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11β-Hydroxyandrostenedione, the product of androstenedione metabolism in the adrenal, is metabolized in LNCaP cells by 5α-reductase yielding 11β-hydroxy-5α-androstanedione

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ABSTRACT

11β-Hydroxyandrostenedione (11OHA4), which is unique to the adrenal, was first isolated from human adrenal tissue in the fifties. It was later shown in the sixties that 11β-hydroxytestosterone (11OHT) was also produced by the human adrenal. Attention has shifted back to these adrenal androgens once more, as improved analytical techniques have enabled more accurate detection of steroid hormones. In this paper, we investigated the origin of these metabolites as well as their subsequent metabolism and examined a possible physiological role for 11OHA4 in prostate cancer cells. In H295R cells treated with forskolin and trilostane, etomidate, a reported cytochrome P450 11β-hydroxylase (CYP11B1) inhibitor, blocked the production of corticosterone, cortisol, 11OHA4 and 11OHT. The metabolism of androstenedione and testosterone by CYP11B1 and aldosterone synthase (CYP11B2) was assayed. Androstenedione was converted by CYP11B1, while the conversion by CYP11B2 was negligible. Both enzymes readily converted testosterone. The metabolism of these 11β-hydroxylated metabolites by 11β-hydroxysteroid dehydrogenase (11βHSD) types 1 and 2 was subsequently investigated. 11βHSD2 catalyzed the conversion of both 11OHA4 and 11OHT to their respective keto-steroids, while 11βHSD1 catalyzed the conversion of 11-ketoandrostenedione and 11-ketotestosterone to their respective hydroxy-steroids in Chinese hamster ovary cells. Investigating a functional role, steroid 5α-reductase types 1 and 2 converted 11OHA4 to 11β-hydroxy-5α-androstanedione (11OH-5α-dione), identified by accurate mass detection. UPLC–MS/MS analyses of 11OHA4 metabolism in LNCaP androgen-dependent prostate cancer cells, identified the 5α-reduced metabolite as well as 11-ketoandrostenedione and 11-ketotestosterone, with the latter indicating conversion by 17β-hydroxysteroid dehydrogenase. Downstream metabolism by 11βHSD2 and by 5α-reductase may therefore indicate a physiological role for 11OHA4 and/or 11OH-5α-dione in normal and prostate cancer cells.

Abbreviations: 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OH-5α-dione, 11β-hydroxy 5α-androstanedione; 11OH-AST, 11β-hydroxyandrosterone; 11OHA4, 11β-hydroxyandrostenedione; 11OHT, 11β-hydroxytestosterone; 11βHSD, 11β-hydroxysteroid dehydrogenase; 17OHPREG, 17-hydroxypregnenolone; 17OHPROG, 17-hydroxyprogesterone; 17βHSD, 17β-hydroxysteroid dehydrogenase; 3βHSD, 3β-hydroxysteroid dehydrogenase; A4, androstenedione; ALDO, aldosterone; CORT, corticosterone; CYP17A1, P450 17-hydroxylase; CYP11B1, P450 11β-hydroxylase; CYP11B2, aldosterone synthase; DHEA, dehydroepiandrostenedione; DHEA-S, dehydroepiandrostenedione-sulfate; SULT2A1, sulfotransferase; DHT, dihydrotestosterone; DOC, deoxycorticosterone; H6PDH, hexose 6-phosphate dehydrogenase; P450, cytochrome P450; PREG, pregnenolone; PROG, progesterone; SRD5A1, steroid 5α-reductase type 1; SRD5A2, steroid 5α-reductase type 2; T, testosterone.

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1. Introduction

Although 11β-hydroxyandrostenedione (11OHA4) is a C19 steroid metabolite unique to the adrenal, it was only recently recognized as an integral member of the adrenal androgen pathway [1]. For many decades 11OHA4 was the subject of investigations surrounding clinical conditions linked to increased adrenal androgen production as observed in virilization [2]. Investigations surrounding excessive androgen production led to the unraveling of adrenal steroidogenesis, in which hormones are synthesized from the common precursor, cholesterol. Steroid hormone production is catalyzed by two distinct groups of enzymes – the cytochrome P450 (P450) enzymes and the hydroxysteroid dehydrogenases, giving rise to the unique bio-reactivity of each of the steroid hormones produced. In the androgen pathway, CYP17A1 catalyzes the conversion of pregnenolone (PREG) to 17OH-pregnenolone (17OHPREG), followed by the lyase reaction. The resulting product, dehydroepiandrostenedione (DHEA), can be converted to either dehydroepiandrostenedione-sulfate (DHEA-S) by sulfotransferase (SULT2A1) or to androstenedione (A4) by 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) [3]. DHEA and A4 both exhibit low androgenic activity, however, they serve as precursor metabolites to the more potent androgens, testosterone (T) and dihydrotestosterone (DHT) in peripheral tissue [4,5]. We recently showed that recombinant baboon CYP11B1, expressed in COS-1 cells, catalyzed the conversion of A4 to 11OHA4, a P450 activity which had, up until then, only been assigned to the conversion of deoxycorticosterone (DOC) and deoxycortisol in the mineralocorticoid and glucocorticoid pathways [1]. In these pathways, CYP11B1 catalyzes the conversion of deoxycortisol and DOC to cortisol and corticosterone (CORT) respectively, while CYP11B2 catalyzes the conversion of DOC to aldosterone (ALDO), via CORT and 18-hydroxycorticosterone (18OHCORT).

11OHA4 is, however, not the only steroid metabolite synthesized from A4 in the adrenal. While virilizing adrenal adenomas have been shown to produce more T (2.6-fold) and A4 (4.6-fold) than the normal adrenal [6], earlier investigations also identified 11β-hydroxytestosterone (11OHT) and 11-ketoandrostenedione (11KA4) in normal adrenal tissue [7]. It was shown as early as 1963 that, in normal adrenal tissue homogenates, T is metabolized to 11OHT, and while being identified as the predominant metabolite, low levels of A4, 11OHA4 and 11KA4 were also shown, leading to the deduction that 17β-hydroxysteroid dehydrogenase (17βHSD) was present in the adrenal [8].

Since the biosynthetic pathways for these metabolites in the adrenal remain unclear, the question arises as to the identity of the enzymes involved in the production of these steroids. Since CYP11B1 and 17βHSD type 5 (17βHSD5) are both expressed in the zona reticularis (ZR) [9–11], it is possible that A4, T and/or 11OHA4 may be the likely precursor candidates for 11OHT production. Immunohistochemical analyses of normal male adrenal tissue showed that while 17βHSD5 was expressed in the ZR and in clusters in the zona glomerulosa (ZG), 17βHSD type 3 (17βHSD3) was present in the ZR as well as in medullary cells [11]. In addition, since both 11β-hydroxysteroid dehydrogenase (11βHSD) isoforms are expressed in the adrenal [12,13], the inter-conversion of the hydroxylated- and keto-metabolites of A4 and T is also possible.

Renewed interest in the adrenal C19 steroid metabolites arose, in part, due to the emergence of improved analytical techniques. New developments in liquid chromatography mass spectrometry (LC–MS) technology have significantly lowered the limits of detection and quantification of current analytical methods, enabling accurate identification and quantification of steroid metabolites produced at low levels. Using LC–MS/MS, adult adrenal primary cultures were shown to produce A4 and 11OHA4 in large amounts under basal conditions, which increased significantly after ACTH stimulation, with 11OHA4 levels increasing 16.6-fold [14]. We have previously shown in forskolin stimulated H295R cells that A4 and 11OHA4 production increased by 1.9- and 4.5-fold, respectively, while the change in T levels remained negligible. Quantification using ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) identified high levels of 11OHA4 under basal (90 nM) and forskolin stimulated (388 nM) conditions [1]. CYP11B1 expression is stimulated by forskolin, which mimics the effect of ACTH in the adrenal [15], while it appears to have no influence on 17βHSD in H295R cells. 11KA4, 11OHT and 11-ketotestosterone (11KT) levels were, however, not analyzed in either of these two studies. In a recent study by Rege et al. [16], the effects of ACTH stimulation on adrenal C19 steroid production was investigated in women. Adrenal vein blood samples were collected, pre and post ACTH administration, and analyzed using LC–MS/MS. 11OHA4, 11KA4, T, 11OHT and 11KT were detected in basal samples, with ACTH stimulation resulting in a 5.2-, 3.2-, 7.3-, 5.5-, and 1.3-fold increase in the respective steroid metabolite levels. 11OHA4, T and 11OHT levels rose to 811, 5.71 and 2.62 nmol/L, respectively [16]. It was, however, shown in male primary adrenal cells that, while ACTH also stimulated A4 production, T levels remained unchanged [11].

