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Simultaneous profiles of sulfonated androgens, sulfonated estrogens and sulfonated progestogens in postpubertal boars (sus scrofa domestica) measured by LC–MS/MS

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ABSTRACT

Sulfonated steroids (s-St) have been usually regarded as inactive metabolites but are progressively considered as precursors for the intra-tissue formation of bioactive steroids. Moreover, independent effects without preceding removal of the sulfate group have been observed. We use the porcine testicular-epididymal compartment as a model to investigate the still largely unknown s-St physiology as the boar exhibits an intriguingly broad s-St spectrum predominantly originating from the testis. The application of LC–MS/MS in steroidomics enables the determination of unconjugated and intact sulfonated steroids with currently highest specificity and good sensitivity, allowing the concurrent measuring of numerous analytes in larger quantities of samples. Profiles (6 h, 20 min intervals) were generated for sulfonated 5-androstene-3ß,17ß-diol (Adiol-S), androsterone (A-S), dehydroepiandrosterone (DHEA-S), epiandrosterone (EA-S), epitestosterone (ET-S), estrone (E1-S), estradiol-17β (E2-S), pregnenolone (P5-S), 17αOH-pregnenolone (OHP5-S) and unconjugated testosterone (T) in four unstimulated and four hCG-stimulated boars. Moreover, concentrations were measured in individual samples collected from testicular afferent and efferent blood to differentiate between testicular vs. extratesticular origin. Highest concentrations were found for EA-S, followed by ET-S, Adiol-S and DHEA-S, which mostly exceeded the levels of E1-S and A-S. Lowest concentrations were obtained for E2-S, P5-S and OHP5-S. The analytical profile also included sulfonated T, 5α-dihydrotestosterone and cholesterol. However, their concentrations were below the limit of quantification. Profiles of quantifiable s-St were consistent with a wave-like pattern associated with T pulses. In postpartal females (n = 5) concentrations of all analytes assessed were undetectable, suggesting that in pigs the adrenals are not a quantitatively significant source of s-St.

1. Introduction

Sulfonation of steroids has been commonly considered as an important mechanism bringing about inactivation and facilitating excretion. However, as sulfonated steroids frequently circulate at significantly higher concentrations compared to the levels of their unconjugated counterparts, they have been progressively recognized as a pool of substrates for the local production of bioactive steroids in specific target tissues expressing the enzyme steroid sulfatase [1–4]. This sulfatase pathway was described as the predominant pathway for the production of estrogens in human hormone-dependent breast cancer tissue in comparison to the de novo synthesis [2,5,6]. However, aside from the role of sulfonated precursors in human placental estrogen biosynthesis [7,8], knowledge on the relevance of sulfatase pathways in physiological settings is still sparse. In addition to a role as substrates for the intra-tissue production of bioactive unconjugated steroids evidence has been obtained for direct effects of sulfonated steroids in brain [9] and testis [10,11]. However, in general concerning the physiology of sulfonated steroids there are still many open questions.

Abbreviations: ACN, acetonitrile; A-S, androsterone sulfate; Adiol-S, 5-androstene-3β,17β-diol-3-sulfate; C-S, cholesterol sulfate; DHEA-S, dehydroepiandrosterone sulfate; DHT, 5α-dihydrotestosterone; DHT-S, 5α-dihydrotestosterone sulfate; EA-S, epiandrosterone sulfate; ET-S, epitestosterone sulfate; E1-S, estrone sulfate; E2-S, 17β-estradiol-3-sulfate; GC–MS, gas chromatography-mass spectrometry; LC–MS/MS, liquid chromatography-tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; OHDHEA-S, 16α-hydroxydehydroepiandrosterone-3-sulfate; OHP5-S, 17α-hydroxypregnenolone sulfate; P5-S, pregnenolone sulfate; SB, boar stimulated with hCG; T, testosterone; T-S, testosterone sulfate; UB, unstimulated boar; V/A, concentration ratio venous/arterial

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A special feature of boars among domestic mammalians is their intriguingly broad spectrum of sulfonated steroids [12–17], making this animal an attractive model for the study of synthesis, transport, metabolism and functions of this class of steroids. Besides sulfonated estrogens [13–15,17], in the boar sulfonated steroids apparently arise from the synthesis of androgens [12,14,15,17–19] and steroidal pheromones [16,20,21] encompassing numerous hydroxylated C19- and C21-steroids. However, the metabolic steps bringing about the broad spectrum of sulfonated steroids in the boar are still incompletely characterized and the cell types where eventually sulfonation takes place remain to be definitely identified for a significant proportion of steroids. Results from comparative measurements in testicular artery and vein [12,16–18,20,22,23] or in lymph fluid of the spermatic cord [22] clearly identified the testicular-epididymal compartment as the predominant source of several sulfonated steroids. High concentrations found in testicular tissue point to the gonads as their predominant site of production [16,24], and observations from studies in cultured porcine Leydig cells indicate that in boars sulfonated estrogens and several sulfonated C19-steroids mainly originate from this type of cells [21,25–27]. According to the traditional concept of sulfonated steroids as waste products, sulfonation of steroids predominantly takes place in target cells or in organs involved in metabolism or excretion such as liver or kidney [4]. However, concerning the porcine testis it is still fully unclear why this organ produces considerable amounts of inactive hormones (e.g. sulfonated estrogens) or why it removes important precursors or intermediates of steroidogenesis such as pregnenolone, dehydroepiandrosterone or 5-androstene-3β,17β-diol from the pool of unconjugated steroids. As a basis to understand the physiology of sulfonated steroids in the boar, one aim of our project was to further characterize the secretion patterns of sulfonated steroids and to evaluate the relationship between the secretion pattern of unconjugated and sulfonated steroids. Thus, in a previous study an LC–MS/MS method was applied to establish for the first time simultaneous profiles for testosterone (T), androstenedione, pregnenolone sulfate (P5-S), dehydroepiandrosterone sulfate (DHEA-S), estrone sulfate (E1-S) and 17β-estradiol-3-sulfate (E2-S) [17,28]. In this follow-up study a recently developed LC–MS/MS based method [29] was applied which focusses on the quantification of sulfonated androgens and their metabolites (androsterone sulfate [A-S], 5-androstene-3ß,17ß-diol-3-sulfate [Adiol-S], DHEA-S, 16α-hydroxydehydroepiandrosterone sulfate [OHDHEA-S], 5α-dihydrotestosterone-17-sulfate [DHT-S], epiandrosterone sulfate [EA-S], epitestosterone-17-sulfate [ET-S], testosterone-17-sulfate [T-S]; for important pathways in androgen metabolism see Fig. 1). Moreover, this method was also validated for the quantification of cholesterol sulfate (C-S), pregnenolone sulfate (P5-S), 17α-hydroxypregnenolone sulfate (OHP5-S) and enables the measurement of E1-S and E2-S. The sample workup applied allowed for the isolation of T from sulfonated steroids, which was quantified applying another LC–MS/MS method [30].

