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The Effects of Adrenocorticotrophic Hormone on Steroid Metabolomic Profiles in Human Adrenal Cells

Yewei Xing[1], **Michael A. Edwards**[1], **Clarence Ahlem**[2], **Mike Kennedy**[2], **Anthony Cohen**[1], **Celso E. Gomez-Sanchez**[3], and **William E. Rainey**[1,*]

[1]Department of Physiology and Surgery, Medical College of Georgia, Augusta, GA

[2]Hollis-Eden Pharmaceuticals Inc., San Diego, CA

[3]University of Mississippi Medical Center, Jackson, MS

Abstract

The adrenal glands are the primary source of mineralocorticoids, glucocorticoids and the so-called adrenal androgens. Under physiological conditions, cortisol and adrenal androgen synthesis are controlled primarily by adrenocorticotrophic hormone (ACTH). Although it is well established that ACTH can stimulate steroidogenesis in the human adrenal gland, the effect of ACTH on overall production of different classes of steroid hormones has not been defined. In this study, we examined the effect of ACTH on the production of twenty-three steroid hormones in adult adrenal (AA) primary cultures and 20 steroids in the adrenal cell line, H295R. Liquid chromatography/tandem mass spectrometry analysis (LC-MS/MS) revealed that in primary cultures that cortisol and corticosterone were the two most abundant steroid hormones produced with or without ACTH treatment (48 h). Cortisol production responded the most to ACTH treatment, with a 64-fold increase. Interestingly, the production of two androgens, androstenedione and 11β-hydroxyandrostenedione, that were also produced in large amounts under basal conditions significantly increased after ACTH incubation. In H295R cells 11-deoxycortisol and androstenedione were the major products under basal conditions. Treatment with forskolin increased the percentage of 11β-hydroxylated products including cortisol and 11β-hydroxyandrostenedione. This study illustrates that normal adrenal cells respond to ACTH through the secretion of a variety of steroid hormones, thus supporting the role of adrenal cells as a source of both corticosteroids and androgens.

Keywords

Adrenal cortex; ACTH; Steroidogenesis; LC-MS/MS

Introduction

The human adult adrenal cortex can be divided into three functionally distinct zones, namely the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR). Each zone exhibits independent regulation and function as illustrated by the selective production of glucocorticoids in the ZF, mineralocorticoids in the ZG and adrenal androgens in the ZR. Adrenal androgens include mainly DHEA, DHEA-S and androstenedione, which exhibit only weak sex hormone activity but can serve as precursors for estrogens and active androgens such as testosterone (Branchaud *et al*. 1983; Haning *et al*. 1985; Chen *et al*. 1996;

[*]**Corresponding author**: William E Rainey, Ph.D., Address: Department of Physiology, Medical College of Georgia, 1120 15th Street, Augusta, GA 30912, Phone: 706-721-7665, Fax: 706-721-8360, wrainey@mcg.edu.

Labrie *et al.* 1998; Burger 2002). In addition to the end products of steroid hormone synthesis, adrenal steroid intermediates can also be detected in blood and used as biomarkers for adrenal disease diagnosis.

Adrenocorticotrophic hormone (ACTH) by binding to its specific G-protein coupled receptor MC2R has been shown to induce adrenal cortex proliferation and steroidogenesis (Ney *et al.* 1969; Sharma 1973; Mahaffee *et al.* 1974; Hornsby & Gill 1977; Di Blasio *et al.* 1990). ACTH can stimulate aldosterone, cortisol and DHEA production in the adrenal gland (Hornsby *et al.* 1973; Hung & LeMaire 1988; Arvat *et al.* 2000; Sirianni *et al.* 2005). However, it is not clear if these steroids represent the most abundant steroids secreted in response to ACTH. O'Hare *et al.*, using high-pressure liquid chromatography (HPLC), demonstrated the production of cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, androstenedione, 11β-hydroxyandrostenedione (11OHA) and 16α-hydroxyprogesterone in primary cultures of human adrenocortical cells (O'Hare *et al.* 1976; Morgan & O'Hare 1979). However, the amounts of individual steroids were not quantified and effects of ACTH were examined only for a few steroids.