Androgen metabolites of adrenal origin are further metabolized in peripheral tissues such as the prostate, in which DHEA is converted to A4, catalyzed by 3βHSD. A4 can either be metabolized to T, and subsequently to DHT, by 17βHSD and 5α-reductase, or alternatively, A4 may be reduced to 5α-dione and hence converted to DHT by the same set of enzymes [8]. In addition, it has been suggested that virilization in patients with P450 21-hydroxylase (CYP21) deficiency is associated with 17OH-allopregnanolone being converted to androsterone by CYP17A1 and subsequently to DHT by 17βHSD3 and 17βHSD type 6 [17]. It is therefore evident that metabolites arising from adrenal steroidogenesis feed into the androgen pathways in peripheral tissues generating DHT or other steroid metabolites capable of exhibiting androgenic activity.

The aim of this study was to investigate the metabolism of A4 and T by adrenal enzymes as well as the downstream metabolism of 11OHA4 in prostate cancer cells. We firstly examined the production of 11OHA4 and 11OHT in H295R cells, after which the 11β-hydroxylation of A4 and T by CYP11B1 and CYP11B2 was assayed. The subsequent conversion of the hydroxylated products, 11OHA4 and 11OHT, by 11βHSD and 17βHSD, which are both expressed in the human adrenal, albeit at low levels, was examined. We explored the possibility that 11OHA4, which exhibits low androgenic activity, may be converted by 17βHSD and/or 5α-reductase type 1 (SRD5A1) and type 2 (SRD5A2) and assayed the metabolism of 11OHA4 in LNCaP cells.

2. Materials and methods

2.1. Materials

Nucleobond® AX plasmid preparation kits were purchased from Machery–Nagel (Duren, Germany). COS-1 and CHO-K1 cells were obtained from the American Type tissue Culture Collection (Manassas, VA, USA) and Mirus TransIT®-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Penicillin–streptomycin, fetal bovine serum and trypsin–EDTA were obtained from Gibco BRL (Gaithersburg, MD, USA). Deuterated cortisol (9,11,12,12-D4-cortisol) was purchased from Cambridge isotopes (Andover, MA, USA). Steroids, forskolin, trilostane, Dulbecco's modified Eagle's Medium (DMEM), RPMI-1640, and an MTT assay kit were purchased from Sigma–Aldrich (St. Louis, MA, USA). Etomidate was supplied by Jannsen Pharmaceuticals (Titusville, NJ, USA) and DMSO was obtained from Merck

(Darmstadt, Germany). DMEM/F_{12} and gentamicin were purchased from Invitrogen/Gibco (Grand Island, NY, USA). Cosmic calf serum was supplied by HyClone®, Thermo Scientific (South Logan, Utah, USA). A bicinchoninic acid (BCA) protein determination kit was purchased from Pierce (Rockford, IL, USA). The Kinetex PFP column was obtained from Phenomenex. All other chemicals were of the finest quality and supplied by trustworthy scientific supply houses.

2.2. Substrate conversion assay in H295R cells

H295R cells were grown to confluency at 37 °C and 5% CO_2 in DMEM/F_{12}, supplemented with L-glutamine, 15 mM HEPES, pyridoxine, 1.125 g $NaHCO_3$/L, 1% penicillin streptomycin, 0.01% gentamicin and 10% cosmic calf serum, after which cells were plated into 12 well dishes (1 ml/well, 4×10^5 cells/ml). Steroid metabolism was assayed as follows: 24 h after plating the cells, the culture medium was replaced with medium containing 10 μM trilostane. After 24 h, the media was replaced with medium containing 10 μM trilostane and 10 μM forskolin, together with 1 μM of the appropriate steroid substrate (DOC, deoxycortisol, A4 or T), in the absence and presence of 10 μM etomidate. Control assays included the addition of 10 μM trilostane; 10 μM trilostane and 10 μM forskolin; and 10 μM trilostane, 10 μM forskolin and 10 μM etomidate. After 48 h, 500 μl aliquots were removed and the steroids extracted using a 10:1 volume of dichloromethane to culture medium. D4-cortisol (15 ng) was added as an internal standard prior to extraction. The medium was removed and the dichloromethane phase dried under N_2. The steroids were resuspended in 150 μl methanol and subjected to UPLC–MS/MS analyses. After each experiment, the cells were washed and collected in phosphate buffer (10 mM, pH 7.4), disrupted via sonication and the total protein content determined by the Pierce BCA method according to the manufacturer's instructions.

2.3. Enzyme assays in transiently transfected COS-1 cells

COS-1 cells were grown at 37 °C and 5% CO_2 in DMEM containing 0.9 g/L glucose, 0.12% $NaHCO_3$, 10% fetal bovine serum and 1% penicillin–streptomycin. The cells were plated into 12 well dishes (1 ml/well, 1×10^5 cells/ml), 24 h prior to transfection. Cells were transiently co-transfected with 1 μg DNA (0.5 μg human CYP11B1/pCMV or 0.5 μg human CYP11B2/pCMV and 0.5 μg human ADX/pCIneo) and 3 μl Mirus TransIT®-LT1 transfection reagent per ml according to the manufacturer's instructions. Control transfection reactions were performed using the pCIneo vector containing no DNA insert. Cells were incubated for 72 h after which the medium was replaced with medium containing 1 μM steroid substrate, DOC, deoxycortisol or A4.

In a subsequent experiment, COS-1 cells were transfected with human SRD5A1/pCMV7 (1 μg/ml) and SRD5A2/pCMV7 (1 μg/ml) as described above. Cells were incubated for 72 h after which the medium was replaced with medium containing 1 μM steroid substrates, 11OHA4 and T. Steroids were extracted from the medium (500 μl) and analyzed as described in Section 2.2.

2.4. Enzyme assays in transiently transfected CHO-K1 cells

CHO-K1 cells were grown at 37 °C and 5% CO_2 in DMEM containing 0.9 g/L glucose, 0.12% $NaHCO_3$, 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were plated into 12 well plates (1 ml/well, 1×10^5 cells/ml) 24 h prior to transfection. CHO-K1 cells were transiently co-transfected with 1 μg DNA (0.5 μg human CYP11B1/pCMV or CYP11B2/ pCMV and 0.5 μg human ADX/pCIneo) and 3 μl *Mirus TransIT®*-LT1 transfection reagent per ml according to the manufacturer's instructions. Cells were incubated for 72 h after which 1 μM T was added to the medium.

In a subsequent experiment, CHO-K1 cells were co-transfected with 0.5 μg human 11βHSD1/pCR3 and 0.5 μg human H6PDH/pcDNA3.2 plasmid DNA using the same protocol as described above. CHO-K1 cells were also transfected with human 11βHSD2/pCR3 plasmid DNA (1 μg) using 3 μl *Mirus TransIT®*-LT1 transfection reagent per ml according to the manufacturer's instructions. Cells were incubated for 72 h after which the appropriate substrate (1 μM), cortisol, cortisone, 11OHA4, 11KA4, 11OHT or 11KT was added to the medium. Control transfection reactions were performed using the pCIneo vector containing no DNA insert in the presence of 1 μM A4, T, 11OHA4, 11OHT, 11KA4 and 11KT. Steroids were extracted from the medium (500 μl) and analyzed as described in Section 2.2.

2.5. 11OHA4 conversion assay in LNCaP cells

LNCaP cells were grown to confluency at 37 °C and 5% CO_2 in RPMI-1640, supplemented 10% fetal bovine serum and 1% penicillin streptomycin. The cells were plated into 12 well dishes (1 ml/well, 1×10^5 cells/ml) and incubated for 24 h. The medium was replaced with low serum medium (1% fetal bovine serum) prior to substrate addition and incubated for 16 h. 11OHA4 (5 μM) was added and the cells were incubated for 48 h. The medium (500 μl) was removed and D4-cortisol (15 ng) was added as an internal standard prior to steroid extraction. Steroids were extracted and the total protein content determined as described in Section 2.2.

