Reduced concentration of androstenone and up-regulation of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase mRNA levels by active immunisation against gonadotropin releasing hormone I

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Abstract

The aim of this study was to investigate the efficacy of active immunisation against recombinant maltose binding protein-gonadotropin releasing hormone I (MBP-GnRH-I6) in preventing boar taint. The concentrations of testosterone and anti-GnRH-I antibodies in serum were determined by radioimmunoassay and enzyme linked immunosorbent assay, respectively. The mRNA levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) in testes were analysed by real time quantitative PCR. The concentrations of androstenone and 3-methylindole in backfat were assayed by HPLC. Active immunisation against MBP-GnRH-I6 increased the serum concentration of anti-GnRH-I antibodies (P<0.05) and the levels of 3β-HSD and 17β-HSD mRNA in testes. However, it reduced the serum concentrations of testosterone (P<0.05) and androstenone. The concentration of 3-methylindole in backfat (P=0.05) was not changed. Thus, we conclude that vaccination against MBP-GnRH-I6 is a practical and effective method to suppress the synthesis of androstenone.

Introduction

Nowadays in China, surgical castration of male piglet is a common practice in pig farming to prevent boar taint in the meat. The unpleasant odour of meat is caused by the accumulation of androstenone and skatole (3-methylindole) in fat (Gunawan et al., 2013). Androstenone is a testicular steroid that accumulates in fat tissue (Kantas et al., 2014). Skatole is a tryptophan metabolite produced by intestinal bacteria in gut and catabolised in liver (Gunawan et al., 2013).

A quantitative trait loci (QTL) of fat androstenone has been detected on pig chromosome 4 (SSC4) (Quintanilla et al., 2003; Lee et al., 2005). The gene encoding 3β-hydroxysteroid dehydrogenase (3β-HSD) has been mapped within this QTL to the position SSC4q16-q21 (Chen et al., 2007). 3β-HSD is a bifunctional enzyme, which catalyses the oxidative conversion of both delta (5)-ene-3 beta-hydroxy steroid and ketosteroid. Moreover, the 3β-HSD enzymatic system plays a crucial role in the biosynthesis of all kinds of hormonal steroids. 17β-hydroxysteroid dehydrogenase (17β-HSD), which catalyses the reversible reactions between estrone and estradiol and between androstenedione and testosterone, is essential for the regulation of the levels of bioactive steroids in tissue (Suzuki et al., 2000). Both 3β-HSD and 17β-HSD are involved in testicular steroid biosynthesis such as androstenone production (Simard et al., 2005; Chen et al., 2007). Therefore, it is interesting to investigate their mRNA abundance and association with other testicular steroids, particularly androstenone that is implicated in boar taint.

Currently, the best alternative method to prevent boar taint appears to be immunocastration by vaccination against gonadotropin releasing hormone I (GnRH-I) (Gutzwiller et al., 2000). A recombinant fusion protein was developed in our laboratory as a vaccine for immunocastration of boar (Fang et al., 2009, 2010b). The recombinant fusion protein consists of 6 copies of GnRH-I in linear sequence (GnRH-I6) and a maltose binding protein (MBP) (MBP-GnRH-I6) (Fang et al., 2009, 2010b). Its effectiveness in suppressing reproductive function has been demonstrated in mice (Fang et al., 2009) and boars (Fang et al., 2010a).

The purpose of this study was to investigate the efficacy of MBP-GnRH-I6 in the levels of androstenone and skatole in fat and regulating the androstenone biosynthetic pathway involving 3β-HSD and 17β-HSD, and in eliminating boar taint.