Given the critical role of ACTH in adrenal development, steroidogenesis and disease, it is appropriate to further determine the detailed effects of ACTH on steroid biosynthesis. In this study, we used liquid chromatography/tandem mass spectrometry analysis (LC-MS/MS) to measure the production of twenty steroid hormones in primary cultures of adrenocortical cells under basal conditions and in response to ACTH treatment. The study has characterized the metabolomic pattern of adrenocortical cells and defined a family of ACTH-responsive steroids thus expanding our knowledge of ACTH action in the human adrenal.

Materials and methods

Tissue collection

Human adult adrenal glands were obtained from cadaveric kidney donors that were transplanted at the Medical College of Georgia (Augusta, GA). Informed consent was obtained from the family of the donor by LifeLink of Georgia. The use of these tissues was approved by the Institutional Review Board of Medical College of Georgia.

Cell culture and treatment

Adult adrenocortical cells were isolated with collagenase-dispase digestion as described previously (Bassett *et al.* 2004). Briefly, adult adrenals were minced and dissociated into a single cell suspension by repeated exposure of the tissue fragments to DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 1 mg/mL collagenase dispase and 0.25 mg/mL DNase-1 (F. Hoffmann-La Roche Ltd., Switzerland). Digestion and mechanical dispersion were carried out four times for 1 h each, at 37°C. Cells were collected between each digestion and combined, then plated at a density of 300,000 cells/well in 24-well Falcon cell culture plates.

Steroid production was measured AA cells grown for 5 days in complete growth medium [DMEM/F12 medium with 10% Cosmic Calf serum (Hyclone, Logan, UT) and antibiotics]. Prior to ACTH stimulation, the cells were cultured in experimental medium (0.1% Cosmic Calf serum and antibiotics) overnight and then treated with vehicle or 10 nM ACTH (Organon, Bedford, OH) for 48 h. The cells were lysed for protein assay and media were collected for steroid assays as described below.

H295R cells were cultured using the same complete growth medium as AA cells, while HAC13 and HAC15 cells were grown in DME/F12 medium supplemented with 10% cosmic

calf serum, 1% insulin/transferrin/selenium Premix (ITS, BD Biosciences) and antibiotics. All three lines were plated at a density of 400,000 cells per well in 12 well dish and treated with vehicle or forskolin (10 μM, Sigma-Aldrich, St. Louis, MO) following the same protocol.

Protein extraction and protein assay

Cells were lysed in 100 μl Mammalian Protein Extraction Reagent (Pierce Chemical Co., Rockford, IL), and the protein content determined by the bicinchoninic acid (BCA) protein assay using the micro BCA protocol (Pierce Chemical Co.).

Liquid chromatography/tandem mass spectrometry (LC-MS/MS)

Deuterium labeled 4-androstenedione, corticosterone, estradiol, pregnenolone, and testosterone were purchased from CDN Isotopes (Pointe-Claire, Canada). All other steroids were purchased from Steraloids (Newport, RI). HPLC solvents and water were of HPLC analytical grade and filtered (0.2 μm) before use. All other reagents were purchased from Sigma (St. Louis, MO) and were of analytical grade or better.

Calibration curves were prepared by serial dilution of analytical standards in water. Quality control (QC) samples were prepared for each steroid analyzed. QC samples were prepared in experimental medium (0.1% Cosmic Calf serum, 1% antibiotics) spiked with analyte at concentrations near the lower, mid, and upper region of the range of quantification. Calibration curves and QC samples were analyzed in parallel with experimental samples. Steroid concentrations were calculated by Masslynx software (Waters, Beverly, MA), with the assumed concentrations for the standards calculated from the calibration curve regression parameters in comparison to theoretical values. The accuracy of QC samples was determined after first subtracting the endogenous concentration determined in experimental medium from the total concentration found in the QC sample.

