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Studies on 5α-androst-16-en-3-one binding to porcine serum, plasma and testicular cytosolic fraction and to human serum

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ABSTRACT

The present study evaluated whether a specific androstenone-binding protein is present in porcine and human serum, and in the cytosolic fraction of porcine testis. The binding of [^{3}H]-androstenone to serum and testicular cytosol was measured in the absence (total binding) and presence (non-specific binding) of unlabelled androstenone. The optimization of the assay is described. As a part of the assay validation, the binding of [^{3}H]-dihydrotestosterone ([^{3}H]-DHT) to porcine and human serum was also examined. As expected, specific binding of [^{3}H]-DHT was detected in human serum, but not in porcine serum. No specific androstenone-binding protein was detected, either in porcine or human serum, or in the cytosolic fraction of porcine testis. The amount of non-specific binding of [^{3}H]-androstenone was slightly lower in porcine serum compared to human serum. Between-animal variations in [^{3}H]-androstenone binding were studied in plasma samples from 15 animals with androstenone concentrations ranging from 1.1 to 23.1 ng/mL. Mean values ± standard deviations of binding in these samples were 15.2 ± 0.9% for total binding and 15.9 ± 0.8% for non-specific bindings. Low between-animal variations indicate that androstenone binding does not affect androstenone accumulation in fat.

1. Introduction

The accumulation of 5α-androst-16-en-3-one (androstenone) in porcine adipose tissue causes a urine-like odour in meat from some entire male pigs, which is a strong contributor to boar taint. The mechanisms by which changes in androstenone levels are regulated remain incompletely understood and are the subject of a huge research effort [1]. It is generally believed that androstenone levels are primarily dependent on testicular synthesis and liver metabolism, and to lesser extent affected by external factors such as raising conditions and feeding system. Additionally, the amount of androstenone that is produced is under genetic control. Recently, it was shown that androstenone accumulation in fat is affected by sulfoconjugation and that sulfoconjugated androstenone did not accumulate in fat due to the higher polarity of the sulfoconjugated-form compared to its free form [2,3]. Variations in the expression of the androstenone-metabolising enzyme hydroxysteroid sulfotransferase (SULT2A1) can affect fat androstenone levels [4]. Similarly, binding of steroids to plasma proteins effectively increases their polarity and reduces their availability to target tissues. The presence of a plasma binding protein for androstenone is of interest since it might regulate the amount of free androstenone which readily accumulates in fat.

Androstenone synthesis in the testis of entire male pigs increases as sexual maturation begins, and androstenone is released into the blood stream and accumulates either in saliva acting as a pheromone, or in fat causing boar taint. In humans, androstenone is a component of body odour and might be responsible for odour-mediated communication [5]. Androstenone synthesis in boar testis and metabolism in the liver has been extensively studied [2,3,6–8]. However, little is known about the circulation of androstenone. In the majority of species, endogenous sex steroids are transported to target tissues complexed with sex hormone-binding globulins (SHBGs). The physiological role of SHBGs includes regulating the bioavailability of steroids. Surprisingly, no SHBGs were detected in porcine plasma [9]; however, the existence of corticosteroid-binding globulin (CBG) [10] and thyroxine-binding protein [11] in pigs have been well described. A specific binding protein for androstenone has been reported in submaxillary glands and saliva of male pigs [12] and porcine olfactory tissue [13]. In humans, spe-

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cific binding of androstenone has been reported in the cytosolic fraction of testis [14]. To our knowledge, androstenone binding to porcine plasma/serum or testicular cytosol has not been studied.

An understanding of androstenone transport in blood is an important issue from both physiological and methodological perspectives. Recent studies have demonstrated that the accuracy of androstenone measurements in plasma requires considerable improvement [15]. An analytical problem became evident during the efforts to compare androstenone concentrations in plasma determined by enzyme-linked immunosorbent assay (ELISA) with and without extraction of the plasma with ethyl acetate. Androstenone concentrations obtained after extraction were much lower compared to those without extraction [15]. The variability in androstenone concentrations obtained by different analytical methods might be due to androstenone interaction with serum binding proteins. The objective of the present study was, therefore, to evaluate the possible existence of a specific androstenone-binding protein in porcine and human serum and in the cytosolic fraction of porcine testis.

2. Materials and methods

2.1. Reagents and stock solutions

[^{3}H]-dihydrotestosterone ([^{3}H]-DHT) with a specific activity 110 Ci/mmol was obtained from Life and Analytical Science (Boston, USA), and [^{3}H]-androstenone with specific activity 53 Ci/mmol was obtained from Amersham Biosciences (UK). Non-radioactive DHT and androstenone were purchased from Sigma–Aldrich Ltd. (Steinheim, Germany).

Stock solutions of unlabelled androstenone and DHT were prepared in 100% ethanol to a final concentration of 10 mg/mL. Working solutions were prepared by dilution of stock solutions with assay buffer to a final concentration of 18 nmol/mL for unlabelled steroid (final concentration of ethanol 0.05%) and 55 pmol/mL for [^{3}H]-steroid (final concentration of ethanol was 0.3% for [^{3}H]-androstenone and 0.6% for [^{3}H]-DHT). Incubations were performed with freshly prepared solutions.