Materials and methods

Animals and handling

The Animal Care and Use Committee of Anhui Agricultural University, China, approved all procedures involving animals, and the study was conducted at the Dadun pig farm. Thirty Chinese Taihu boars from a commercial pig farm were kept according to a well-managed hog farm. The animals were fed a different diet according to the different weight, that is, 6 MJ metabolisable energy/kg of feed and 18.0% crude protein at an amount of 1.86 kg per day for 20 to 50 kg, 8.4 MJ metabolisable energy/kg of feed and 15.5% crude protein at an amount of 2.58 kg per day for 50 to 80 kg, 10 MJ metabolisable energy/kg of feed and 13.2% crude protein at an amount of 3.0 kg per day, which was offered in two portions in the morning and late afternoon. Boars were assigned randomly to three groups. The first group of ten male piglets was surgically castrated before 1 week of age. The second group of ten male pigs was immunised with 2 mL vaccine containing MBP-GnRH-I6 (Fang et al., 2010b) containing Al(OH)3 adjuvant (Sigma Aldrich, St. Louis, MO, USA) (250 mg MBP-GnRH-I6 protein/mL). Vaccination was performed twice at 9 and 17 weeks of age by intramuscular injection. The remaining 10 male pigs were kept gonadally intact and without MBP-GnRH-I6 treatment throughout the study.
Collection of samples
All pigs were weighed before first immunisation at 9 weeks of age and euthanised at 25 weeks of age. A blood sample (5 mL) one day before euthanisation was collected into a heparinised Vacutainer tube via jugular venipuncture. Serum was separated by centrifugation at 2000 × g for 15 min at 4°C and kept at −80°C until analysis. Total RNA was isolated from testes samples taken immediately and frozen in liquid nitrogen; then, they were transported to the laboratory within 1 h, and stored at −80°C until analyses.

Determination of anti-gonadotropin releasing hormone I antibody titer
The GnRH-I antibody titer in serum was determined by ELISA as described in Fang et al. (2010a).

Steroid analysis
Testosterone concentration in serum was measured using a commercial RIA kit (Furui Bioengineering, Beijing, China) according to the manufacturer’s instructions. Sensitivity of the assay was 0.002 ng/mL. The intra- and inter-assay CV was less than 9.5 and 10.3%, respectively. Correlation coefficient of standard curve was 0.99936.

Fat androstenone and 3-methylindole were measured by HPLC (Hansen-Møller, 1994) with the following modifications. In brief, samples were subjected to microwave and tissue-determination by ELISA (140 750 at 2000 × g for 15 min at 4°C and kept at −80°C until analysis). The concentration of testosterone was determined by gel electrophoresis with 1% agarose containing formaldehyde. The concentration of total RNA was measured by absorbance at 260 nm with a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Real-time PCR
First strand cDNA was synthesised from total RNA (0.5 μg) by EnergeticScript (Synthesis Kits; ShineGene, Shanghai, China). Real-time PCR on the first strand cDNA was performed on a FTC2000 instrument (Funglyn, Scarborough, Canada) using ShineSybr Real Time qPCR Master Mix Kit (ShineGene) and. Each reaction well in a 96-well plate contained 30 pmol each of the forward and reverse primers, 1×Hotstart Fluoro-PCR mix and 1 μL cDNA. The final reaction volume was 50 μL. All samples were run in triplicate. PCR parameters were as following: preliminary denaturation at 94°C for 3 min and 40 cycles of 94°C for 20 s, 60°C for 20 s and 72°C for 20 s. The absence of nonspecific products was confirmed by analysis of the melting curves. Primer information for 3β-HSD gene (accession number: NM_001004049.1), 17β-HSD (Chen et al., 2007) and the internal control β-actin (accession number: U07786.1) were shown in Table 1.

Calculations and statistical analyses
Relative mRNA quantification was performed using the relative standard curve method. The relative 3β-HSD and 17β-HSD expression values were obtained by dividing the value of 3β-HSD and 17β-HSD with the value of β-actin, respectively. Comparison of the levels of 3β-HSD and 17β-HSD mRNA, serum testosterone, anti-GnRH-I antibody, fat androstenone and 3-methylindole among groups of animals were performed using Student’s t-test. Differences were considered to be significant at P<0.05.

Results
Body weight
At 9 weeks of age, there was no difference in the mean live weight among groups (P>0.05). At 25 weeks of age, however, the MBP-GnRH-I6 immunised male pigs and gonadally intact male pigs were heavier than the surgically castrated pigs (P<0.05; Table 2).

Anti-gonadotropin releasing hormone I antibody titer
The titer of anti-GnRH-I antibody one day before euthanisation was measured by ELISA. The titer of anti-GnRH-I in vaccinated boars was significantly higher than that in the other groups (P<0.01) (Figure 1), suggesting that MBP-GnRH-I6 induced a strong anti-GnRH-I response.

Concentrations of steroids and 3-methylindole
Concentrations of serum testosterone, fat androstenone and 3-methylindole in all pigs at 25 weeks of age are shown in Table 3. Serum testosterone levels in surgical castrated animals were below the limit of detection. In MBP-GnRH-I6 immunised male pigs, serum testosterone levels were much lower (P<0.05) than that of gonadally intact male animals.

Fat androstenone concentrations were below the limit of detection (0.05 μg/g) in surgical castrated animals and MBP-GnRH-I6 immunised male pigs. However, the fat androstenone concentration was high in gonadally intact male animals. Concentrations of 3-methylindole did not show any difference (P>0.05) among groups.