Steroids of interest were assigned to one of three categories for quantification by LC-MS/MS based on the need for pre-column derivatization to achieve an appropriate lower limit quantification (LLQ) and/or chromatographic resolution: 1) no pre-column derivatization needed, 2) keto-steroids amenable to condensation with hydroxylamine, and 3) estradiol and estrone. Each method used one or more deuterium-labeled internal standards. Calibration curves and QC samples used for each method were prepared with the corresponding pre-column derivatization method. (See supplemental information Tables 3.2.S1, S2, and S3 for details regarding internal standards, retention times, quantified mass transitions, assay performance parameters and statistics, and detection limits for steroid measurement.)

Method 1—LC-MS/MS without pre-column derivatization (5-androstenediol, androstenedione, corticosterone, cortisol, DHEA, 11-deoxycorticosterone, 11-deoxycortisol, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, and testosterone). Samples (100 μL) were extracted with 4 mL methyl-tert-butyl-ether (MTBE). The organic phase was evaporated to dryness and reconstituted in 200 μL of water/acetonitrile (75:25, v:v). 20 μL of the reconstituted samples was applied to an Xterra C18 column (2.1×250 mm, 5 μm, Waters, Beverly, MA), and eluted with a mobile phase gradient of 30–85% acetonitrile in water, with 0.1% formic acid. The column temperature was maintained at 40°C. The column eluent was subjected to positive-mode electrospray ionization (ES^+), and the analytes were detected with a tandem quadrupole mass spectrometer (Waters, Beverly, MA).

Method 2—LC-MS/MS analysis of oxime derivatives of keto-steroids (aldosterone, dihydrotestosterone, 11OHA, 7α-DHEA, 16α-DHEA, 17α-hydroxypregnenolone, pregnenolone, and progesterone). Samples (100 μL) were extracted with 4 mL MTBE, and

the organic phase was evaporated to dryness. The residue was dissolved in 1 M aqueous hydroxylamine hydrochloride, and incubated for 1 h at 60˚C. The reaction mixtures were extracted with 2.5 mL MTBE, dried, and reconstituted in 200 μL of water/acetonitrile (75:25, v:v), and analyzed as above using a mobile phase gradient of 25–60% acetonitrile in water, with 0.1% formic acid.

Method 3—LC-MS/MS analysis of dansylated estradiol and estrone. Samples (100 μL) were extracted and dried as above, dissolved in 0.2 mL of 1 mg/mL dansyl chloride in 1 M aqueous sodium bicarbonate, and incubated for 1 h at 60˚C. The reaction mixtures (20 μL) were analyzed by LC-MS/MS as above using an Xbridge Phenyl column (2.1×150mm, 5 μm, Waters, Beverly, MA) with a mobile phase gradient of 35–90% acetonitrile in water, with 0.1% formic acid.

Steroid Immunoassay

Steroid concentrations in the medium were determined using enzyme immunoassay (EIA) for cortisol (Alpco, Salem, NH), and corticosterone (DRG International Inc., Mountainside, NJ) following the manufacturer recommendation, except that standard curves were prepared in the experimental cell culture medium. Immunoassay for 11OHA was developed in our laboratory. Briefly, plates were coated with rabbit IgG 1 μg/100 μL (LAMPIRE Biological Laboratories, Inc. Pipersville, PA) overnight in a 1 M NaCl, 10 mM phosphate pH 7.4 buffer. Plates were then washed four times in PBS buffer and incubated with 100 μL goat anti-rabbit IgG antisera (LAMPIRE Biological Laboratories) 1/500 in PBS for one hour. Before each assay, coated plates were washed four times in PBS and 50 μL of standards and sample were added to each well. 50 μL of 11OHA antibody was added to each well at a 1:10,000 dilution (in PBS buffer) and mixed on shaker for 5 min. 50 μL of biotin-labeled 11OHA (1:12,000,000 dilution in PBS) was added to each well and mixed for another 5 min. The plate was then placed at 4˚C and allowed to incubate for 18 h. Plates were washed four times in PBS and 150 μL of avidin-HRP (Vector laboratories, Burlingame, CA) was added at a 1:2500 dilution to each well. Plates were shaken for 30 min, followed by four-time washes in PBS and developed for 15 min with 100 μL per well of tetramethylbenzidine substrate (Thermo scientific, Waltham, MA). The reaction was stopped with 50 μL per well of 1 N sulfuric acid. Absorbance was measured at 450 nm. Results were normalized to protein per tissue culture well and shown as fold changes compared to basal.