2.6. UPLC–MS/MS separation and quantification of steroid metabolites

Stock solutions of DOC, CORT, 18OH-CORT, ALDO, 11-DHC, deoxycortisol, cortisol, cortisone, DHEA, DHEA-S, A4, T, 11OHA4, 11KA4, 11OHT, 11KT and DHT were prepared in ethanol (2 mg/ml). A series of standards (0.2, 2, 20, 100, 200, 1000, and 2000 ng/ml were prepared in methanol from the stock solutions. In addition, each standard contained an internal standard, D4-cortisol (final concentration, 100 ng/ml).

Steroid metabolites were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Phenomenex Kinetex PFP (2.1 mm × 100 mm, 2.6 μm) column as previously described [1], with modifications to the gradient of the LC system (supplementary Table 1). The total run time was 5 min and the injection volume was 5 μl. A Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. All steroids were analyzed in multiple reaction monitoring (MRM) mode using an electrospray probe in the positive ionization mode (ESI+). The following settings were used: capillary voltage of 3.5 kV, source temperature 120 °C, desolvation temperature 400 °C, desolvation gas 900 L h^{-1} and cone gas 50 L h^{-1}. Other relevant information including the parent and daughter ions, cone (V) and collision (eV) voltages, retention times (RT) and the validation of the UPLC–MS/MS assay is shown in supplementary Table 2. Calibration curves were constructed using weighted ($1/x^2$) linear least squares regression. Data were collected with the MassLynx 4.1 software.

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The limit of detection (LOD) for each steroid metabolite was defined as the lowest concentration at which a signal-to-noise (S/N) ratio greater than 3 was detected. The limit of quantification (LOQ) was defined as the lowest concentration of each steroid on the standard curve at which a S/N ratio greater than 10 was detected and which could be measured with acceptable precision, expressed as percent relative standard deviation (%R.S.D.).

Method validations were carried out by replicate analysis ($n = 3$) of cell culture media (0.5 ml), DMEM, DMEM/F_{12} and RPMI-1640,

spiked with each steroid at a low (2 ng/ml) and high concentration (2000 ng/ml), followed by the addition of D4-cortisol (15 ng) as an internal standard. Steroids were extracted and samples were prepared for UPLC–MS/MS as described in Section 2.2. The relative standard deviation for each steroid was less than 10% at a concentration of 2 ng/ml (<10% R.S.D.) and less than 20% at a concentration of 2000 ng/ml (<20% R.S.D.). Three independent samples were prepared for each concentration (biological repeats) and each sample was analyzed 5 times (instrumental repeats). Absolute recoveries of the steroids extracted from spiked cell culture media were calculated and the peak areas of extracted samples subsequently compared with standard solutions. The recoveries of the steroids (T, 11OHT, 11KT, A4, 11OHA4 and 11KA4) ranged from 73.95% to 122.50% (average: 97.57 ± 14.64%). Standard curves were generated for each steroid metabolite using the following concentrations: 0.2, 0.4, 2, 4, 20, 200 and 2000 ng/ml. The calibration curves were linear over these concentration ranges, with regression correlation coefficients (r^2) always being greater than 0.99.

Accurate mass determinations were preformed on a Synapt G2 quadrupole time-of-flight mass spectrometer (Waters, Milford, USA) using ESI+. Steroids were introduced into the MS by an ACQUITY UPLC (Waters, Milford, USA) using the same LC conditions as described above. The capillary voltage was 2.5 kV, cone voltage 15 V, source temperature 120 °C, desolvation temperature 275 °C, desolvation gas 650 L h^{-1} and cone gas 50 L h^{-1}. The rest of the settings were optimized for best sensitivity. The instrument was calibrated with sodium formate and leucine enkephalin was used as the reference standard as lock mass.

2.7. Statistical analysis

All experiments were performed in triplicate and results are presented as means ± SEM. The data were analyzed with an unpaired *t*-test using GraphPad Prism (version 5) (GraphPad Software, San Diego, California). A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

Androgen production in the adrenal is generally defined to include the C19 steroids – DHEA, A4, androstenediol and T. Although it had been firmly established by earlier studies that the human adrenal was capable of synthesizing hydroxy- and keto-metabolites of A4 and T, these steroids are seldom included as adrenal androgens. This study was therefore undertaken to investigate the production and metabolism of 11OHA4 and 11OHT during adrenal steroidogenesis.

3.1. UPLC–MS/MS analysis of C19 adrenal steroids

Significant advances in analytical techniques have enabled accurate analyses of steroids produced at low levels by the adrenal, allowing the identification and quantification of metabolites such as 11KA4, 11OHT and 11KT. In 2011, Xing et al. [14] reported that ACTH stimulated 11OHA4 production in primary adrenal cultures and that the metabolite was one of the major steroids produced by the human adrenal. LC–MS/MS analyses in H295R cells showed that deoxycortisol and A4 were the major steroids produced under basal conditions and that the addition of forskolin stimulated, amongst others, the production of 11OHA4 [14]. What sets this study apart from previous investigations is that, for the first time, all the known steroid metabolites produced in the adrenal cell were analyzed by LC–MS/MS, together with limited steroid derivitisation, allowing analyses of all the intermediates and end products in the steroidogenic pathway, which up until now had remained challenging. Subsequent to this study, using an UPLC–MS/MS, we quantified all the metabolites produced in H295R cells, which had been identified to date, and showed that 11OHA4 was a major product of steroidogenesis [1]. High concentrations of A4, 0.585 μM and 11OHA4 0.811 μM were also detected in adrenal vein samples collected from women after ACTH administration [16]. UPLC–MS/MS analyses of C19 steroid levels in H295R cells showed significantly increased levels of A4 and 11OHA4 to 1.338 μM (1.4-fold) and 0.330 μM (3.5-fold), respectively upon forskolin stimulation (Table 1). We quantified the steroid metabolites of interest, using our previously published method with minor changes to the LC elution gradient, which enabled the separation of all the C19 steroids of interest as well as of the glucocorticoids, using a single chromatographic separation. UPLC–MS/MS chromatographic separation of 11OHA4, 11KA4, 11OHT and 11KT is shown in Fig. 1. Other relevant information regarding the retention times, cone voltages, collision energies, precursor and product ions, LOD, LOQ, calibration ranges and linearities are shown in Table 2.

Table 1
Analyses of C19 steroids produced in H295R cells. Cells were incubated in the absence and presence of forskolin (10 μM) for 48 h and steroids were quantified by UPLC–MS/MS. Fold change ± SEM in response to forskolin treatment was calculated from the changes in absolute values of individual steroids compared to basal values. Data was analyzed using an unpaired *t*-test and results are representative of three independent experiments, each performed in triplicate (*$P < 0.05$, ***$P < 0.001$).

Steroid metabolite	Basal	Forskolin	
	Total ± SEM (nM)	Total ± SEM (nM)	Fold change
DHEA-S	3.6 ± 0.2	5.3 ± 0.3	↑1.6*
A4	913.2 ± 29.2	1338.0 ± 81.1	↑1.4***
11OHA4	100.4 ± 5.2	329.9 ± 27.2	↑3.5***
11KA4	2.8 ± 1.0	3.4 ± 0.8	
T	46.0 ± 3.8	50.0 ± 2.4	
11OHT	N.D.	N.D.	
11KT	N.D.	N.D.	

3.2. DOC, deoxycortisol, A4 and T metabolism in H295R cells

Based on our previous findings which showed that 11OHA4 was the product of A4 hydroxylation in H295R cells [1] and previous

Table 2
Parameters for the detection and quantification of six C19 steroids by UPLC–MS/MS: retention times (RT, min), cone voltages (CV), collision energy (CE), limit of detection (LOD) and limit of quantification (LOQ).

Steroid metabolite	RT (min)	Precursor ion	CV	Product ion A	CE	Product ion B	CE	LOD[a] (ng/ml)	LOQ[b] (ng/ml)	Calibration range (ng/ml)	Linearity (r^2)
11KT	2.24	303.2	30	121.0	20	267.0	20	0.7	2	2–2000	0.9966
11OHT	2.25	305.3	35	121.0	20	269.0	15	0.07	0.2	0.2–2000	0.9941
11OHA4	2.36	303.2	30	121.0	30	267.2	15	0.07	0.2	0.2–2000	0.9989
11KA4	2.46	301.2	35	241.0	30	257.0	25	0.2	0.4	0.4–2000	0.9988
Testosterone	3.07	289.2	30	97.2	22	109.0	22	0.07	0.2	0.2–2000	0.9931
A4	3.23	287.2	30	96.9	15	108.8	15	0.07	0.2	0.2–2000	0.9990

[a] Limit of detection was defined as a *S*/*N* ratio >3.
[b] Limit of quantification was defined as a *S*/*N* ratio >10.