2.2. Radiochemical purity determination and storage

The present study was performed during March–April 2006. Radiochemical purity of [^{3}H]-DHT, as declared by the manufacturer, was greater than 97% at the day of production (December 21, 2005). Two HPLC methods were used: (1) Zorbax ODS column with methanol:tetrahydrofuran:water (45:15:40) as a mobile phase, and (2) Zorbax Silica column with hexane–isopropanol (95:5) as a mobile phase. Radiochemical purity of [^{3}H]-androstenone was assessed by HPLC [2] using an on line radioactivity detector (Beckmann model 171) twice within an eight month interval and was found to be 90%.

Both [^{3}H]-androstenone and [^{3}H]-DHT were stored at −20 °C in ethanol according to the manufacturer's instructions.

2.3. Steroid binding assay

Prior to performing binding studies, the assay was first validated with respect to substrate, incubation time, sample dilution, and assay buffer. Three assay buffers were used in the incubations; phosphate buffered saline containing 0.1% gelatine (PBS–G) pH 7.4, TRIS–HCl containing 0.4% bovine serum albumin (TRIS–BSA) pH 7.5, and DELFIA assay buffer (PerkinElmer product 1244-111) containing 0.5% BSA (DELFIA–BSA) pH 7.75. A buffer without added protein could not be used in these studies due to the low polarity of androstenone. Assay incubation times from 0 up to 72 h were evaluated. Analysis of binding of DHT to human plasma and serum was performed as a positive control. The differences in the binding between serum, stripped and un-stripped plasma were evaluated.

Steroid binding assays were performed using pooled porcine plasma from 20 young boars. Plasma from 15 of these boars (mean live weight ± standard deviation, 109 ± 4.6 kg) were also analysed individually. In addition, pooled human plasma from 2 healthy male and female volunteers, serum from a single sexually mature boar and serum from a male volunteer were analysed. To remove endogenous steroids, plasma was stripped prior to analysis with charcoal (0.2 g of charcoal Norit A in 20 mL of plasma) at 4 °C overnight, with the charcoal subsequently removed by centrifugation at 4000 × *g* for 20 min.

Aliquots (100 μL of plasma or serum) were incubated with [^{3}H]-androstenone diluted in assay buffer (100 μL, 5.5 pmol per tube) in the absence (total binding) or the presence (non-specific binding) of 1.8 nmol of unlabelled androstenone. Incubations were performed in glass tubes. Additionally, 1.8 nmol of unlabelled 3α-androstenol was investigated as a cold ligand with porcine plasma. The binding of various amounts of [^{3}H]-androstenone from 0.1 to 11 pmol was also measured. Incubations were terminated by the addition of 500 μL ice-cold dextran-coated charcoal solution (DCC) followed by incubation at 4 °C for 20 min. DCC was prepared by continuously stirring 0.5 g Norit A and 0.05 g of dextran in 100 mL of distilled water prior to use. The DCC-bound steroids were removed by centrifugation at 4000 × *g* at 4 °C for 10 min. The supernatants were then added to 8 mL of scintillation liquid, vigorously mixed and counted under standard tritium conditions. The difference between total and non-specific binding represents the specific binding of steroid by plasma. The same procedure was applied to measure dihydrotestosterone (DHT) binding in porcine and human serum with 5.5 pmol of [^{3}H]-DHT per tube. Serum samples were not stripped prior to analysis.

Testicular tissue was obtained from two mature purebred boars (Duroc and Landrace). Testes (1 g) were cut into small pieces and homogenised in 5 mL of 50 mM TRIS–HCl, pH 7.4, containing 1 mM EDTA. Cytosolic fractions were prepared from the homogenate by differential centrifugation as described [14]. The binding of [^{3}H]-androstenone was measured using the same procedure as for plasma.

The total amount of added radioactivity was determined by counting the same amount of [^{3}H]-steroid that was used in the incubation. The amount of radioactivity in the incubations was determined in the steroid/serum or plasma mixture before adding the DCC. Thus, the same amount of buffer was present in the scintillation fluid containing the total radioactivity and in the scintillation fluid containing the samples. The radioactivity in the incubations was considered as 100% when calculating the percentage of binding. Total radioactivity added directly to the scintillation fluid was compared to the radioactivity in the incubations to determine the percentage of non-specific binding of [^{3}H]-androstenone to the test tubes at various incubation times, 0 h, 1 h, 6 h and 20 h. The radioactivity remaining in the incubations averaged 75.8% ± standard deviation 4.4%, suggesting that approximately 25% of the [^{3}H]-androstenone non-specifically bound to the test tubes. The non-specific binding of [^{3}H]-DHT to the test tubes was below 2% (remaining radioactivity 98.9 ± 4.2%). The amount of binding to the test tubes did not vary with incubation time. To increase the accuracy of the steroid-binding assay, the remaining radioactivity in the incubations was considered as 100%.