Statistical Analysis

Individual experiments were repeated three times, using cells isolated from adrenal glands obtained from different donors. Results are given as means ± S.E.M. Statistics were calculated using the two-tail paired Student’s t test (GraphPad Prism 3.0,GraphPad Software, Inc. San Diego, CA). $P<0.05$ was considered statistically significant.

Results

The major steroidogenic pathways for adrenal and gonadal tissues are shown in Figure 1. Twenty-three steroid hormones were quantified in the medium from AA cells with and without ACTH stimulation, using LC-MS/MS and RIA (Table 1).Under basal conditions, the three most abundant steroids produced by AA cells were cortisol (31%), cortisone (15%) and corticosterone (13%). Interestingly, two androgens, androstenedione and 11OHA were also produced by adrenal cells in relatively high amounts (4% and 9 %).

Following treatment with ACTH for 48 h, the production of aldosterone, cortisol, DHEA and all detectable intermediate products of each steroidogenic pathway increased by approximately 4–60 fold. (Table 1). The most highly stimulated steroids were cortisol (63-

fold), corticosterone (37-fold) and 11-deoxycortisol (23-fold). Production of the adrenal androgens, androstenedione (26-fold), DHEA (18-fold) and 11OHA (17-fold), was also stimulated by ACTH treatment. The results of individual experiments using AA cells isolated from three different donors, are shown for cortisol, corticosterone, 11-deoxycortisol, and 11OHA (Fig 2). EIA was used to confirm the effects of ACTH on the steroid production in AA cells (Fig 3). The relative increases of cortisol, corticosterone and 11OHA were approximately 2-fold lower with EIA compared to LC-MS/MS, but both analytical methods indicated significant increases in the production of all the three steroids after ACTH treatment.

Comparison of the ratio of individual steroids produced by AA cell primary culture shows that cortisol represents 30% of total steroids made under basal conditions and 61% after ACTH treatment (Figure 4). Increased cortisol production following treatment confirmed that ACTH is a potent stimulator of glucocorticoid synthesis that greatly increases the capacity of the adrenal to produce cortisol under the control of the HPA axis. The global decrease in the production of other steroidogenic intermediates after ACTH treatment were consistent with the known effects of ACTH on steroidogenesis. The pie chart summarizing the ratio of individual steroids to total steroids before and after ACTH treatment is shown in Table 4.

Similar experiments were performed in three different H295R cell models, followed by the same analysis of metabolomic profile (Figure 5). The only experimental change was the use of forskolin instead of ACTH as an agonist for steroidogenesis, because the HAC15, HAC13 and H295R only have minor response to ACTH. Different from primary cultures, H295R cells mainly produced 11-deoxycortisol under both basal and stimulated conditions (42 % versus 30 %), while androstenedione was also produced in large amount (29 % at basal versus 21 % after forskolin treatment). In addition, cortisol was a significant product of the H295R cells, representing 10 % of the steroids measured under basal conditions. The reason for a greater output of 11-deoxycortisol than cortisol may relate to the low basal expression of 11β-hydroxylase (CYP11B1) in H295R cells (data not shown). Treatment with forskolin increased the relative output of cortisol production to 26%, which corresponded to increases in CYP11B1 expression (data not shown). The forskolin stimulating effects on CYP11B1 was also supported by increases in other 11-hydroxylated steroids (11-fold for cortisol, 13-fold for corticosterone and 5-fold for 11-hydroxyandrostenedione). Although these steroids represent only a small portion of the total steroid made by the H295R (Figure 5), the two steroids with the greatest forskolin stimulated fold increase were estrone (20-fold) and estradiol (19-fold), confirming that forskolin activates aromatase expression and activity (Watanabe & Nakajin 2004). Cortisone and 11-dehydrocorticosterone concentrations were not quantified in this experiment.