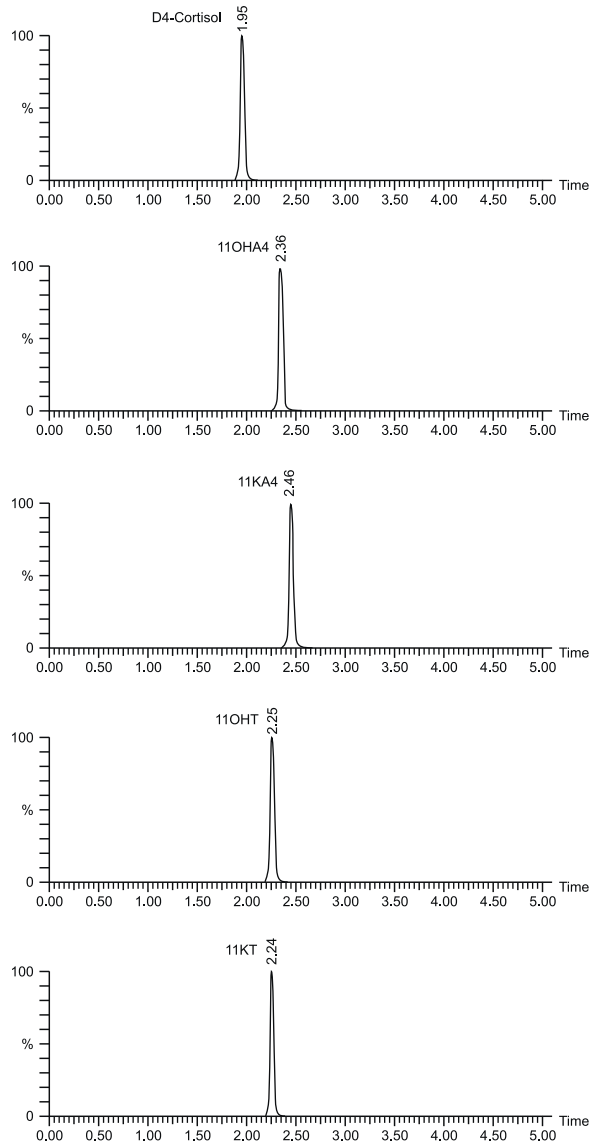

Fig. 1. UPLC–MS/MS chromatographic separation of 11OHA4, 11KA4, 11OHT, 11KT and D4-cortisol are shown. Retention times are indicated on the chromatograms of steroid metabolites (5 μl of a 2 μg/ml standard solution) shown in multiple reaction monitoring (MRM) mode.

reports showing that forskolin stimulates CYP11B1 expression [18], we compared C11-hydroxylated product formation by assaying the conversion of DOC (Fig. 2A), deoxycortisol (Fig. 2B), A4 (Fig. 2C) and T (Fig. 2D) in the presence of trilostane (10 μM), a selective inhibitor of 3βHSD, in forskolin-stimulated (10 μM) H295R cells. In these assays, conversions were investigated using 1 μM substrate, thus facilitating the accurate detection and quantification of all the metabolites formed. Negligible amounts of CORT, cortisol, 11OHA4 and 11OHT were detected after 48 h, indicating that basal 11β-hydroxlated metabolite production was successfully suppressed in the presence of trilostane. The addition of appropriate substrates (1 μM) together with trilostane (10 μM) resulted in the production of cortisol (646 nM), CORT (361 nM), 11OHA4 (239 nM) and 11OHT (133 nM), which in turn was inhibited by the addition of etomidate (10 μM), a CYP11B1 inhibitor.

Of interest were the levels of 11OHT (±50 nM) detected in stimulated cells in the presence of trilostane as well as upon addition of T and etomidate. Similar levels of 11OHT were also detected under basal conditions in the presence of trilostane. In both basal and stimulated conditions T was not detected in the presence of trilostane, indicating that the available T was converted to 11OHT. Neither T nor 11OHT were detected upon addition of etomidate, under basal or stimulated conditions. It is generally reported that etomidate is a selective inhibitor of 11β-hydroxylation, decreasing cortisol plasma levels, while increasing 17OHPROG and deoxycortisol plasma levels [19,20]. In addition, it has been reported that etomidate, administered as a single dose, may influence cortisol biosynthesis to a greater degree during ACTH-stimulation than under basal conditions [21]. In our analyses of the steroid profiles, we detected that etomidate did not only decrease the levels of cortisol, CORT and 11OHA4 under basal and forskolin stimulated conditions, but also the levels of their precursors, suggesting that etomidate is perhaps not such a selective inhibitor of 11β-hydroxylase activity as reported to date (data not shown). Our data nevertheless indicated that 11OHA4 and 11OHT are both products of 11β-hydroxylation of A4 and T. Low levels of 11OHT (59 nM) were detected in the conversion of A4, indicating that 11OHA4 may serve as a substrate for 17βHSD5. We have, however, shown that 11OHA4 is not readily converted by 17βHSD5 (unpublished data). It is thus possible that the 11OHT produced in the conversion of A4 (Fig. 2C) arises from the conversion of A4 to T by 17βHSD5 and the subsequent hydroxylation of T.

3.3. DOC, deoxycortisol, A4 and T conversion by human CYP11B1 and CYP11B2

The above findings, together with the report that the conversion of ^{14}C-T in normal human adrenal homogenates yielded 11OHT as the major product [8], led us to examine the conversion of A4 and T by human CYP11B1 and CYP11B2. Although earlier studies had identified 11OHA4 in human and bovine adrenal mitochondria, there was no clear indication that the hydroxylation of A4 could be attributed to CYP11B1 and/or CYP11B2 activity [22,23]. However, purified bovine CYP11B1 in reconstituted systems and recombinant bovine CYP11B1 expressed in COS-1cells had been shown to convert A4 to 11OHA4, albeit at a lower rate than DOC [24,25]. In this study, we compared the conversion of four substrates, DOC, deoxycortisol, A4 and T, all at a concentration of 1 μM, by human CYP11B1 (Fig. 3A) as well as the conversion of DOC, A4 and T by human CYP11B2 (Fig. 3B) in cells transiently co-transfected with the appropriate human cDNA and human adrenodoxin (ADX). DOC, deoxycortisol and A4 conversion assays were conducted in COS-1 cells, while T conversion was assayed in Chinese hamster ovary (CHO-K1) cells, as endogenous 17βHSD2 in COS-1 cells [26] would convert the T substrate to A4, therefore confounding results in these cells. Conversion assays using 1 μM substrate enabled the accurate detection of product formation by UPLC–MS/MS, as some substrates were converted to more than one product. CYP11B1 and CYP11B2 catalyzed the conversion of DOC and deoxycortisol with little substrate remaining after the incubation period. Although CYP11B1 readily catalyzed the conversion of A4 to the 11β-hydroxylated product, conversion by CYP11B2 was negligible, with 82% substrate remaining even after the extended incubation period of 12 h. The conversion assays of A4 also yielded negligible levels of 11KA4 due to 11βHSD activity, which has been shown in COS-1 cells [27]. In contrast, the 11β-hydroxylation of T was catalyzed to the same degree by both CYP11B1 and CYP11B2. Clearly T is a substrate for CYP11B and would be converted to 11OHT in the adrenal. However, A4 may also contribute toward 11OHT production, specifically in females, since 17βHSD3 and 17βHSD5 are expressed in the adrenal, and together with CYP11B1, A4 may thus be converted to T and subsequently hydroxylated by CYP11B1. In this study we observed that the conversion of T was not as efficient as that of A4 by CYP11B1, even after 16 h. In stimulated H295R cells we detected 11OHT (Fig. 2) in the presence of trilostane as well as

