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INCREASED IMMUNOREACTIVE 11-KETOTESTOSTERONE CONCENTRATIONS IN SHEEP FECES AFTER ACTH CHALLENGE

OMER SID-AHMED, NINO ARIAS, RUPERT PALME, and ERICH MÖSTL*
Department of Biomedical Sciences, Institute of Medical Biochemistry, University of Veterinary Medicine, Vienna, Austria

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Abstract: 11-Oxoetiocholanolone and related substances are important metabolites of cortisol and are excreted via feces in ruminants. To investigate whether 11-ketotestosterone (11-KT) or its immunoreactive metabolites are formed and excreted in ruminant feces, an enzyme immunoassay (EIA) was developed and validated. The antibody was raised in rabbits against 11-KT-3-CMO:bovine serum albumin with biotinylated 11-KT as a label. The assay showed a sensitivity of 0.3 pg/well. To validate the assay biologically, 6 rams were injected with a synthetic analogue of the adrenocorticotropic hormone (Synacthen, 2 μg/kg body wt). An aliquot was collected of each fecal portion spontaneously defecated 8 h before Synacthen injection to 24 h after injection and stored at −20 °C until analysis. Samples (0.5 g) were extracted using 80% methanol and immunoreactive metabolites measured using the 11-KT EIA and an already established 11,17-dioxoandrostane (11,17-DOA) EIA. High-performance liquid chromatography separation revealed no peak in the same elution position as authentic 11-KT; therefore, reacting substances were referred to as 11-KT equivalents. In the case of 11-KT immunoreactive substances, the values increased from baseline (median, 136 ng/g feces) to a peak concentration (median, 424 ng/g) 10 to 14 h after Synacthen injection and declined afterwards. Concentrations of 11,17-DOA showed the same pattern, but the values were 2 to 4 times higher. From this data, the authors conclude that 11-KT-like substances, specifically $C_{19}O_3$-androgens with a 17ß-hydroxy group, were present in the feces. These substances originate from the adrenals and are most likely cortisol metabolites. *Environ Toxicol Chem* 2013;32:1332–1336. © 2013 SETAC

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INTRODUCTION

In mammals, androgens (19-carbon steroids) are produced by the gonads and adrenal glands. These hormones can be grouped into $C_{19}O_2$-androgens (e.g., dehydroepiandrosterone, androstenedione, and testosterone) and 11-oxygenated androgens ($C_{19}O_3$-androgens, e.g., 11ß-hydroxyandrostenedione and 11-ketotestosterone). The 11-oxygenated androgens are important in teleost fish and produced mainly by the gonads, whereas 4-androstene-11ß-ol-3,17-dione is predominantly of adrenal origin and considered a weak androgen in humans [1]. However, the gonadal pathway to produce 11-ketotestosterone (11-KT) appears to be active in mammals; for example, 11-KT is produced in the gonads of immature mice [2].

Androgens are metabolized and excreted in the urine and feces [3]. Using the chicken-comb test, androgen activity was reported [4] while studying the feasibility of using bovine manure as a source of B vitamins for chickens. Furthermore, androgenic activity in the feces of cows—but not bulls—was described after 24 h incubation at 45 °C [5]. These findings were confirmed and evidenced by the excretion of androgenic metabolites in pregnant and progesterone-injected cows [6]. Later, the androgenic activity in cow feces was reported to be dependent on the presence of androgen precursors and microbial activity [7].

Cortisol is a potential precursor of fecal 11-oxygenated 19-carbon steroids because the glucocorticoid is converted by anaerobic intestinal bacteria [8], and the side chain of cortisol can be cleaved, resulting in the formation of $C_{19}O_3$-androstanes. These molecules are reabsorbed, metabolized again in the liver, and excreted into the gut via the entero-hepatic circulation [8–10].

In ruminants, appreciable amounts of steroid hormone metabolites are excreted in the feces [3]. Radiometabolism studies of ^{14}C-cortisol in sheep have shown that authentic cortisol is not present in the feces, but a multitude of radioactive metabolites, including $C_{19}O_3$-androgens, are excreted [11,12]. Consequently, an assay for measuring 11-oxoetiocholanolone was developed [11], and the concentrations of these immunoreactive substances are now widely used to measure adrenocortical activity in ruminants, reflecting the production of glucocorticoids in the adrenal glands [12,13]. Steady increases in C_{19} metabolites, such as 5ß-$C_{19}O_3$-androstanes, have been reported for fecal samples stored at room temperature for up to 24 h, which can be explained as an indicator of side-chain cleavage by fecal microorganisms [12].

