0.55	0.02	0.70	0.30
$B_{(SLLW)Pi}$	21	0.61	0.39	-	0.80	0.20

[1] A – Asn (AAU); G – Asp (GAU)

There was no difference between tested crossbreeds in allele and genotypes frequencies, but significant deviation from Hardy-Weinberg equilibrium was observed indicating that the locus is under selection pressure. Both, crossbreed A and B, contain predominantly wild type allele G (0.7 in A and 0.8 in B) that could be explained by using a Pietrain as terminal sires in breeding schemes, breed the mostly homozygotic for the allele G, associated with low backfat thickness (Burgos et al., 2006). Due to only one pig in crossbreed A and none in crossbreed B AA was homozigotic. This genotype was not included in the analysis. Increase in concentrations of biochemical and hematological parameters investigated in relation to MC4R genotype and crossbreed are shown in Table 2.

Table 2. Mean values (standard deviation) of blood parameters increase after pre-slaughter handling in the different MC4R genotypes and crossbreeds

Crossbreed	$A_{(DuSL)\ Pi}$		$B_{(SLVJ)Pi}$	
Genotype	AG $\bar{x}$ ±SD	GG $\bar{x}$ ±SD	AG $\bar{x}$ ±SD	GG $\bar{x}$ ±SD
Blood metabolites				
Cortisol	2.57 (0.16)a	3.03 (0.32)b	2.63 (0.21)a	3.27 (0.44)b
Lactate	2.08 (0.14)a	2.41 (0.24)b	2.79 (0.30)b	3.63 (0.31)c
Glucose	1.24 (0.13)	1.25 (0.15)	1.38 (0.19)	1.39 (0.24)
Enzyme activity				
CK	5.42 (0.21)A	7.72 (0.50)B	4.39 (0.15)C	9.27 (0.67)D
ALT	1.28 (0.15)	1.25 (0.20)	1.29 (0.18)	1.22 (0.20)
AST	2.01 (0.09)A	2.33 (0.18)B	1.78 (0.10)A	2.28 (0.11)B
LDH_{-total}	2.24 (0.54)AB	2.11 (0.35)AB	1.43 (0.19)A	2.84 (0.44)B
LDH_1	0.63 (0.07)	0.54 (0.05)	0.65 (0.08)	0.66 (0.07)
LDH_2	1.9 (0.37)	1.84 (0.62)	2.15 (0.68)	2.4 (0.72)
LDH_3	0.98 (0.33)	0.75 (0.28)	1.46 (0.51)	1.5 (0.69)
LDH_4	1.02 (0.65)	0.75 (0.52)	0.82 (0.56)	1.43 (0.54)
LDH_5	1.59 (0.21)	2.03 (0.30)	1.67 (0.29)	1.48 (0.35)
Hematological parameters				
WBC	1.18 (0.22)	1.15 (0.18)	1.13 (0.09)	1.17 (0.11)
RBC	1.17 (0.10)ac	1.06 (0.02)b	1.28 (0.07)a	1.12 (0.07)bc
HGB	1.17 (0.08)	1.06 (0.03)	1.21 (0.06)	1.10 (0.07)
PCV	1.18 (0.07)AB	1.07 (0.08)A	1.32 (0.09)B	1.14 (0.09)A

CK-lactate dehydrogenase; AST-aspartate aminotransferase; LDH-lactate dehydrogenase; ALT-alanine aminotransferase; WBC-blood cell; RBC- red blood cell; HGB- hemoglobin concentration; PCV-packed cell volume. Means with different letters in the same row are significantly different at $P<0.05$ (A, B) and $P<0.10$ (a, b, c)