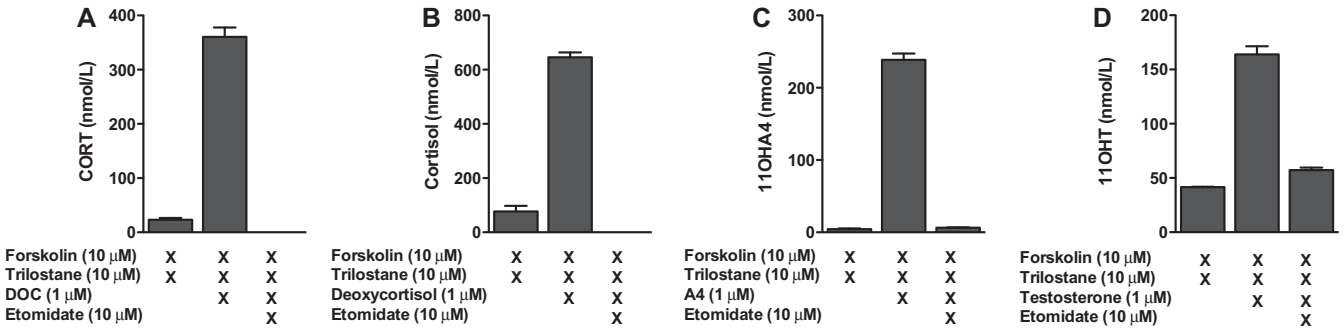

Fig. 2. Analysis of 11β-hydroxylation in H295R cells. Conversion of 1 μM (A) DOC, (B) deoxycortisol, (C) A4 and (D) T was assayed after 48 h in cells treated with forskolin (10 μM) and trilostane (10 μM) in the absence and presence of etomidate (10 μM). Results are representative of two independent experiments performed in triplicate, and are expressed as the mean ± SEM.

upon addition of etomidate, and although we showed that 11OHA4 is not converted to 11OHT in CHO-K1 cells which express 17βHSD1 and 17βHSD7, it is possible that, after 48 h, basal 11OHA4 may have been converted to 11OHT due to the catalytic activity of 17βHSD. Although our data on steroid conversion indicate that both A4 and T are 11β-hydroxylated, the production of 11OHT via A4 and T would also depend on the level of expression of CYP11B1 and 17βHSD5 in the adrenal. Our analyses of basal and forskolin stimulated H295R cells (Table 1) show that forskolin does not increase T, 11OHT or 11KT levels, which is in agreement with the findings by Hofland et al. [11] who showed that ACTH did not increase T levels or stimulate 17βHSD3 and 17βHSD5 in normal adrenal cells. Although CYP11B1 and CYP11B2 are primarily expressed in the adrenal cortex [28], CYP11B1 is also expressed in the gonads of mice [29], while both CYP11B1 and CYP11B2 are expressed in human primary prostate carcinomas and metastatic primary prostate carcinomas [30,31]. In rat Leydig cells, the 11β-hydroxylase activity shown by Wang et al. [32] was linked to a modulatory role in which the authors proposed that the 11β-hydroxylation of steroid metabolites yield endogenous inhibitors of 11βHSD1, thus affecting either the protective function of the dehydrogenase activity toward glucocorticoid metabolites or the reductase activity toward inactive metabolites in these cells. The hydroxylation of T at C11 in the testes would result in the formation of 11OHT, which was shown to inhibit 11βHSD1 in whole cell preparations of rat Leydig cells. With the dual activity of 11βHSD1 in Leydig cells modulating glucocorticoid action in the testis, the hydroxylation of T in these cells and the subsequent formation of 11KT, may indicate opposing roles for these metabolites regarding their effect on the dehydrogenase/reductase activities of 11βHSD1 [32].

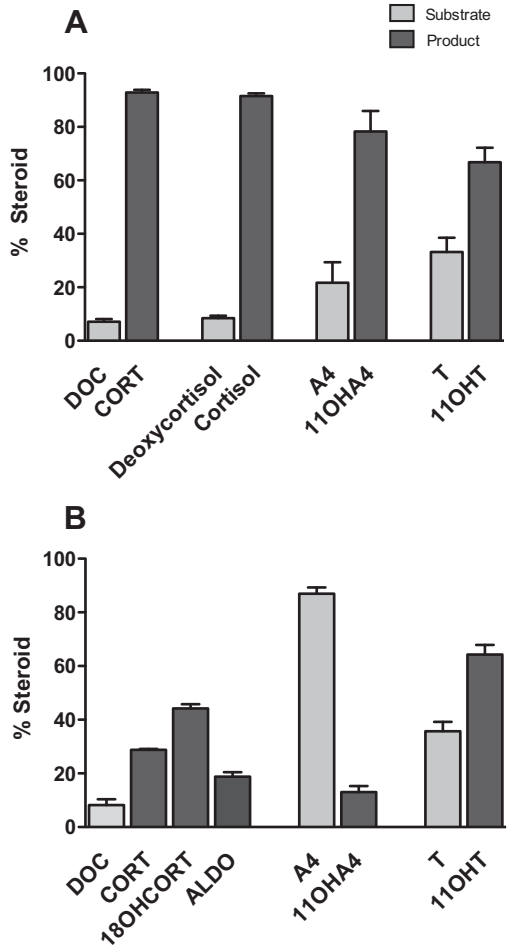

Fig. 3. Analysis of 11β-hydroxylation in transiently co-transfected COS-1 and CHO-K1 cells. (A) CYP11B1 and ADX conversion of 1 μM DOC, deoxycortisol and A4 after 6 h in COS-1 cells, and T after 16 h in CHO-K1 cells; (B) CYP11B2 and ADX conversion of 1 μM DOC and A4 after 12 h in COS-1 cells, and T after 16 h in CHO-K1 cells. Results are representative of two independent experiments performed in triplicate and are expressed as the mean ± SEM.

Our previous findings, in which cortisol was not cleaved at C17 to form 11OHA4 in H295R cells, were confirmed in this study, since 11OHA4 was not detected in the analyses of metabolites formed in the conversion assay of deoxycortisol in COS-1 cells expressing CYP11B1. Although the H295R cell line is a carcinoma cell model, we showed that 11OHA4 and 11OHT were products formed by the hydroxylation of A4 and T at C11, verified by the homologous expression of CYP11B1 and CYP11B2. Earlier reports of investigations regarding the origin of adrenal 11OHA4 implicated the C17,20-lyase activity of CYP17 toward cortisol being responsible for the production of adrenal 11OHA4 [33]. Subsequent studies have shown the side-chain cleavage of cortisol resulting in the formation of 11OHA4 to take place in vascular smooth muscle (VSM) cells, and in liver and kidney tissue [34–36]. In the VSM cells, both 11OHA4 and 11KA4 formed with NAD^+ as the preferred cofactor for the lyase reaction. However, 11KA4 was also detected in the presence of $NADP^+$ and indicating that the metabolite was also a product of 11OHA4 conversion by 11βHSD1 [34].

3.4. 11OHA4, 11KA4, 11OHT and 11KT conversion by 11βHSD1 and 11βHSD2

In our analyses of steroid metabolites in H295R cells, we detected 11KA4 at low levels under all experimental conditions. We also detected low levels of 11KA4 (3–7%) in our conversion assays of A4 by CYP11B1 and CYP11B2 in COS-1 cells described above, indicating that endogenous 11βHSD2 in COS-1 cells

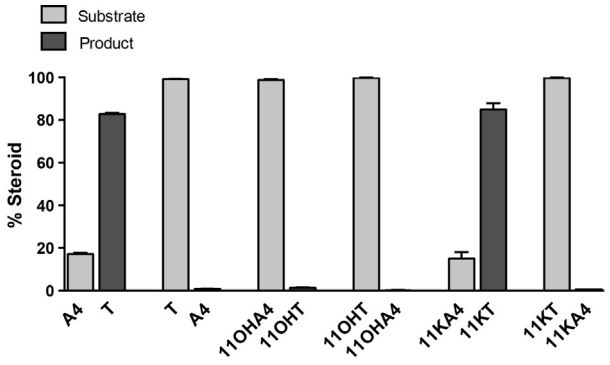

Fig. 4. Substrate (1 μM) conversion by endogenous 17βHSD in CHO-K1 cells. A4, T, 11OHA4, 11OHT, 11KA4 and 11KT conversion was assayed after 24 h in CHO-K1 cells transfected with pCIneo. Results are representative of two independent experiments performed in triplicate and are expressed as the mean ± SEM.