Some of the excreted gonadal metabolites are suspected to be androgenic substances; therefore, the contribution of livestock and humans to the environmental load of steroid hormones has become an international concern. Most research work thus far has focused on the quantification and estimation of excreted estrogens, gonadal androgens, and gestagens in different farm animals [14], municipal biosolids, and animal manure [15], but the presence of $C_{19}O_3$-androgens was neglected, even though they are the active androgens in fish. Data concerning the androgenic activity of $C_{19}O_3$-androstanes in mammals remain elusive.

In rats, 11ß-hydroxyandrostenedione has been found to have an osteotropic effect but little androgenic activity [16]. In contrast to reports in mammals, 11-KT is the most potent androgen in fish. Interestingly, the $C_{19}O_3$-cortisol metabolite 5α-androstane-3, 11, 17-trione was described recently to act

* Address correspondence to erich.moestl@vetmeduni.ac.at.
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similarly to 11-KT in medaka fish, causing the masculinization of females [17]. In addition, 5α-reduced $C_{19}O_2$-androstanes appear to act as androgens in some fish species. For example, the potency of 5α-dihydrotestosterone as an androgenic compound in the fathead minnow (*Pimephales promelas*) has been described [18]. Furthermore, an 11-oxygenated 5ß-androstane (i.e., 11-oxoetiocholanolone) has been shown to act as a putative pheromone in some fish species [19]. Until now, only 19-carbon 5ß-steroids with an oxo group at position 17 of the molecule have been described in the feces of ruminants and used as noninvasive parametric values for animal welfare. The objective of the present study was to investigate whether ruminants should be considered as sources of 5α-androstanes with a 17ß-hydroxy group configuration. Therefore, an enzyme immunoassay against 11-KT was developed and validated in fecal samples of sheep.

MATERIALS AND METHODS

Animal treatment and fecal sample collection

Feces from 6 male Austrian mountain sheep (age, 3–5 yr; weight, 74–107 kg) at the Teaching and Research Farm of the University of Veterinary Medicine Vienna were analyzed. Permission to conduct the animal experiments was obtained from the local authority (GZ 68.205/0108-II/10b/2009). The animals were apparently healthy and housed in metabolic cages (1.5 × 1.2 m). Hay and fresh water were provided ad libitum, and all animals had access to a mineral lick.

All animals were intravenously injected with the synthetic Adrenocorticotropic hormone analogue (Synacthen, Novartis; 2 μg/kg body wt). Fecal samples were collected in plastic bags after each spontaneous defecation, homogenized, and stored immediately at −20 °C.

The fecal samples containing ^{14}C-cortisol metabolites were collected in an earlier experiment [3] in which ^{14}C-cortisol was injected into rams to monitor excretion of the steroid hormone. Those samples were then stored at −20 °C.

Feces extraction (0.5 *g* feces) was made on both Synacthen treated ram feces and on ^{14}C-cortisol feces using 80% methanol method [11]. After centrifugation, an aliquot of the supernatant was transferred to a new vial, diluted (1:5 v/v) with assay buffer (Tris-hydroxyl-aminomethane [20 mmol/L], NaCl [0.3 mol/L], bovine serum albumin [1 g/L], Tween 80 [1 mL/L], pH 7.5), and analyzed by 11-KT and 11-oxoetiocholanolone enzyme immunoassays (EIAs).

Enzyme immunoassay

Both EIAs were performed as described previously [11], with the exception that protein A was used to coat the microtiter plates instead of antirabbit immunoglobulin G (IgG). The microtiter plates (F96 MaxiSorp, No. 442404, Co. Nunc) were coated with 50 μg protein A (Sigma Aldrich) dissolved in 25-mL coating buffer (0.25 mL/well) and left overnight at room temperature. The second coating used a buffer containing bovine serum albumin plus sodium azide, and the plates were stored at room temperature until use (up to 4 wk).

