Plasma leptin levels in pigs with different leptin and leptin receptor genotypes

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Introduction

The fundamental roles of the leptin (Lep) and leptin receptor (Lepr) hormonal axis in fat deposition, energy expenditure and reproduction have prompted the characterization of Lep and Lepr gene polymorphisms in diverse mammalian species and the analysis of their functional effects (Van der Lende et al. 2005). In human and cattle, polymorphisms in the Lep and Lepr genes have been found to influence serum Lep concentration (Thompson et al. 1997; Strobel et al. 1998; Liefers et al. 2004, 2005). In pigs, diverse Lep and Lepr polymorphisms have been associated with phenotypic variation of a variety of growth, fatness and carcass traits, although their effects on Lep expression have not been explored yet. One of the most studied pig Lep polymorphisms is a silent C3469T substitution at exon 3. The association of this mutation with production traits has been investigated in a variety of porcine breeds leading to controversial results (reviewed in Świtowski et al. 2003). Kręnkowa et al. (1999) reported that the TT genotype is associated with an increased growth rate when compared with the TC genotype, whereas Kulig et al. (2001) described the opposite relationship. Szydlowski et al. (2004) undertook the association analysis of the C3469T polymorphism in several pig breeds concluding that the associations found are population-specific. Recently, De Oliveira Peixoto et al. (2006) reported significant associations between C3469T polymorphism and diverse growth, fatness and carcass traits in a composite pig population. With regard to the Lepr gene, Óvilo et al. (2002) genotyped an

Summary

A C3469T mutation at exon 3 of the pig leptin (Lep) gene has been genotyped in diverse pig breeds yielding controversial results with regard to its association with growth, fatness and carcass traits. A similar situation has been reported for a HpaII restriction fragment length polymorphism (RFLP) in the pig leptin receptor (Lepr) gene, where associations were found depending on the statistical model employed. The main objective of our work was to investigate if leptin plasma concentrations differ in pigs with different C3469T and Lepr HpaII RFLP genotypes. With this aim, we have measured plasma leptin levels at 160 days in 68 Landrace pigs with different Lep C3469T and Lepr HpaII RFLP genotypes. Neither Lep (TT: 11.68 ng/ml, TC: 10.71 ng/ml) nor Lepr (AA: 12.6 ng/ml, AB: 10.93 ng/ml, BB: 11.74 ng/ml) genotypes influenced significantly plasma Lep concentration. Moreover, we did not find any association between Lep and Lepr genotypes and phenotypic variation at growth and fatness traits in a commercial population of 320 Landrace pigs.
intron 4 HpaII RFLP in an F₂ Iberian × Landrace cross and found significant associations depending on the statistical analysis employed. Moreover, Óvilo et al. (2005) analysed the effects of three Lepr missense polymorphisms in an Iberian by Landrace cross and found significant associations between Lepr genotype and backfat thickness and carcass traits. Similarly, Maćkowski et al. (2005) identified a significant association between a Tsp509I RFLP Lepr genotype and backfat over shoulder. In the current work, we have investigated if Lep and Lepr genotypes are associated with a differential level of plasma Lep concentration as well as with phenotypic variation at several production traits in a Landrace population.

Materials and methods

Animal material and phenotypic records

Pigs used in the experiment were taken from a random sample of 320 halothane free Landrace males and females belonging to the experimental farm of Nova Genética S.A. (Lleida, Spain). Pigs had ad libitum access to a commercial diet and they were weighed at 28 days (n = 120, from 18 to 30 days) and at 160 days of age (n = 320, from 152 to 164 days). At 160 days, ultrasonic backfat was measured at 5 cm off the midline with a Renco Lean-Meater ultrasonic device (Renco Corp., Minneapolis, MN, USA) and a 10-ml blood sample was obtained with a syringe and kept in tubes containing EDTA. Blood collection took place during the morning (approximately between 8 and 10 AM) and pigs had not been fasted. Plasma was obtained by centrifugation at 3000 g for 10 min and frozen at −40°C until required.

All pigs were slaughtered in the same commercial slaughterhouse, where carcass traits were measured. Live weight was recorded 24 h prior to slaughter. Hot carcass weight was used to calculate the killing out percentage. Carcass length was measured from the anterior edge of the symphysis pubis to the recess of the first rib. After chilling for 24 h at 2°C, both sides of each carcass were divided into standardized commercial joints. Weight of untrimmed hams was collected according to the customary procedure used in the Spanish slaughterhouses. At 24 h postmortem, a slice of about 200 g of semimembranous muscle was taken from the left ham of the carcass, vacuum packaged and stored in a deep freeze until required. Once defrosted, vacuum drip losses were eliminated and, after trimming, the excess of fat was minced. A representative aliquot of the freeze-dried muscle was used to determine intramuscular fat content by ether extraction (AOAC 1990) in a Soxhlet apparatus (Kimble/Kontes, Vineland, NJ, USA).