oxidized some of the 11OHA4 formed. Since both 11βHSD1 and 11βHSD2 are expressed in the human adrenal, we investigated the activity of these enzymes toward the 11β-hydroxylated metabolites in CHO-K1 cells, which do not express endogenous 11βHSD. However, endogenous 17βHSD in CHO-K1 cells [37,38] would confound interpretation of results and even though we established that the endogenous 17βHSD did not exhibit oxidative activity (only 11OHT was detected in the conversion assay of T (Fig. 3)), the reductive activity had not been established. Various 17βHSD enzymes have been identified in the ovaries of rodents, which catalyze either the reduction or oxidation of C17 keto- or hydroxy-steroids, respectively, or both, depending on substrate and cofactor availability [38]. We therefore assayed the basal conversion of A4, T, 11OHA4, 11OHT, 11KA4, and 11KT in CHO-K1 cells. Only T and 11KT were detected after 24 h, indicating that the endogenous 17βHSD exhibited reductive activity toward A4 and 11KA4 only, since no 11OHA4 was converted to 11OHT (Fig. 4). While endogenous 17βHSD2 in COS-1 cells readily converted T, 11KT and 11OHT to A4, 11KA4 and 11OHA4, respectively (unpublished data), endogenous 17βHSD did not convert 11OHA4 in CHO-K1 cells. It is possible that the hydroxy- and not the keto-group at C11 hinders the binding of the steroid substrate in a position favorable for the reduction of the keto-group at C17. Interestingly, both 11OHA4 and 11KA4 have been shown to be converted to 11OHT and 11KT by 17βHSD3 in the presence of NADPH in HEK-293 Ebna cells [39]. HEK-293 cells also express 11βHSD2 and would convert 11OHA4 to its keto-product, which would in turn be converted to 11KT if the reaction conditions were optimal. It is, however, possible that 17βHSD has a higher catalytic activity for 11OHA4 than 11βHSD2.

Having established the basal 17βHSD activity, we assayed the conversion of 11OHA4, 11KA4, 11OHT and 11KT in CHO-K1 cells transiently transfected with human 11βHSD1 and 11βHSD2. We co-transfected 11βHSD1, a bi-directional enzyme catalyzing both the oxo-reductase and dehydrogenase reactions, with the cofactor hexose 6-phosphate dehydrogenase (H6PDH) to simulate conditions in which the oxo-reductase activity of 11βHSD1 predominates [40]. 11βHSD1 converted 11KA4 to 11OHA4 (46%) (Fig. 5A) and 11KT to 11OHT (66%) (Fig. 5C). Neither 11KA4 or 11KT were detected when 11OHA4 and 11OHT were added as substrates. The predominant reductase activity was confirmed in the control conversion assay in which no cortisone was detected when cortisol was added as substrate, while ±45% cortisone was converted to cortisol (Fig. 5E). In addition, 11βHSD2 activity was confirmed in the conversion of cortisol to cortisone (65%) (Fig. 5F).

In the analyses of 11βHSD1 activity toward 11KA4 we also detected 11OHT (45%). This was due to the intrinsic activity of 17βHSD in CHO-K1 cells, which converted the substrate to 11KT,

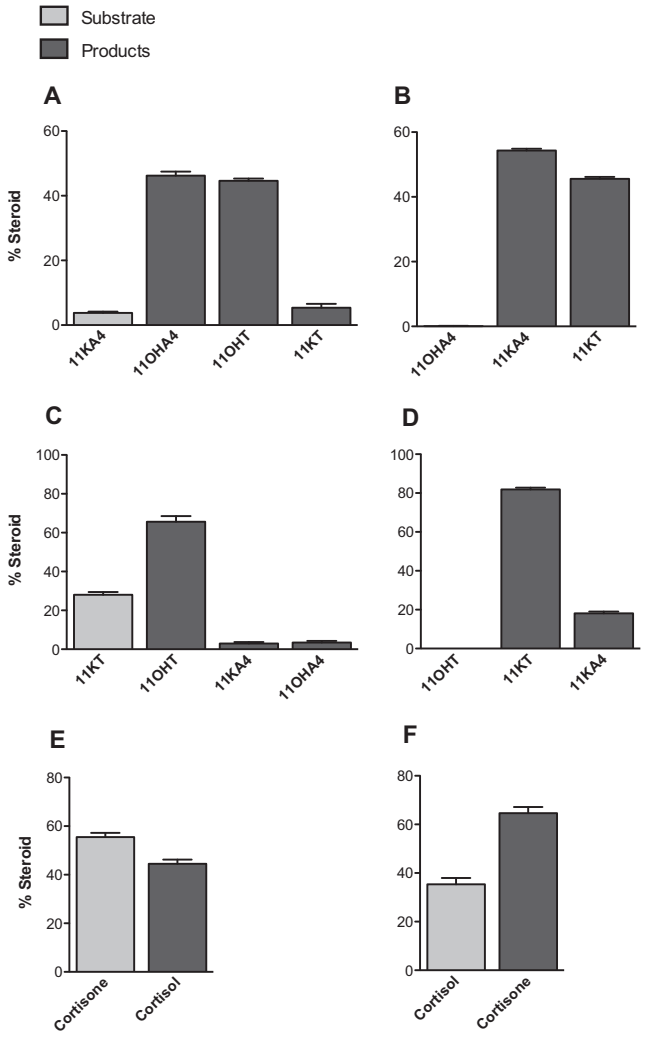

Fig. 5. Substrate (1 μM) conversion by human 11βHSD1 and 11βHSD2 in CHO-K1 cells. (A) 11KA4 conversion by 11βHSD1 and H6PDH; (B) 11OHA4 conversion by 11βHSD2; (C) 11KT conversion by 11βHSD1 and H6PDH; (D) 11OHT conversion by 11βHSD2; (E) cortisone conversion by 11βHSD1 and H6PDH (F) cortisol conversion by 11βHSD2 after 48 h. Results are representative of two independent experiments performed in triplicate and are expressed as the mean ± SEM.

followed by the subsequent formation of the hydroxylated product catalyzed by 11βHSD1. Our analyses of the metabolites formed when 11KT was added as substrate showed negligible levels of 11KA4 (3%) and 11OHA4 (4%). Although the catalytic activity of 11βHSD1 appeared to be greater toward 11KT, this would have to be determined in the absence of 17βHSD, which competes for 11KA4 as substrate.

Conversion of 11OHA4 by 11βHSD2 yielded 11KA4 (54%) as well as 11KT (46%) (Fig. 5B). No 11OHT was detected, indicating that the 11KT formed was a product of 11KA4 conversion by endogenous 17βHSD. 11βHSD2 also converted 11OHT to 11KT (82%), with no remaining substrate being detected (Fig. 5D). Low levels of 11KA4 (18%) were, however, detected indicating that oxidation at the C17 keto-group of 11KT was taking place. This may possibly be attributed to the substrate concentration favoring the reverse reaction, since 17βHSD enzymes are able to catalyze the reverse reactions in vitro, under conditions that are optimal and in which cofactors are not limited [38].

The data therefore clearly shows that 11KA4 and 11KT as well as 11OHA4, and 11OHT are metabolized by 11βHSD1 and 11βHSD2, respectively. These enzymes have previously been

shown to catalyze the inter-conversion of cortisol and cortisone as well as that of CORT and 11-dehydrocorticosterone (11-DHC) only. In addition, while endogenous 17βHSDs expressed in CHO-K1 cells did not catalyze the conversion of 11OHA4 to 11OHT, 11KA4 was readily converted to 11KT.

The biosynthesis of 11OHA4 and 11OHT by CYP11B1 and CYP11B2, together with the contribution by 11βHSD, lends further complexity to the adrenal steroidogenic pathway. Whereas CYP17A1, 3βHSD and cytochrome b_5 are enzymes involved at a branch point in adrenal steroidogenesis in terms of metabolites committing to a specific pathway, perhaps CYP11B2, with its restriction to the zona glomerulosa (ZG), and CYP11B1, with its expression in the ZF and ZR, could be viewed as a second zone specific branch point within adrenal steroidogenesis, serving as a fine-tuning mechanism, determining the levels and ratios of end products. Immunohistochemical analyses identified the expression of 17βHSD3 and 17βHSD5 in the ZR [11], which would convert A4 to T, delivering the latter as substrate for 11β-hydroxylation, and in turn, 11OHT becomes available as substrate for 11βHSD2, yielding 11KT. Conversely, the hydroxylation of A4 yielded 11OHA4, which may be converted to 11KA4, a metabolite which in turn may serve as a substrate for 17βHSD, yielding 11KT.