The pre-slaughter handling practices caused a marked increase in cortisol concentration (P<0.01), the most frequently monitored blood indices to acute stress response. This finding is in agreement with former investigations (Kannan et al., 2000) and the idea that the loading, transport and unloading can be significant stressors. As shown in table 2, a trend toward lower increase in cortisol concentration (P<0.10) was observed in AG then in GG pigs. In addition, AG pigs showed trend toward lower increase in lactate concentration than GG pigs (P<0.10), but no differences were observed in glucose concentration. The elevation of blood metabolites, such as lactate is a characteristic of a metabolic challenge or physical stress (Warris et al., 1992). High concentrations of both lactate and hydrogen ions, the end product of anaerobic glycolysis, can cause changes in muscle membrane permeability and muscle cell damage (Payne and Payne 1987; Heinze and Mitchell, 1989). Typical signs of these processes are elevated of blood CK, LDH, AST and ALT activity (Payne and Payne, 1987). In this study the higher increase of CK and AST activity was observed in GG compared to AG pigs (P<0.05). Trend toward lower increase of cortisol and lactate level and lower increase of CK and AST activity suggest higher resistance to excessive muscular activity and physical stress and lower response to physical stress in heterozygote AG pigs compared to GG homozygote. Significant interaction between MC4R genotype and crossbreed was observed for total LDH activity. Higher increase in LDH activity was observed in GG homozygous pigs from group B, but not in pigs from group A (P<0.05). Analysis of LDH isoenzymes showed that increases in total LDH levels after preslaughter handling is due mainly to increases in the LDH_5 form, occuring mainly in skeletal muscle and increases in blood after muscle damage or exposure to disturbing conditions in pigs (Broom and Johnson, 1993). The changes in hematological parameters showed higher increase in RBC (P<0.10) and haemoglobin content (P<0.05) in heterozygous AG compared to homozygous GG pigs.

Table 3. Effect of MC4R genotypes and crossbreed on some carcass and meat quality traits

	$A_{(DuSL)\ Pi}$		$B_{(SLVJ)Pi}$	
	AG LSM (SE)	**GG** LSM (SE)	**AG** LSM (SE)	**GG** LSM (SE)
Hot carcass weight (kg)	78.75 (1.45)	78.62 (1.81)	81.0 (1.57)	80.75 (1.8)
Backfat thickness (mm)				
BF-1	20.89 (1.28)	21.00 (1.35)	22.25 (1.91)	23.12 (1.35)
BF-2	11.56 (1.19)A	9.12 (1.26)B	14.5 (1.26)C	12.1 (1.18)AD
Diameter of MLD (mm)	67.44 (1.94)A	65.12 (2.06)A	72.75 (2.21)C	71.44 (2.01)B
Lean meat content (%)	58.51 (1.24)A	60.62 (1.02)B	56.91 (1.32)A	58.52 (1.07)A
Meat quality				
pH_i	6.27 (0.09)A	6.39 (0.11)A	5.95 (0.10)B	6.05 (0.08)B
pH_u	5.66 (0.04)A	5.59 (0.04)A	5.53 (0.06)B	5.60 (0.04)AB
Drip loss (%)	5.47 (0.52)A	6.70 (0.46)B	6.72 (0.43)B	6.27 (0.67)B

LSM (SE) - last square means (standard errors); BF-1 – backfat thickness on the last rib level; BF-2 – backfat thickness above the m. gluteus medius; Means with different letters in the same row are significantly different (P<0.05)

The results in Table 3 show that MC4R genotype had significant effect on backfat dept measured above the *m. glutes medius* and estimated lean meat content (P<0.05). GG homozygotes had lower backfat thickness and consequently higher lean meat content than AG heterozygotes. Also, the result showed that crossbred B had significantly higher diameter of MLD (P<0.05) but lower backfat thickness (P<0.10). These results are in agreement with previous study which suggest that G allele is associated with low backfat thickness (Kim et al., 2000b; Hernandez-Sanchez et al., 2003, Houston et al., 2004) and significant decrease of backfat in order GG>AG>AA (Jokubka et al., 2006).

CONCLUSION

The results obtained from this pilot study indicate lower response to physical stress in heterozygote AG pigs in comparison to GG homozygote which support a possible role of the

MC4R Asp298Asn polymorphism in the genetic basis of stress response. Also, the results confirm that GG pigs have a lower backfat thickness and higher estimated lean meat content, but these traits are additionally influenced by crossbreed.

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