Plasma leptin concentration was determined in 68 Landrace males by using an ELISA kit based on a polyclonal antibody raised against mouse leptin (Crystal Chem. Inc., Downers Grove, IL, USA). Recombinant porcine leptin (Diagnostic Systems Laboratories, Inc., Webster, TX, USA) was used to standardize this test. Standard curves were calculated by linear regression in duplicate concentrations of 12 800, 6400, 3200, 1600, 800, 400 and 200 pg/ml with r² ≥ 0.98. Plasma samples were assayed in duplicate. In our hands, the leptin ELISA kit had an intra-assay coefficient of variation (CV) of 5.7–7.2% and an inter-assay CV of 12.27–15.76% (at concentrations of 200 and 12 800 pg/ml). Intra-assay and interassay repeatability coefficients were 0.97 and 0.91 respectively.

Genotyping of the pig Lep and Lepr genes

Genomic DNA isolation from muscle samples was performed by incubating 300 mg samples in 5 ml lysis buffer (Tris 50 mm pH = 8, EDTA 20 mm and 5% SDS) and 50 μl protease K (10 mg/ml) at 37°C overnight and gentle agitation. Five hundred microlitre of the lysate was phenol–chloroform extracted and ethanol precipitated. The DNA pellet was resuspended in 50 μl of ultrapure water and 2 μl RNase (10 mg/ml). The isolation of genomic DNA from blood samples was performed as previously reported (Vidal et al. 2005). We genotyped a C3469T mutation at exon 3 of the pig Lep gene in 320 Landrace pigs (Stratil et al. 1997). Primer sequences were Lep-FW, 5′-CTG TCT CCT CCA AAC AGA GGG TCA-3′ and Lep-REV, 5′-CAG CAG CCA GGG CTG AGG TCC A-3′. The PCR mixture contained 1.5 mm MgCl₂, 100 μm dNTP, 0.5 μm of each primer, 100 ng genomic DNA and 0.625 U Tαq DNA polymerase (Ecogen SRL, Barcelona, Spain) in a final 25 μl volume. The thermal profile included 30 cycles of 94°C for 1 min, 69°C for 1 min and 72°C for 1 min. The 353 bp PCR product was digested with 5 U HinfI for 4–5 h and electrophoresed in a 3% agarose gel. The HinfI digestion of the Lep amplicon yielded two restriction patterns named as T (355 bp) and C (293 and 62 bp). A 2-kb fragment corresponding to intron 4 of the pig Lepr gene was amplified by following the procedure reported in Stratil et al. (1998). Digestion of the 2-kb amplicon with 5 U HpaII for 4–5 h yielded two restriction patterns A (2 kb) and B (1.45 and 0.55 kb). Restriction fragments were electrophoresed in a 1.5% agarose gel allowing to identify the three Lepr genotypes.
Statistical methods

A mixed model was used to describe the data. The model was as follows:

\[ Y_{ijkl} = \mu + \text{batch} \times \text{sex}_j + \text{genotype}_k + b \times \text{age}_{ijkl} + a_{ijkl} + \epsilon_{ijkl} \]

where batch*sex\(_j\) is the fixed effect of batch \(i\) (1–11); by sex \(j\) (male or female); genotype\(_k\) is the effect of \(\text{Lep}\) (TT, TC or CC) or \(\text{Lepr}\) (AA, AB or BB) genotype \(k\); age\(_{ijkl}\) is a covariate to account for the deviations from the mean age at which controls were made; \(a_{ijkl}\) is the random polygenic additive effect associated to each pig; and \(\epsilon_{ijkl}\) is the residual term. Carcass weight was used as a covariate for the traits registered after slaughter instead of the age at control. Polygenic additive effects were assumed to be multivariate normally distributed \(N(0, \Sigma \sigma^2)\), in which \(\Sigma\) is the numerator relationship matrix, and \(\sigma^2\) is the polygenic additive variance. A two-generation pedigree was considered to calculate \(\Sigma\). Residuals were assumed to be uncorrelated with variance \(\sigma^2\). The variance–covariance matrices have been estimated by residual maximal likelihood (REML) using the EM algorithm, as applied in the REMLF90 programs (Misztal 1999). Iterations were performed until the criterion of convergence was less than \(1 \times 10^{-7}\). Estimated fixed effects and predicted random effects were obtained by considering the estimated variance–covariance parameters as the true parameters. After performing the Bonferroni correction for multiple tests, associations were considered significant when \(p \leq 0.01\).