Discussion

Adrenal steroidogenesis has been extensively studied over the past 50 years (Grant 1962; Mason & Fraser 1975; Lebrethon *et al.* 1994a; Lebrethon *et al.* 1994b; Fottner *et al.* 1998), however the breadth of analytes considered in previous studies was limited by the available analytical technology. Previously reported *in vitro* studies have used isolated human adrenal cells as short term dispersed cultures as well as long-term monolayer cultures. Short term experiments using suspension cultures of human adrenocortical cells found a dose-dependent stimulatory effect of ACTH on cortisol, corticosterone, androstenedione and DHEA/DHEAS synthesis (Keymolen *et al.* 1976). Long-term monolayer cultures have been useful to study steroid production, however the steroid synthesis profile in those cells were not well characterized. Fottner, et al. tested the stimulating effects of ACTH only on cortisol and DHEA-S (Fottner *et al.* 1998), while Guillon tested four steroids (aldosterone,

corticosterone, cortisol and 17-hydroxyprogesterone), but only after short term ACTH treatment (2 h) (Guillon *et al*. 1995). The studies by Lebrethon, et al. both used 2 h treatment times for ACTH and only changes in cortisol, aldosterone or DHEA were measured in individual studies (Lebrethon *et al*. 1994a; Lebrethon *et al*. 1994b). In the current study, we used primary cultures of human adrenocortical cells as a model to describe the broad effects of ACTH on steroidogenesis. Our LC-MS/MS quantification of 23 steroids in response to ACTH stimulation is a significant extension of previous reports with regard to the model, the effects of ACTH, and the analytical methodology, and provides a comprehensive overview of chronic ACTH effects on the steroid profile.

Our LC/MS-MS data coincides with previous reports that cortisol is the major steroid stimulated by ACTH (O'Hare & Neville 1974; Israeli *et al*. 1975; Kolanowski & Crabbe 1976). All the intermediates of the cortisol-producing pathway, including 17α-hydroxypregnenolone, 17α-hydorxyprogesterone and 11-deoxycortisol, were detectable under basal conditions and the presence of high levels of 11-deoxycortisol (147 pmol/mg protein) suggested that CYP11B1 is a late rate-limiting step in controlling cortisol production as has been suggested for the enzyme aldosterone synthase in aldosterone biosynthesis. After ACTH treatment for 48 h, cortisol production increased more than 60-fold, far exceeding that of its precursors, confirming the activating effect of ACTH on CYP11B1. Analysis of steroid production pre and post stimulation in the H295R cells also support a role for CYP11B1 expression as a gate-keeper for cortisol biosynthesis. Both cortisol and 11-hydroxyandrostenedione were significantly up-regulated by 48 h forskolin treatment and represented two of the major steroids produced by H295R cells after stimulation.

Human adrenocortical cells *in vitro* produce approximately 1/5 as much cortisone as cortisol in response to ACTH (Kolanowski & Crabbe 1976). Higher levels of cortisone and 11-dehydrocorticosterone were reported in adrenal slices (Mazzocchi *et al*.1998). Our data confirm that primary human adrenal cells can produce significant amounts of cortisone (16%) and 11-dehydrocorticosterone (6%) under basal condition, indicating the presence of functioning 11β-hydroxysteroid dehydrogenase in normal adrenocortical cells. LC-MS/MS data from adrenal vein samples confirmed the ability of adrenal to make cortisone (unpublished data). However, the relatively higher production of cortisone *in vitro* suggested the possibility of alternations in the 11-dehydrogenase activity that has been reported to occur during the isolation process (O'Hare 1973).