The steroid shunt in the adrenal may therefore contribute metabolites other than the reported A4 and low levels of T to the androgen pool, which, together with further enzymatic conversions in peripheral tissues, may yield potent androgenic steroids. Although limited studies have been conducted with 11OHA4, it is widely accepted that this metabolite exhibits low androgenic activity. Bélanger et al. [41] hypothesized that the biosynthesis of 11OHA4, which is the major C19 steroid metabolite in the guinea pig, may be a mechanism by which adrenal androgens are inactivated when they showed that 11OHA4 had low androgenic activity [41]. While guinea pig CYP17A1 utilizes both 17OHPROG and 17OHPREG as substrates for the lyase reaction [41,42], humans are a Δ5 lyase specific species, with CYP17A1 preferentially catalyzing the conversion of 17OHPREG to DHEA. The low expression levels of 3βHSD in the ZR [10] results in the formation of DHEAS as the primary C19 steroid produced in the adrenal. The guinea pig is, nevertheless, a specie in which the adrenal produces high levels of DHEA, A4 and 11OHA4. It has been shown that ACTH administration in guinea pigs resulted in the accumulation of C19 steroids with a higher formation of A4 and 11OHA4, with 11OHA4 being the only C19 steroid released into circulation [41]. This group had previously suggested that increased adrenal plasma C19 levels may be a source of potent androgens when DHEA and A4 were shown to increase prostate weight in castrated rats, with DHT accumulating in prostatic tissue [43]. In our previous study, we showed that in H295R cells, basal levels of A4, 11OHA4 and T are high, with the former two metabolites being increased significantly upon forskolin stimulation [1]. Although the H295R cell line is an adrenocortical carcinoma cell line producing higher levels of adrenal androgens, a study by Xing et al. [14] showed that high levels of both A4 and 11OHA4 were also produced in adult adrenal primary cell cultures under basal conditions. In addition, while ACTH stimulation increased both DHEA and DHEAS levels, significant increases were detected in the levels of A4 (25.7-fold) and 11OHA4 (16.6-fold) [14]. The conversion of 11OHA4 to the keto-form, and subsequently to T derivatives, may place 11OHA4 in an important position in these metabolic pathways due to the amounts produced in comparison to 11OHT and its conversion to 11KT in the adrenal. In the study by Rege et al. [16] it was shown that 11OHT and 11KT exhibited substantially greater androgenic activity toward the AR than A4, 11OHA4 and 11KA4. Although 11OHT and 11KT may appear to be important steroids, these steroids were detected at very low levels in human adrenal vein samples (0.48 nM and 0.39 nM pre ACTH administration). Furthermore, the concentration of A4 was 100-fold higher than that of T in the adrenal vein samples. The metabolism of these two substrates by CYP11B1 resulted in 11OHA4 being ±300-fold higher than 11OHT before and after ACTH administration. The levels of 11OHA4, being significantly higher than those of T, may reflect the relative expression of these two enzymes in the adrenal as well as the possible competition between CYP11B1 and 17βHSD for A4. Microarray analyses showed that CYP11B1 was highly expressed in normal adult adrenals and that while the expression levels of 17βHSD5 were also substantial, 17βHSD3 as well as 11βHSD1 and 11βHSD2 were only detected at very low levels. The low levels of 11βHSD2 expression resulted in low levels of the keto-metabolites being produced even after ACTH administration [16]. Although the endogenous levels of 17βHSD and 11βHSD are as such that only low levels of these androgen metabolites are produced in the adrenal, the peripheral conversion of these steroid metabolites together with 11OHA4 cannot be ignored.

3.5. 11OHA4 metabolism in LNCaP cells

Perhaps the hypothesis put forward by Bélanger et al. in 1993 [41] is one that may still stand in terms of AR interaction with the conversion of excess A4 to 11OHA4 by CYP11B1, decreasing circulating levels of A4, which would otherwise be channeled to produce DHT via the formation of 5α-androstanedione or via the formation of T in the prostate. Excess adrenal androgen production, raising A4 levels in circulation, could have implications in castrate resistant prostate cancer, since the metabolite is metabolized by 17βHSD and by steroid 5α-reductase. However, 11OHA4 and its adrenal metabolites may be converted to more potent androgens in peripheral tissues and the production of the metabolite can therefore not be regarded as an inactivation process. In addition, T may also be hydroxylated at C11 in prostate cancer cells as CYP11B2 has been shown to be present in LNCaP cells, with its expression being upregulated in the presence of dutasteride, a selective 5α-reductase inhibitor, suggesting a mechanism for the production of alternative androgens, such as 11OHT, in the absence of DHT in prostate cancer [31]. We firstly assayed the conversion of 11OHA4 by steroid 5α-reductase type 1 (SRD5A1) and steroid 5α-reductase type 2 (SRD5A2) in transiently transfected COS-1 cells (Fig. 6A). UPLC–MS/MS analysis showed that both isoenzymes readily metabolized 11OHA4 to 11β-hydroxy-5α-androstanedione (11OH-5α-dione), with negligible substrate remaining after 3 h, comparable to the conversion of T to DHT (Fig. 6B). 11OH-5α-dione was identified by accurate mass detection and is shown in Fig. 7. With the 5α-reduction of 11OHA4 by both isoenzymes, indicating a possible role for this metabolite in prostate cancer, we assayed the metabolism of 11OHA4 (5 μM) in LNCaP cells after a 48 h incubation period. UPLC–MS/MS analysis of steroid metabolites (Fig. 8) detected 11OH-5α-dione, however, quantification was not possible since the steroid is not commercially available. In the analyses of unconjugated metabolites, high levels of 11KA4 (1.8 μM) were detected while 11KT (174 nM) and negligible 11OHT (6 nM) were also detected. In an assay conducted by Byrns et al. [44], in which 5 μM A4 was assayed in LNCaP cells, the levels of unconjugated steroids were ±15%, while glucuronidated steroids comprised ±45% of the A4 metabolites analyzed [44]. We subsequently assayed the metabolism of 1 μM 11OHA4 and in the analyses of both unconjugated and conjugated steroid metabolites (Fig. 9) did not detect 11OHT. In the analyses of the metabolism of A4 and T, neither 11OHA4 nor 11OHT were detected, indicating that the basal expression of CYP11B2 may be negligible.

Our data shows that while 11OHA4 is metabolized to 11OH-5α-dione, the production of high levels of 11KA4 indicates that 11OHA4 is certainly a substrate for 11βHSD2 in these cells. The lower levels of 11KT and negligible 11OHT levels show that while conversion by 17βHSD takes place to a lesser degree, the production of these

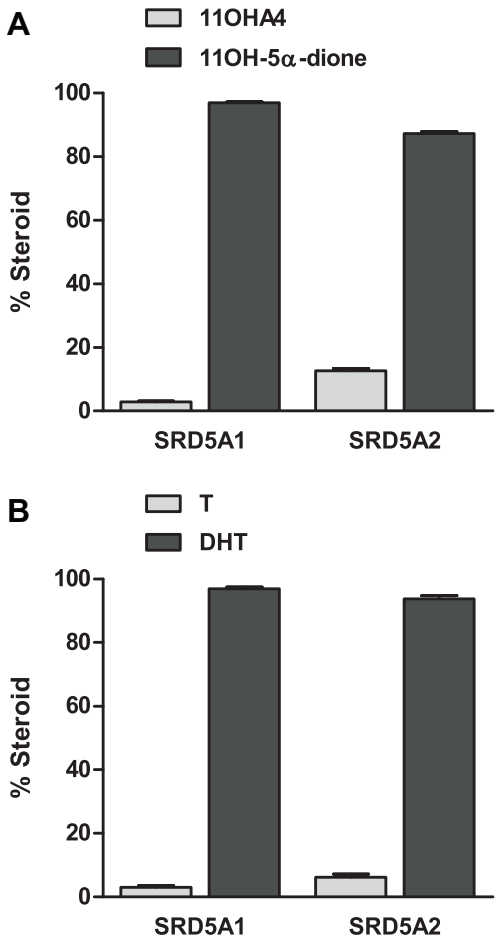

Fig. 6. Substrate (1 μM) conversion by human SRD5A1 and SRD5A2 expressed in COS-1 cells. (A) 11OHA4 conversion by SRD5A1 and SRD5A2, (B) T conversion by SRD5A1 and SRD5A2 after 3 h. Results are representative of two independent experiments performed in triplicate and are expressed as the mean ± SEM.