3β- and 17β-hydroxysteroid dehydrogenase mRNA levels in testis
Both 3β-HSD and 17β-HSD mRNA levels of the testis samples in the gonadally intact male animals were lower than that in the MBP-GnRH-I6 immunised male pigs (P<0.05; Figure 2).

Table 1. Primer sequences for real-time PCR and size of the amplified products for aim gene and β-actin house-keeping genes.

| Gene name | Forward primer (5’–3’ ) | Reverse primer (5’–3’ ) | Product (bp) |
|-----------|--------------------------|--------------------------|--------------|
| 3β-HSD    | TGGGCCGTCGCTCATGTTGAT   | CTGCCGCTGCGCTCTGTGTA   | 184          |
| 17β-HSD   | AGTCTCTCAATCCCTGTTGAAATC | TCTCCTACTAGGCTCATCTTTTG | 91           |
| β-actin   | GCCGACAGGATGCAGAAGG     | TGGAAAGTGGACACGGAGG    | 131          |

3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.
Discussion

Immunisation with MBP-GnRH-I6 increased the concentrations of anti-GnRH-I antibody in the serum and mRNA levels of 3β-HSD and 17β-HSD in the testis, and reduced the concentrations of serum testosterone and fat androstenone, suggesting that MBP-GnRH-I6 treatment of boars can prevent boar taint effectively. Therefore, this method represents an effective and practical alternative to surgical castration.

Immunisation with MBP-GnRH-I6 is effective in increasing the anti-GnRH-I antibody and reducing the testosterone level, as reported previously (Fang et al., 2010a). Several other studies have also demonstrated that successful immunological castration reduced testosterone levels to a very low level (Oonk et al., 1998; Zamaratskaia et al., 2008; Claus et al., 2008; Bilskis et al., 2012). In the current study, the first immunisation took place at the age of 9 weeks, which is rather different from procedures with other products (Meloen et al., 1994; Zamaratskaia et al., 2008; Claus et al., 2008). The reason is that the time of puberty onset varies with pig breed. Generally, the time of puberty onset varies with pig breed. Generally, the time of puberty onset in Chinese local pig breed is earlier than the exotic breed, so the first immunisation time in our study was earlier than the other reports (Zamaratskaia et al., 2008; Claus et al., 2008).

The mean body weight of the MBP-GnRH-I6 immunised pigs is not significantly different from that of gonadally intact male pigs, but greater than that of surgically castrated male pigs. It was shown that GnRH-I immunised animals had a significantly higher body weight than control animals (Cronin et al., 2003; Zeng et al., 2001). Therefore it is likely that the MBP-GnRH-I6 immunised animals had the benefits of male steroid hormones. The androgens could have stimulated growth (Zeng et al., 2001).

Androstenone has been known for years to be responsible for the unpleasant urine-like odours in boars (Claus, 1979). Androstenone is a male-specific hormone. Production of androstenone is dependent on sexual maturity (age and weight) and genotype of the animal (Claus, 1979; Oonk et al., 1995). In the present study, active immunisation reduced fat.

Table 2. Change of body weight in surgically castrated male pigs, maltose binding protein-gonadotropin releasing hormone I immunised boars and gonadally intact male pigs.

| Age, week | GI, kg   | SC, kg    | MI, kg   |
|-----------|----------|-----------|----------|
| 9         | 28±3.85  | 29±3.21   | 28±4.09  |
| 25        | 112.56±9.02 | 96.90±14.09* | 109.63±8.22 |

GI, gonadally intact male pigs (n=10); SC, surgically castrated male pigs (n=10); MI, MBP-GnRH-I6 immunised male pigs (n=10). *P<0.05. Values are presented as mean±standard deviation.

Table 3. Effect of maltose binding protein-gonadotropin releasing hormone I immunisation on the concentrations of serum testosterone, backfat androstenone and 3-methylindole.

|          | GI                | SC                | MI                |
|----------|-------------------|-------------------|-------------------|
| Serum testosterone, ng/mL | 3.55±1.40         | <0.002°           | 1.01±0.91*        |
| Fat androstenone, μg/g     | 1.161±0.631       | 0.095±0.053       | 0.127±0.064       |
| Fat 3-methylindole, μg/g   | 0.102±0.075       | 0.095±0.053       | 0.127±0.064       |

GI, gonadally intact male pigs (n=10); SC, surgically castrated male pigs (n=10); MI, MBP-GnRH-I6 immunised male pigs (n=10). °Value below the detection limit; the detection limits of the testosterone and androstenone assay were 0.002 ng/mL and 0.05 μg/g, respectively. *Significant difference (P<0.05) between GI and MI. Values are presented as mean±standard deviation.

