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Feeding dried chicory root to pigs decrease androstenone accumulation in fat by increasing hepatic 3β hydroxysteroid dehydrogenase expression

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ABSTRACT

The present study investigated the *in vivo* effect of chicory root on testicular steroid concentrations and androstenone metabolizing enzymes in entire male pigs. Furthermore, the effect on skatole and indole concentrations in plasma and adipose tissue was investigated. The pigs were divided into two groups; one receiving experimental feed containing 10% dried chicory root for 16 days before slaughter, the control group was fed a standard diet. Plasma, adipose and liver tissue samples were collected at slaughter. Plasma was analyzed for the concentration of testosterone, estradiol, insulin-like growth factor 1 (IGF-1), skatole and indole. Adipose tissue was analyzed for the concentration of androstenone, skatole and indole, while the liver tissue was analyzed for mRNA and protein expressions of 3β-hydroxysteroid dehydrogenase (3β-HSD), sulfotransferase 2A1 and heat-shock protein 70 (HSP70). The results showed that the androstenone concentrations in the adipose tissue of chicory fed pigs were significantly ($p < 0.05$) lower and indole concentrations were higher ($p < 0.05$) compared to control fed pigs. Moreover the chicory root fed pigs had increased mRNA and protein expression of 3β-HSD and decreased HSP70 expression ($p < 0.05$). Testosterone and IGF-1 concentrations in plasma as well as skatole concentrations in adipose tissue were not altered by dietary intake of chicory root. It is concluded that chicory root in the diet reduces the concentration of androstenone in adipose tissue *via* induction of 3β-HSD, and that these changes were not due to increased cellular stress.

1. Introduction

Steroids of testicular origin are known for their diverse effect in pigs. Male pigs possess high concentrations of androstenone (5α-androst-16-en-3-one), estradiol and testosterone in plasma [1]. This leads to increased levels of the main boar taint-related compounds androstenone and skatole (3-methylindole) in adipose tissue in sexually mature male pigs and decreased meat quality. Clearance of endogenous compounds and xenobiotics by the liver is usually conducted in two steps: Phase I and II [2]. Phase I typically consists of an oxidation of the compound usually catalyzed by cytochrome P450 (CYP450) enzymes, while Phase II is conducted by a more diverse group of enzymes and consists of conjugation with a hydrophilic group, like glucuronidation, sulfoconjugation or glucosidation. The outcome of Phase I and/or II metabolism is often the elimination of the compound by excretion. In pigs, the endogenous boar taint compounds androstenone and skatole are processed by Phase I and II enzymes before excretion *via* the urine [3]. Hepatic metabolism of androstenone in pigs is mainly mediated by the 3β-hydroxysteroid dehydrogenase (3β-HSD) followed by sulfoconjugation by hydroxysteroid sulfotransferase isoforms 2A1(SULT2A1) or 2B1 [3]. The Phase I metabolism of skatole is mainly mediated by hepatic CYP1A2, CYP2A and CYP2E1 [4,5].

There is a strong interaction between testicular steroids and skatole metabolism in the liver due to involvement of testicular steroids in the regulation of CYP450. *In vitro* experiments with porcine primary hepatocytes have shown that androstenone increases the protein expression of CYP2A6, while estradiol sulfate and testosterone had no effect on CYP2A6 protein expression [6]. Moreover, in porcine primary hepatocytes, androstenone was shown to inhibit a skatole induced increase in CYP2E1 expression [7]. However, androstenone alone did not affect CYP2E1 expression. Kojima et al. [8] showed that administration of testosterone propionate to pigs decreased the expression of CYP1A. The suggestion that CYP450 expression in pigs is affected by steroids is further supported by studies showing difference in CYP450 expression/activity with respect to gender [9–13], castration [14] and immunocastration (active immunization against GnRH) [11,15]. These studies provide strong evidence that androgens and estrogens have a down-regulating effect on CYP450 expression and activity.

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Apart from testicular steroids, dietary composition has been shown to modulate CYP450 expression in humans [16], rats [17] and pigs [18]. Oral administration of chicory root to male pigs was shown to increase the expression and activity of CYP1A2 and 2A. The observed increase in CYP450 expression in chicory fed pig and the suggestion that steroids down-regulate CYP450 expression, led to the hypothesis that chicory fed pigs has decreased concentrations of testicular steroids and increased expression of steroid metabolizing enzymes.

This study is an extension of previous work from our research group showing that administration of chicory root to sexually mature pigs increases CYP2A, 1A2 and 2E1 mRNA expression, as well as CYP2A and CYP1A2 activity and protein expression [18]. The aim of the present study was to investigate the effect of oral administration of chicory root on steroid concentration in plasma and adipose tissue and steroid metabolism in the liver. The mRNA and protein expression of 3β-HSD and SULT2A1 was analyzed. Increased expression of phase I and/or II enzymes are often related to toxicological responses, so to evaluate if this is the reason for the changes related to consumption of chicory we investigated, the mRNA and protein expression of heat-shock protein 70 (HSP70). The expression of HSP70 is related to increased hepatic cellular stress [19,20].