The primary aims of this study were a) to characterize the spontaneous secretion patterns of sulfonated steroids over a 6-h period b) to analyze the temporal relationships between the established profiles among one another and between the individual sulfonated steroids and testosterone c) to similarly characterize the secretion of sulfonated steroids after stimulation with human chorionic gonadotrophin (hCG) to analyze the temporal relationships between increases induced by the treatment d) to obtain an approximate estimate whether the sulfonated steroids assessed are predominantly of testicular origin or rather result from peripheral metabolism as indicated by concentrations measured in efferent vs. afferent testicular blood vessels. In order to characterize the testicular output of unconjugated DHT, it was also included in this part of the study and measured by GC–MS.

DHEA-S, E1-S, E2-S, P5-S and T, for which secretion profiles have been previously described applying a different method [17], have been included again in the measurements to compare their secretion patterns with the profiles of the newly assessed analytes.

2. Material and methods

2.1. Collection of blood samples from boars

All animal experiments were in accordance with the relevant regulations and were approved by the competent authority (Regierungspraesidium Giessen, permit No. V54-19c-20-15(I) Gi 18/14-No. 32/2010). The samples from postpubertal boars used in this study were taken from a larger set of samples collected in a previous study [17], where sample collection was described in detail. Identical names indicate identical animals between the two studies.

2.1.1. Sample collection from unstimulated boars

Blood samples from four unstimulated boars (UB3-6, German Landrace × Pietrain) aged 8–11 months (mean: 9.7 ± 1.2 months) were collected from the jugular vein starting between 9:00–9:45 a.m. From each animal a total of 20 samples were drawn at 20 min intervals and heparin plasma samples were stored at −20 °C until analysis.

2.1.2. Sample collection from hCG-stimulated boars

Sample collection was performed as described above for unstimulated boars. In two 9.5 month old boars (SB2, SB3, German Landrace x Pietrain), two prestimulatory samples were taken 20 min apart. After intravenous injection of 1500 I.U. hCG (Ovogest – Intervet, Unterschleissheim, Germany), blood sample collection was continued at 20 min intervals for another 6 h (protocol 1). In another two 11–12 months old German Landrace x Pietrain crossbred boars (SB5, SB6) four prestimulatory samples were taken at 20 min intervals. During the first hour after application of 1500 I.U. hCG, the sampling interval was reduced to 10 min, followed by the collection of another 16 samples at 20 min intervals (protocol 2). Finally, to exert maximum stimulation, in an 11.5 months old Large White boar (SB8) the hCG dosage was increased to 10,000 I.U. For a better temporal resolution of the hormonal profiles, in this animal sampling was initially at 5 min intervals from 15 min before to 15 min after hCG stimulation, followed by sampling at 3 min intervals during the next 60 min. A last sample was taken 90 min after hCG-application (protocol 3).

2.1.3. Sample collection from local blood vessels of the testis and spermatic cord

Sampling from arterial and venous blood vessels of the testis and the spermatic cord was performed under general anesthesia in four 11–19 month old boars (two German Landrace x Pietrain crossbreds, two Large Whites). Samples were collected from 2 to 3 veins running on the testicular surface (capsular vein), from the terminal branch of the testicular artery near the distal testicular pole (capsular artery) and from the testicular artery and vein running in the spermatic cord. Finally, blood was collected from the ear vein to measure steroid levels in the systemic circulation.

2.2. Sample collection from adult females

In order estimate an adrenal contribution to the steroids under investigation measurements were performed in single samples obtained from five adult sows (three Dan-Hybrid sows, two Large Whites). These samples were leftovers from routine diagnostic sampling 1–2 weeks after farrowing and were provided by the Department of Clinical Veterinary Sciences, Clinic for Swine, Justus-Liebig-University, Giessen, Germany.

2.3. Sample processing and steroid measurements by liquid chromatography-tandem mass spectrometry (LC–MS/MS)

Sulfonated steroids (A-S, Adiol-S, C-S, DHEA-S, DHT-S, EA-S, ET-S, T-S, E1-S, E2-S OHDHEA-S, OHP5-S, P5-S) and T were quantified by LC–MS/MS using a triple quadrupole mass spectrometer (TSQ,