Results and discussion

Genotype frequencies of the \(\text{Lep}\) single nucleotide polymorphism in our Landrace population were TT: 0.521, TC: 0.398, CC: 0.081. The CC genotype is very rare in most of the pig breeds, with frequencies ranging from 0 to 0.02 in Polish Landrace, Piétrain and Large White pigs (Kuryl et al. 2003; Szydlowski et al. 2004). The C allele has also shown low frequencies in the Duroc, Landrace and Yorkshire breeds (Kennes et al. 2001). The frequencies of the \(\text{Lepr}\) genotypes were AA: 0.034, AB: 0.307 and BB: 0.659. These results are similar to the ones reported by Stratil et al. (1998), where the B allele was the predominant one in the Landrace breed as well as in Meishan, Large White and Czech meat pig amongst others.

Our main goal was to investigate if there are differences in plasma \(\text{Lep}\) levels amongst pig \(\text{Lep}\) and \(\text{Lepr}\) genotypes to gain new insights into the molecular basis of the phenotypic associations reported in the literature. The mean levels of plasma leptin we have found in our Landrace population were slightly higher than the ones reported in other studies (Berg et al. 2003; Estienne et al. 2003), but they are completely consistent with what has been published in human (Sone & Osamura 2001, mean plasma leptin concentration: 8 ng/ml), mouse (Gregersen et al. 2003), dog (Sagawa et al. 2002) and cat (Shibata et al. 2003). This discrepancy between our and previous studies focused on pigs might be explained by (i) the existence of a significant variation in plasma \(\text{Lep}\) levels amongst pig populations, as reported by Berg et al. (2003) and (ii) the fact that we have measured \(\text{Lep}\) levels with ELISA whereas previous studies employed a radioimmunoassay technique. In any case, it is worth mentioning that measurement of plasma leptin levels in diverse mammalian species has revealed differences of one and even two orders of magnitude amongst individuals (Sagawa et al. 2002; Friedman-Einat et al. 2003; Shibata et al. 2003). Of outmost importance, the intra-assay and interassay repeatability coefficients of our ELISA technique were very high ensuring that the comparisons of leptin plasma concentrations amongst pigs with different \(\text{Lep}\) and \(\text{Lepr}\) genotypes were not significantly biased by the experimental procedures involved in the measurement of this phenotype. Only one animal had CC genotype in the sample of animals with measured \(\text{Lep}\) plasma levels, so we had not included this genotype in the analysis. We have found that \(\text{Lep}\) plasma levels were very similar when comparing TC with TT \(\text{Lep}\) genotypes (Figure 1a), with a slight (and non-significant) superiority of the TT genotype. Moreover, we did not observe any significant difference in plasma \(\text{Lep}\) levels amongst \(\text{Lepr}\) genotypes (Figure 1b).

Next, we measured growth, fatness and carcass traits in 320 Landrace pigs (Table 1) and performed an association analysis so as to find out if there is any relationship between \(\text{Lep}\) genotypes and phenotypic variation at production traits (Table 2). We found only a weak association (\(p = 0.11\)) between \(\text{Lep}\) genotype and weight of hams at 160 days. The size of our Landrace population is greater than the ones employed in other published studies (Kennes et al. 2001; Kulig et al. 2001, Szydlowski et al. 2004), although we cannot completely rule out the possibility that the absence of significant associations has been produced by a lack of statistical power (particularly for these traits where only 120 registers were taken). This problem is exacerbated by...
the low frequency of the CC genotype, a circumstance that makes difficult the comparison between the two homozygous genotypes which, in principle, under an additive model, would be expected to display the most extreme phenotypes. An optimal approach to tackle this issue in further studies would be to genotype a few thousands of individuals and subsequently to register phenotypic traits in a subsample where all genotypes are equally represented. In any case, our data contrast markedly with the highly significant association between Lep C3469T genotype and average daily weight gain reported by Kennes et al. (2001) in a Landrace population (n = 102). Kulig et al. (2001) genotyped 131 Landrace pigs and found that lean meat content and average daily gains in TC pigs are higher than those of TT individuals. Conversely, Szydlowski et al. (2004) did not find any association between Lep C3469T genotype and any of the 10 growth and fatness traits measured in a population of 120 Polish Landrace pigs, although they found associations with intramuscular fat content in Large White pigs (n = 135) and with loin weight in a synthetic L990 line (n = 184). They concluded that the associations found are population-specific (Szydlowski et al. 2004). However, each of these populations, considered individually, has a rather limited size making difficult to assess the reliability and biological significance of the results found. More recently, De Oliveira Peixoto et al. (2006) genotyped more than 800 pigs from a composite F2 population created by crossing two Brazilian Piau boars with 18 Landrace, Large White and Pietrain sows. In doing so, they found significant associations between C3469T and weight at several ages, feed intake, feed conversion, average daily gain and bacon depth. However, it should be noted that results obtained from divergent F2 crosses cannot always be extrapolated to highly selected commercial populations (Vidal et al. 2005).