2. Materials and methods

2.1. Animals and sampling

Experimental design and slaughter procedure is described in Rasmussen et al. [18]. Briefly, 22 entire male pigs were randomly assessed to experimental (16 pigs) or control (6 pigs) feeding strategies 16 days prior to slaughter. The pigs receiving experimental feed were given a diet containing 10% dried chicory root, while the control group remained fed with the energy matched control diet. The two groups of pigs were kept in separate pens. All pigs were fed *ad libitum* and slaughtered at the same age in a commercial slaughter house. Blood samples were collected at the time of killing, while liver and adipose tissue samples were collected approximately 20 min later at the opening of the carcass. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Due to an error during the slaughter procedure, only 14 tissue samples from the experimentally fed pigs were obtained. Blood samples were kept on ice until arrival at laboratory. At arrival, plasma was separated from blood cells by centrifugation at 2000 × *g* for 15 min and stored at −20 °C.

2.2. Determination of testosterone, estradiol and IGF-1

Testosterone concentration in plasma was measured using a commercial RIA kit (TKTT, Diagnostic Products Corporation, Los Angeles, CA, USA), according to the manufacturer's instructions.

IGF-1 concentration in plasma was measured using a commercial EIA kit (DSL-10-2800, Diagnostic System Laboratories, Webster, TX, USA) in accordance with the manufacturer's instructions.

Estradiol concentration in plasma was measured using a commercial EIA human salivary kit (1-3702, Salimetrics, State College, PA, USA), through an adaptation of the manufacturer's protocol [21]. In brief, plasma samples were diluted with equal volume water and then kept overnight at 4 °C. 200 μl of the plasma mixtures were then added to the pre-coated wells and incubated 1 h at room temperature on a rotator (500 rpm) and, additionally, 1 h without rotation. After the pre-incubation, all following steps were performed in accordance with the manufacturer's instructions.

2.3. Determination of skatole, indole and androstenone

The determination of skatole and indole concentrations in plasma was performed by UHPLC, through an adaptation of our previous method [22]. Chromatography was carried out on a LaChrom Ultra system (Merck), consisting of a binary pumping system (L-2160U), autosampler (L-2200U), column oven (L-2300), fluorescence detector (L-2485U) and EZ Chrom Elite (Version 3.2.1) software. In brief, plasma samples were mixed with equal volume of acetone and mixed for 30 s. The samples were then kept at −20 °C for 20 min and centrifuged for 20 min at 12.000 × *g* (4 °C). The clear supernatants were injected on a Hibar Purospher STAR (50 mm × 2.1 mm, 2 μm) column (Merck). The mobile phase was delivered isocratically, using 45% methanol and 55% H_2O, both with 0.1% acetic acid. At a flow rate of 0.6 ml/min, indole and skatol were eluted after approximately 1.07 and 2.13 min, respectively. Fluorescence detection was performed with an excitation wavelength of 275 nm and an emission wavelength of 360 nm.

Concentrations were calculated using a standard curve of skatole and indole dissolved in H_2O and measured under the same conditions.

In order to analyze skatole, indole and androstenone in fat, the samples were first liquefied in a microwave oven at 300 W for 3 min. Then 150 μl liquid fat was mixed with 750 μl methanol (containing 0.33 μg/ml androstenone as internal standard) and incubated at 60 °C for 5 min before vortexing for 30 s. After 60 min incubation at −20 °C, the samples were centrifuged at 4500 × *g* for 5 min. For analysis of indole and skatole, the supernatant was analyzed by HPLC according to Chen et al. [23]. For analysis of androstenone, 140 μl of supernatant was mixed with 4.4 μl H_2O, 10 μl BF_3 and 30 μl 2% dansylhydrazine (w/v) and incubated for 5 min before performing HPLC analysis according to Chen et al. [23].

2.4. RNA isolation and semiquantitative PCR

RNA isolation, reverse transcription and PCR was done according to Rasmussen et al. [13]. Briefly, RNA was isolated from approximately 10 mg of homogenized liver tissue using a commercial RNA isolation kit (Rnasey Mini Kit, WVR, Herlev, Denmark), according to the manufacture's instruction. The concentration of isolated RNA was estimated by measuring the absorbance at 260 nm, and the purity assessed by the ration between the absorbance at 260 nm and 280 nm, this ratio was always above 1.8. Equal amount of the isolated RNA was converted to cDNA by the use of SuperScript II Rnase H Reverse Transcriptase and Oligo(dT)12–18 Primer (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. The relative mRNA content of the samples was assessed by the use of PCR with specific primers and a TaqMan probes. Equal volumes of cDNA were mixed with specific primers, TaqMan probes and TaqMan® 2× universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The PCR reaction was conducted in duplicates using a ABI 7900 HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Primers and TaqMan probes (Table 1) were designed using Primer Express (Version 2, Applied Biosystems, Carlsbad, CA, USA). To test for unspecific DNA amplification, samples with genomic DNA or H_2O were subject to the PCR. Relative mRNA expression was calculated by the obtained Ct values and normalized against the mRNA expression of GAPDH. The expression of the control group was arbitrarily set to 1.