A new assay was developed to measure 11-KT immunoreactive substances. The antigen (11-ketotestosterone-3-CMO: bovine serum albumin) was kindly donated by A. Scott (Centre for Environment, Fisheries and Aquatic Science, Weymouth, UK) and sent to Pineda Antikörper Service (Berlin, Germany) to immunize rabbits.

As a label, 11-ketotestosterone-3-CMO (10 mg) was linked to 10 mg EZ-Link Biotin PEO-amine-biotinyl-3,6-dioxaoctanediamine (10 mg; Pierce) using the mixed anhydride reaction. Label purification was performed as described previously [12]. Working dilutions of antibody (1:300 000) and label (1:10^6) for the 11-KT assay were made by checkerboard titration with 11-KT as a standard. To monitor the stimulation of glucocorticoid production by the adrenal gland after Synacthen injection, a previously validated 11-oxoetiocholanolone EIA was used [11,13].

Validation of the 11-KT assay

The standard curve for 11-KT ranged from 0.3 pg to 80 pg per well. The 50% intercept was 2 pg/well. The sensitivity of the test was calculated as the least amount of 11-KT that was different from a concentration of zero at the 95% confidence limit (∼0.3 pg/well). The assay showed cross-reactions with 5α-androstan-17β-ol-3,11-dione (39%), 5β-androstane-17β-ol-3-one (3.6%), 5α-androstan-3α-ol-11,17-dione (0.3%), 4-androstene-3,11,17-trione (0.2%), 5α-androstan-3β-ol-11,17-dione (0.2%), 5α-androstan-17β-ol-3-one (0.1%), 4-androstene-17α-ol-3-one (0.1%), and 4-androstene-17β-ol-3-one (0.04%). To test the linearity of the assay in fecal sample extracts, a pooled sample from different animals was used, followed by serial dilution with assay buffer. The concentrations were measured using the 11-KT EIA. Different amounts of feces had no significant influence on the results expressed as nanograms per gram of feces. The intra-assay and interassay coefficients of variation for the low-level pool of the 11-KT assay were 3.9% and 8.7%, respectively.

High-performance liquid chromatography separation of radiolabeled ^{14}C-cortisol metabolites and immunoreactive substances

To characterize the radiolabeled and immunoreactive metabolites in feces, a fecal sample containing the ^{14}C-cortisol metabolites was used from a previous experiment [3]. A high-performance liquid chromatography (HPLC) immunogram was produced as described previously [20]. Briefly, 1 mL of the methanol phase from the extract was diluted in 10-mL water and re-extracted using a Sep-Pak (Waters) C18 cartridge following the manufacturer's instructions. The dried extract was dissolved in 50% methanol and separated using HPLC (Novapac C18, column dimension: 0.39 × 15 cm; pumps: Waters 626; controller: Waters 6005; photodiode array detector: Waters 996) with water/methanol as the mobile phase (starting with 50% and up to 75% methanol within 35 min using a linear gradient) operated at room temperature. The flow rate was 1 mL/min. Three fractions were collected per minute and the immunoreactivity measured in each fraction using the 11-KT and 11,17-DOA assay. The radioactivity was measured in all fractions using liquid scintillation counting. The elution positions of some standards were determined using an ultraviolet detector.

Statistical analysis

Statistical analyses were performed using SigmaStat 3.1 and SigmaPlot 11.0 was used for preparing the figures (SPSS). Due to normality test failure, results are expressed as median and the correlation between the concentrations of 11-KT and 11,17-DOA was calculated as the Spearman correlation test.

RESULTS

The concentrations of immunoreactive 11-KT and 11,17-DOA in fecal samples of two rams are shown in Figure 1. High interindividual variability was seen in the baseline and peak values of all animals. The baseline levels of 11-KT ranged from