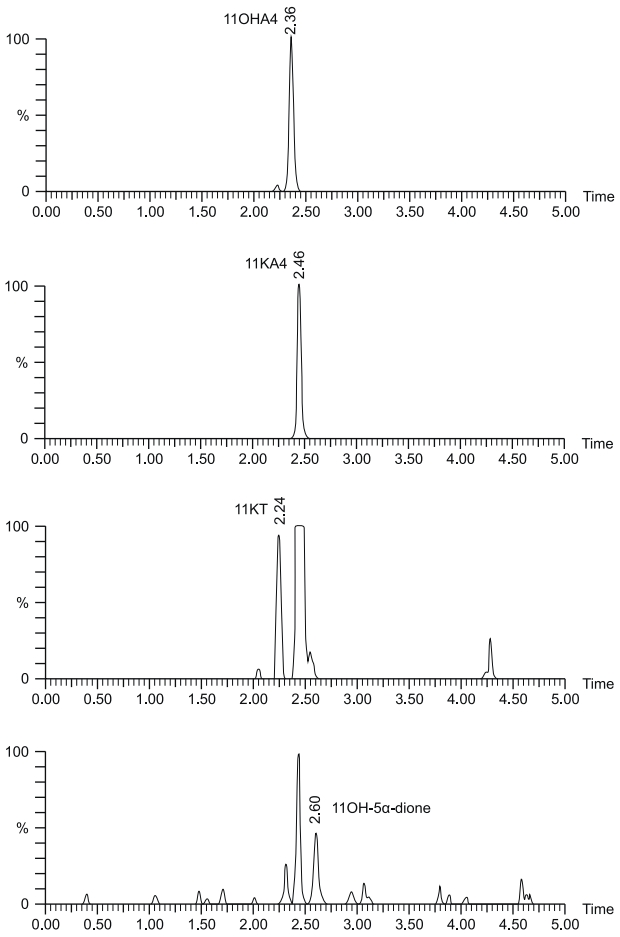

Fig. 8. UPLC–MS/MS analysis of 11OHA4, 5 μM, conversion by LNCaP cells. Representative chromatograms of substrate and metabolites after 48 h are shown in multiple reaction monitoring (MRM) mode. Retention times of steroids are indicated on the chromatograms.

metabolites would depend on the expression levels of the relevant enzymes and cofactor availability in the prostate cells. In a study by Latif et al. [45], it was demonstrated that 11OHT and 11KT strongly inhibited 11βHSD2 activity in sheep kidney microsomes. While 11OHA4 was shown to be a weak inhibitor, no inhibition by 11KA4 was detected [45]. Although we detected high levels of 11KA4 and 11KT while not detecting 11OHT when we assayed 1 μM 11OHA4, it is possible that the presence of 11OHT and 11KT may influence the conversion of 11OHA4 and 11OHT to their keto-derivitives in prostate cells. This would, however, depend on the expression levels of 11βHSD2 and those of enzymes competing for the same substrates as well as the levels of steroid metabolites.

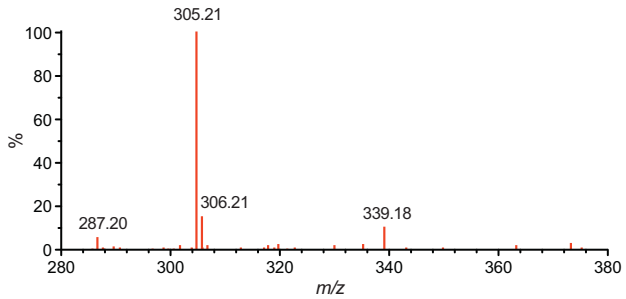

Fig. 7. MS spectrum of 11OH-5a-dione ([M+H]$^+$ m/z 305.21). Data was acquired from m/z 280–380.

The presence of 11βHSD2 activity was first shown in LNCaP cells in 1994, with the conversion of cortisol to cortisone in the presence of NAD$^+$. A functional mineralocorticoid receptor (MR) was also reported, which led the authors to suggest that 11βHSD may act as a "gate keeper" in hormone responsive prostate cancer cells [46]. In a more recent study by Dovio et al. [47], the conversion of cortisol to cortisone by 11βHSD2 was shown in LNCaP cells but not in PC3 cells, an androgen-independent prostate cancer cell line.

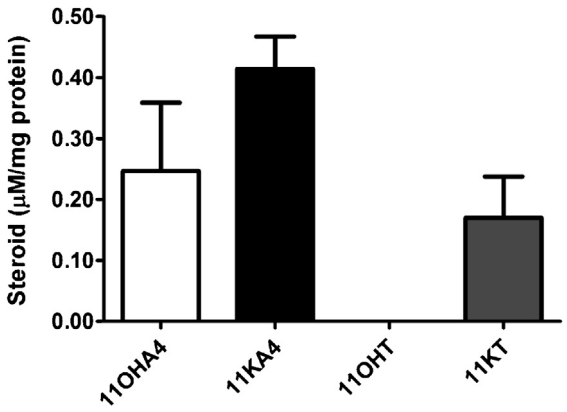

Fig. 9. Analyses of 11OHA4 (1 μM) metabolism in LNCaP cells. Conversion by 11βHSD and 17βHSD after 36 h is representative of three independent experiments performed in triplicate and are expressed as the mean ± SEM (n = 3).

11βHSD1 activity was not detected in either cell line, and while the MR and glucocorticoid receptor (GR) were expressed in both cell lines, GR expression levels were very low in the LNCaP cells. We also assayed 11OHA4 metabolism in PC3 cells and did not detect 11KA4, supporting the findings that 11βHSD2 is not expressed in these cells.

4. Conclusion

Taken together, it is clear that the biosynthesis of both 11OHA4 and 11OHT is catalyzed by both CYP11B isoforms, suggesting that CYP11B1 and CYP11B2 not only play a role in the biosynthesis of mineralocorticoids and glucocorticoids, but also in the biosynthesis of androgens in the adrenal and possibly in prostate cancer. Although the level of 11β-hydroxylation of T by CYP11B2 may be negligible in the adrenal due to zone specific enzyme expression, it is also quite possible that circulating T may be hydroxylated by CYP11B2 in the ZG and thus contribute toward 11OHT production. Furthermore the inter-conversion of 11KA4 and 11OHA4 and of 11KT and 11OHT by 11βHSD1 and 11βHSD2, further adds to the complexity of adrenal androgen production. Although 11OHA4 may also be metabolized to 11KT, due to the expression of 11βHSD2 and 17βHSD5 in the adrenal, the levels of 11KA4 and the T metabolites produced by the adrenal are low in comparison to 11OHA4. In our adrenal cell model and in primary adrenal cultures, T production was low, possibly due to the low levels of 17βHSD expression. While the formed 11OHA4 may contribute negligibly to the 11OHT pool, the low levels of the 11keto-metabolites formed are indicative of the low expression levels of 11βHSD2, since the enzyme readily converted 11OHA4 and 11OHT to 11KA4 and 11KT. Furthermore, the normal adrenal, under basal as well as post ACTH administration, produced higher levels of A4, 11OHA4 and 11KA4 than of T, 11OHT and 11KT, reflecting the relative expression levels of the enzymes catalyzing their production in the adrenal. Given the basal concentrations of the 11OHA4 and 11OHT metabolites, it appears that the shunt to the end products is greater via 11OHA4 in the adrenal. However, the biosynthesis of more potent androgens may lie in the reduction of 11KT and 11OHT in peripheral tissues as the dihydro forms may be produced as readily as 11OH-5α-dione was produced by the reduction of 11OHA4 by both SRD5A1 and SRD5A2. Although the interaction of 11OHA4 and 11OH-5α-dione with steroid receptors is uncertain, there is a tissue-specific role, within this scenario, being played by 11βHSD1 and 11βHSD2. It is furthermore possible that 11OHA4 and/or 11OH-5α-dione may interact, not only with the AR, but also with the MR and/or the GR in normal and prostate cancer cells and that the expression of 11βHSD1 and 11βHSD2 may modulate steroid metabolite levels in androgen responsive cells, affecting receptor occupancy and influencing hormonal responses. Subtle differences in terms of enzyme expression will determine the manner in which the steroids are metabolized as well as subsequent downstream effects, resulting from either wild type AR or mutated ligand-promiscuous AR interaction.

Irrespective of the outcome of future research exploring these new avenues, the data presented clearly places 11OHA4 back in the adrenal steroidogenesis pathway. Further investigations into the biological function will perhaps change the spectator status of this metabolite, and reinstate 11OHA4 as a player in the steroid arena.

Acknowledgements

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