The association analysis between Lepr genotypes and growth, fatness and carcass traits did not reveal subsequently to register phenotypic traits in a subsample where all genotypes are equally represented.

Table 1 Number of records, mean and residual standard deviations (RSD) adjusted for batch for the analysed growth and fatness variables measured at 160 days in a Landrace population

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Mean</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight at 28 days (kg)</td>
<td>120</td>
<td>7.29</td>
<td>1.37</td>
</tr>
<tr>
<td>Weight at 160 days (kg)</td>
<td>320</td>
<td>94.85</td>
<td>9.22</td>
</tr>
<tr>
<td>Backfat depth at 160 days (mm)</td>
<td>320</td>
<td>11.62</td>
<td>1.40</td>
</tr>
<tr>
<td>Weight gain 28–160 days (kg)</td>
<td>120</td>
<td>94.18</td>
<td>9.55</td>
</tr>
<tr>
<td>Carcass weight (kg)</td>
<td>241</td>
<td>79.29</td>
<td>8.29</td>
</tr>
<tr>
<td>Carcass length (cm)</td>
<td>241</td>
<td>81.67</td>
<td>3.26</td>
</tr>
<tr>
<td>Killing out (%)</td>
<td>241</td>
<td>74.49</td>
<td>2.38</td>
</tr>
<tr>
<td>Intramuscular fat (% DM)</td>
<td>241</td>
<td>9.41</td>
<td>3.82</td>
</tr>
<tr>
<td>Intramuscular fat (% FM)</td>
<td>241</td>
<td>2.65</td>
<td>1.07</td>
</tr>
<tr>
<td>Weight of hams (kg)</td>
<td>241</td>
<td>24.15</td>
<td>2.42</td>
</tr>
</tbody>
</table>

DM, dry matter basis; FM, fresh matter basis.

Figure 1 Mean plasma leptin concentration (with its standard deviation) at 160 days in pigs with TC (n = 13) and TT (n = 45) Lep C3469T genotypes (a); and with AA (n = 6), AB (n = 22) and BB (n = 31) Lepr intron 4 HpaII RFLP genotypes (b). None of the genotype comparisons was significant.
any significant association (Table 2). Previously, Óvilo et al. (2002) showed that Lepr genotypes are associated with backfat thickness and intramuscular fat content when an animal model, with the Lepr genotype as a fixed factor, is employed. In contrast, when the genotypes of Lepr and seven flanking microsatellite markers are analysed jointly in a QTL regression model, these effects disappear demonstrating that they are produced not by the Lepr locus but by other nearby QTL. These results were later confirmed by genotyping three missense Lepr mutations and 13 flanking microsatellites in an Iberian × Landrace cross (Óvilo et al. 2005). This experiment allowed to demonstrate the existence of one QTL at 60–100 cM, with effects on backfat thickness and weight of the ribs with sternum, and another one peaking at 130–132 cM with highly significant effects on diverse fatness and carcass traits. The confidence interval of this second QTL encompassed the Lepr locus (122 cM, Óvilo et al. 2005).

In summary, further studies will be required to fully understand the effects of Lep and Lepr genotypes on production traits. The genotyping of markers flanking Lep and Lepr would be needed to ascertain if the associations found are causal or, conversely, they are due to the existence of nearby QTL. This aspect would be particularly critical for the association analysis of Lepr genotype, as this locus is located in a SSC6 QTL-rich region (Óvilo et al. 2005). In addition, functional and expression studies will be needed to evaluate the biological significance of the polymorphisms found. In our study, we provide evidence that the two analysed single nucleotide polymorphism do not have an effect on plasma leptin concentration but it would be interesting to confirm this result by measuring the transcriptional rate of each allele in a representative panel of porcine tissues.

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