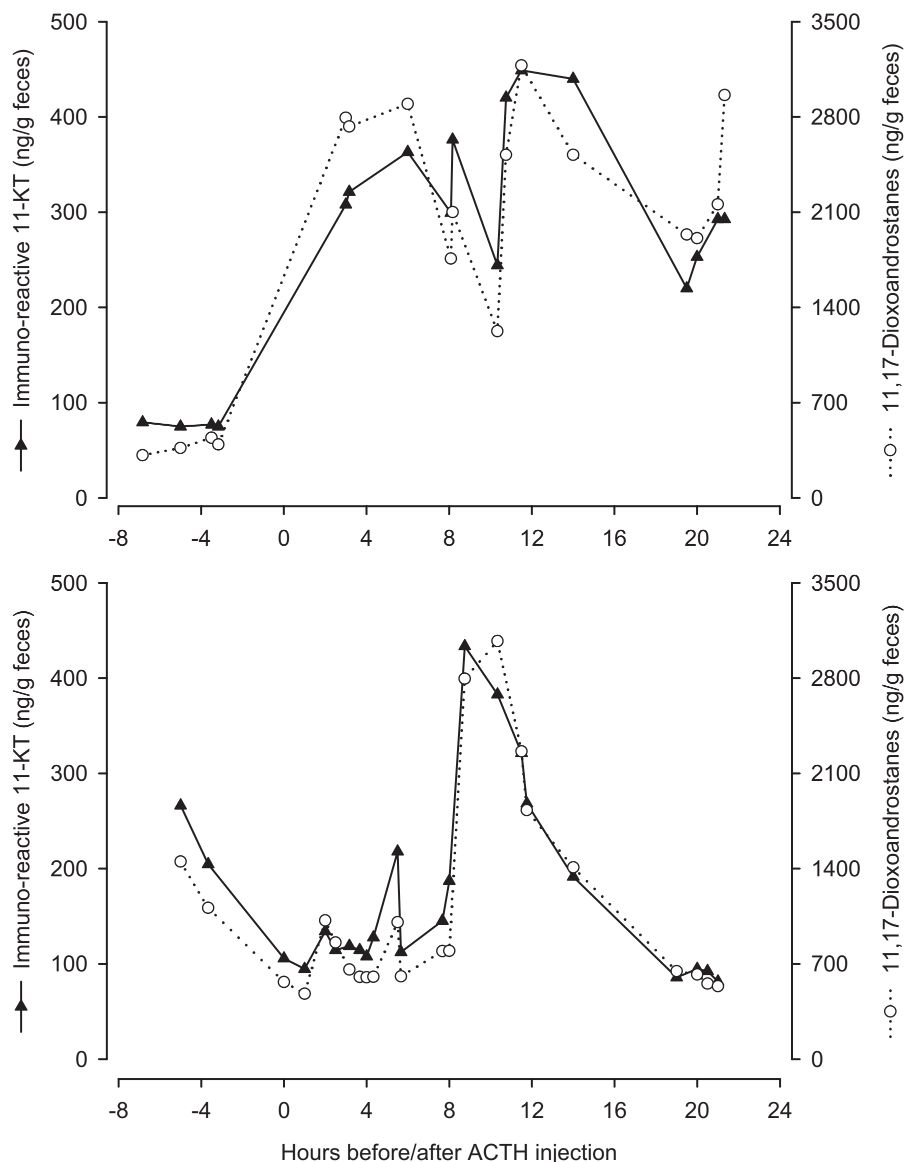

Figure 1. Concentrations of immunoreactive 11-ketotestosterone (11-KT) and 11,17-dioxoandrostane (11,17-DOA) before and after Synacthen injection in two rams.

76 ng/g to 240 ng/g feces (median, 136), and from 188 ng/g to 297 ng/g feces (median, 252) for 11,17-DOA. Following Synacthen injection, the fecal concentrations of both 11-KT and 11,17-DOA reached peak values between 10 and 12 h (Figure 1). Peak concentrations of 11-KT and 11,17-DOA ranged from 352 ng/g to 475 ng/g feces (median, 424) and 1211 ng/g to 1822 ng/g feces (median, 1570), respectively. After Synacthen injection, the increase (median) above baseline values of 11-KT and 11,17-DOA was 293% and 626%, respectively. In all individuals, the concentrations of both groups of metabolites highly correlated ($n = 125$ [total number of fecal samples from all 6 rams]; $r = 0.863$; $p < 0.0001$), but 11,17-DOA values were approximately 2 to 4 times higher. Although concentrations measured by the 2 EIAs started to decline gradually after the Synacthen peak, they were still significantly different from baseline levels ($p < 0.001$) at the end of the experiment.