Figure 1. Detection of antibodies in boars immunized with MBP-GnRH-I6. Microwell plates were coated with 100 μL antigen (10 μg/mL) and reacted with sera collected from MBP-GnRH-I6 immunised male pigs (n=10), surgically castrated pigs (n=10) and gonadally intact male pigs (n=10) at 1 d before slaughter. The results (mean±standard deviation) shown here are for 1:100 to 1:50,000 dilutions of sera of A450 nm value. Significant differences (P<0.05) were observed in the antibody titers in immunised boars using Student's t-test. ■ MBP-GnRH-I6 immunised male pigs; ▲ surgically castrated male pigs; ♦ gonadally intact male pigs.

Figure 2. Relative quantity of 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA and 17β-hydroxysteroid dehydrogenase (17β-HSD) mRNA in testis. GI, gonadally intact male pigs (n=10); MI, MBP-GnRH-I6 immunised male pigs (n=10). Values are means of the relative quantity, with their standard deviation shown by the vertical bars. Asterisk indicates significant difference (P<0.05) between groups.
androstenone to levels below 0.5 μg/g, which is similar to that of surgically castrated male pigs. The findings of the present study are in agreement with previous reports (Bonneau et al., 1994; Meloen et al., 1994; Zeng et al., 2001) and commercial vaccine Improvac® (Gutzwiller et al., 2013; Kantas et al., 2014). A level of androstenone 0.5 μg per gram of fat was considered as a threshold level for the detection of boar taint in fresh pork (Bonneau et al., 1992; Zeng et al., 2001).

In addition to androstenone, skatole (3-methylindole) appears to be another compound contributing to boar taint. Onkø et al. (1995) reported that skatole (3-methylindole) may contribute more to boar taint than steroids when pigs were euthanised at a low weight (<80 kg). Many studies (Babøl et al., 1996; Mølten et al., 2002) showed that active immunisation against GnRH-I reduced the concentrations of fat skatole to a value below 0.2 μg/g, which is the threshold level for the detection of boar taint in fresh pork (Dunshea et al., 2001; Bonneau et al., 2000; Kantas et al., 2014). In the present study, the average body weight was about 100 kg at euthanisation and all animals had low 3-methylindole levels below 0.2 μg/g. Thus, the contribution of androstenone to boar taint appears to be more important.

Androstenone is synthesised in the Leydig cells (Claus et al., 1994; Chen et al., 2007). Both 3β-HSD and 17β-HSD belong to the short-chain alcohol dehydrogenase reductase superfamily (Chen et al., 2007), which is involved in the production of androstenone and testosterone (Simard et al., 2005). The enzyme 3β-HSD catalyses the transformation of androstenone to β-androstenediol (Doran et al., 2004), which is accumulated to only low levels in fat due to its 3-OH hydrophilic structure, and therefore it is less important in causing boar taint (Garcia-Regueiro and Diaz, 1989). Low 3β-HSD enzyme activity is associated with large androstenone accumulation in the fat (Nicolaou-Solano et al., 2006). The enzyme 17β-HSD catalyses the reversible transformation between estrone and estradiol, and between androstenedione and testosterone in humans (Suzuki et al., 2000), but its function in pigs has not been determined. In our study, MBP-GnRH-I6 immunisation caused a significant increase of the levels of 3β-HSD and 17β-HSD mRNA in the testis as compared with the control pigs. These findings are consistent with other reports on the effect of a commercialised GnRH-I vaccine, Improvac, which increases the levels of 3β-HSD and 17β-HSD mRNA in testis (Chen et al., 2007). The increase of the 3β-HSD and 17β-HSD mRNA levels in the MBP-GnRH-I6 immunised pigs was most likely due to the decreases levels of testicular steroids. Indeed, Clark et al. (1996) reported that steroid negatively influenced the regulation of the expression of 3β-HSD. Endogenously produced testosterone repressed 3β-HSD expression (Payne and Sha, 1991).

Conclusions

In conclusion, this study has shown that immunisation with the MBP-GnRH-I6 increased the levels of 3β-HSD and 17β-HSD mRNA in testis. MBP-GnRH-I6 was very effective in eliminating boar taint and could be a potential candidate of vaccination for immunological castration.

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