HPLC separation of glucocorticoid metabolites

Reverse phase-HPLC analysis revealed that ^{14}C-cortisol was extensively metabolized. All immunoreactive substances eluted between fractions 20 and 50, indicating that they are nonconjugated steroids. The ^{14}C-cortisol metabolites eluted in 6 major peaks, 5 of which were in the same range as immunoreactive substances measured with the 11-KT and 11-oxoetiocholanolone EIA (Figure 2). Though the peaks were not positively identified, three major immunoreactive peaks of 11-KT (30–33, 37–41, and 46–49) coincided with the ^{14}C-cortisol peaks. The RP-HPLC separation system did not detect authentic 11-KT at its respective elution position (fraction 22).

DISCUSSION

Endogenous steroid hormones of human or animal origin have been introduced to the environment for a long time [14]. However, the recent rise of intensive animal husbandry and urbanization has dramatically increased the amounts of steroid released into the environment. From a toxicological point of view, most research has focused on sex steroids and synthetic anabolic hormones in sewage treatment plants, which act as endocrine disruptor compounds (EDCs) to aquatic organisms. Glucocorticoid metabolites received less attention, with the

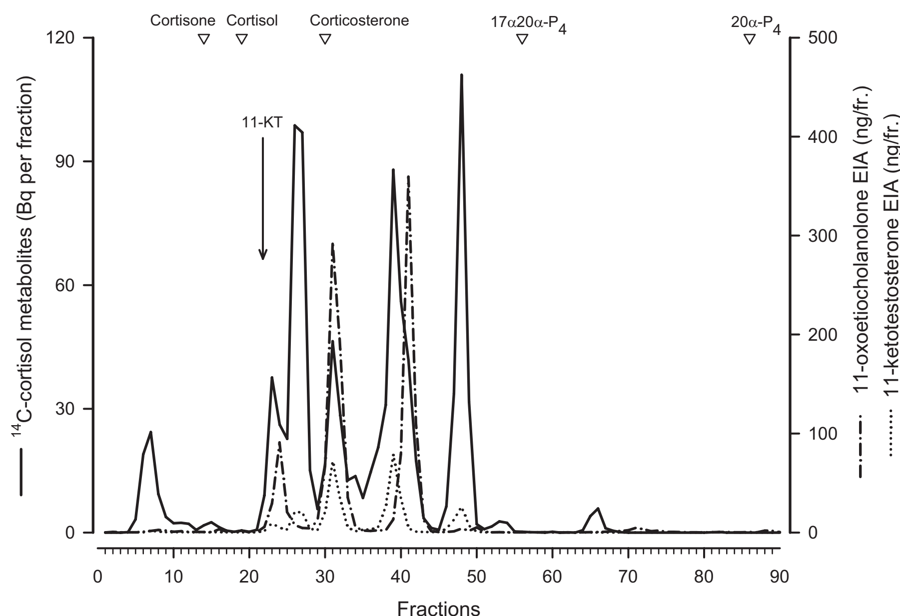

Figure 2. High-performance liquid chromatography elution pattern of radioactivity and immunoreactive substances. The black arrow indicates the elution position of 11-ketotestosterone (11-KT; fraction 22). The elution positions of cortisone, cortisol, corticosterone, 17α,20α-dihydroxyprogesterone (17α20α-P4), and 20α-hydroxyprogesterone (20α-P4) are indicated by inverted triangles. EIA = enzyme immunoassay.

exception of their wide use as a noninvasive measure of animal welfare. However, the possible role of glucocorticoid metabolites as EDCs remains unclear.

To demonstrate that immunoreactive 11-KT metabolites are present in animal manure and from adrenal origin, an 11-KT EIA was developed and validated. The 11-KT-EIA exhibited very high sensitivity (0.3 pg/well) compared to currently available commercial kits, providing a useful tool for noninvasive monitoring of androgen production in fish aquaria, where low amounts of steroids constitute a major drawback for the applicability of these assays. A second advantage of this EIA is that it does not need the use of radioactive tracers that constitute major environmental biohazards. The highest cross reactivity (39%) shown by 5α-androstan-17β-ol-3,11-dione (11-ketoandrostanolone), which is 5α-reduced 11-KT, would be quite useful and should not present a problem as far as the biological activity is concerned because 5α-reduced androgens (e.g., dihydrotestosterone) are more potent than 4-androstene (e.g., testosterone) [20].

Two points regarding the excretory profile of fecal 11-KT immunoreactive substances merit discussion. First, the 11-KT EIA detected immunoreactive substances, and their concentrations increased after stimulation by Synacthen, indicating that the substances or their precursors were produced by the adrenal glands. Second, peak values were reached between 10 and 12 h (Figure 1), paralleling those of the 11,17-DOA EIA and showing the same excretory pattern (gut passage time) within a comparable time in sheep [13]. Reverse phase-HPLC separation revealed different immunoreactive metabolites with polarity similar to unconjugated steroids, but none of the peaks showed the same elution position as authentic 11-KT. Despite 11-KT EIA being raised against the 4-androstene structure, the assay showed cross reactions with 5α-reduced KT. As far as the linking position of the antigen at position C-3 in the new 11-KT EIA is concerned, it would make it difficult for the antibody to differentiate between 5α-reduced and delta-4 steroids as demonstrated by the cross reactions as described earlier [21].

Our new 11-KT EIA has to be considered as group specific for 11-oxo and 17ß-hydroxy configuration, and the authentic 11-KT was absent in our samples. Though the HPLC peaks were not positively identified, some showed chromatographic behavior (±2 fractions) with 11,17-DOA identified by Ganswindt et al. [22] using the same separation system for ^{14}C-cortisol metabolites: 11ß-hydroxyetiocholanolone at fractions 22 to 24 and 11-oxoetiocholanolone at fractions 36 to 40. Furthermore, using HPLC coupled with mass spectrometry, Möstl et al. [12] reported a mixture of C-21 and C-19 metabolites coincided in a single radioactive peak. Therefore, due to steroid metabolite complexity in fecal matter, the 11-oxoetiocholanolone EIA [11,13] picked a group of unconjugated metabolites (11,17-DOA) similar to the new 11-KT EIA. However, the major peak of 11-KT immunoreactive substances in fractions 36 to 40 coincided only with ^{14}C-cortisol metabolites.

Based on our results for cross reactivity and HPLC chromatographic separation, we can assume that rams excrete $C_{19}O_3$-androgen metabolites with a 5α-configuration plus a 17ß-hydroxy group in the feces. The origin of these metabolites or their precursors is the adrenal gland, as the concentrations of these substances were indicated after Synacthen injection and the 11-KT EIA coincided with some ^{14}C-cortisol metabolites in the HPLC immunogram (Figure 2). These steroids may be a product of the sheep itself, or most by side-chain cleavage induced by micro-organisms in the gut using adrenal steroids as precursors. If adrenal androgens are the cause of the fecal androgen concentrations, one has to expect a certain amount of 17α-hydroxylated androgens and lower concentrations of 11-oxoetiocholanolone. It has been shown that 11-oxoetiocholanolone is rapidly converted by red blood cells of ruminants into 17α- and 17ß-hydroxylated steroids [23]. A similar scenario of C-17 oxygenated metabolite transformation would be expected, as other steroids have been reported in ruminant feces [24]. Measurement of 11,17-DOA in feces is an established assessment of adrenocortical activity in response to stressful situations in various species, including ruminants [11–13],

cats [25], monkeys [26], and nonhuman primates [27]. However, quantification of biologically active glucocorticoid metabolites in animal manure has received no attention, though available evidence confirmed that mammalian fecal glucocorticoid metabolite (5α-androstane-3, 11, 17-trione) masculinized the sex characteristics of the anal fin in female medaka in a dose-dependent manner, and this effect was comparable to the effects of the major fish androgen, 11-KT [17].

In conclusion, the present study did not show the presence of 11-KT in animal manure after Synacthen injection, but cross-reacting metabolites were detected. However, metabolites could constitute a major problem for aquatic organisms; therefore, the assessment of the androgenic potential of 11-oxoetiocholanolone and 17ß-hydroxymetabolites originating from animal manure is important. It may also be of interest to measure the amount of $C_{19}O_3$-androgens in sewage water.

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