While both DHEA and DHEA-S responded well to ACTH stimulation, the relative production of these steroids was lower than might be expected from their reported abundance in systemic circulation. Several factors may influence the production of Δ5 C19 steroids by primary adrenal cell cultures, including alterations that occurred in vivo prior to cell isolation and the relative proportion of zona fasciculata and zona reticularis cells in the culture. According to the clinical data supplied for two of the adrenal donors, the adrenal glands came from young patients (age range 21–22 year old) who had sustained severe automobile accident injuries and died of intracranial hemorrhage/stroke. Adrenal glands were collected 2–3 days after brain death. Studies from several independent groups have reported decreased DHEA and DHEA-S levels in severely ill patients, while androstenedione and cortisol concentrations remained stable (Parker *et al*. 1985; Luppa *et al*. 1991; Beishuizen *et al*. 2002). Our data from primary cultures coincide with previous findings by showing decreased levels of DHEA in both basal and ACTH treated groups. Regarding cell types, zona reticularis cells mainly release DHEA (Endoh *et al*. 1996), which during our incubation period may be transformed by in the mixed fasciculata / reticularis cultures to Δ4 steroids (androstenedione and 11OHA), decreasing the apparent production of

DHEA. However, the potential contribution of this activity to our results was not established.

Interestingly, androstenedione and 11OHA were produced in large amounts even in unstimulated AA cells (84 and 174 pmol/mg protein, respectively). Compared to the data of Fearon and colleagues (Fearon *et al.* 1998), our cells have a lower cortisol/androstenedione ratio (6 versus 20). The difference may relate to their use of freshly isolated cells or their treatment time of only 2 h. Alternatively, AA cells in monolayer culture may produce more androstenedione under chronic ACTH treatment.

Androstenedione is more readily converted to testosterone than DHEA or DHEA-S (Zipser *et al.* 1981). Testosterone functions as a more active androgen than androstenedione in circulation. Several studies have shown that the adrenal gland contributes to testosterone production either by direct secretion or peripheral conversion of adrenal-derived precursor (Keymolen *et al.* 1976; Macdonald & Matt 1984; Fenske 1986; Canonaco *et al.* 1989; Nakamura *et al.* 2009). In addition, the contribution of adrenal gland to circulating testosterone in women is particularly important. However, in the present study, we were not able to detect testosterone in the medium. However, this may relate to our relative low limit for detection of 50 ng/ml. The lack of detection in the primary cultures contrasts with our findings in the H295R adrenocortical cell line, which show detectable levels of testosterone even under unstimulated conditions (Nakamura *et al.* 2009). Our data support the concept that the H295R cancer cells produce more testosterone and estrone than the primary adrenal cell cultures. This is likely due to the high expression of 17β-hydroxysteroid dehydrogenase type 5 (HSD17B5) and significant expression of aromatase (CYP19) in the H295R cells compared to normal adrenal cells (Staels *et al.* 1993; Nakamura *et al.* 2009). It should also be noted that our primary cultures of adrenal cells only produced low levels of DHEA, suggesting that the mixed cortical cells may exhibit more of a fasciculata phenotype, while H295R produces a variety of adrenal steroids, including mineralocorticoids, glucocorticoids, estrogens and androgens. Further experiments will be needed to differentiate the role of ZF and ZR in testosterone production in human adrenal glands.

In summary, using the LC-MS/MS for steroid analysis we characterized the relative production of a wide range of steroids in primary cultures of normal adrenal cells and the H295R adrenal cell lines. This represents the first comprehensive study using LC- MS/MS to examine the production of steroids by adrenal cells in culture. The current report should serve as a reference for researchers studying adrenal steroidogenesis using either normal cells in culture or the H295R adrenal cell line.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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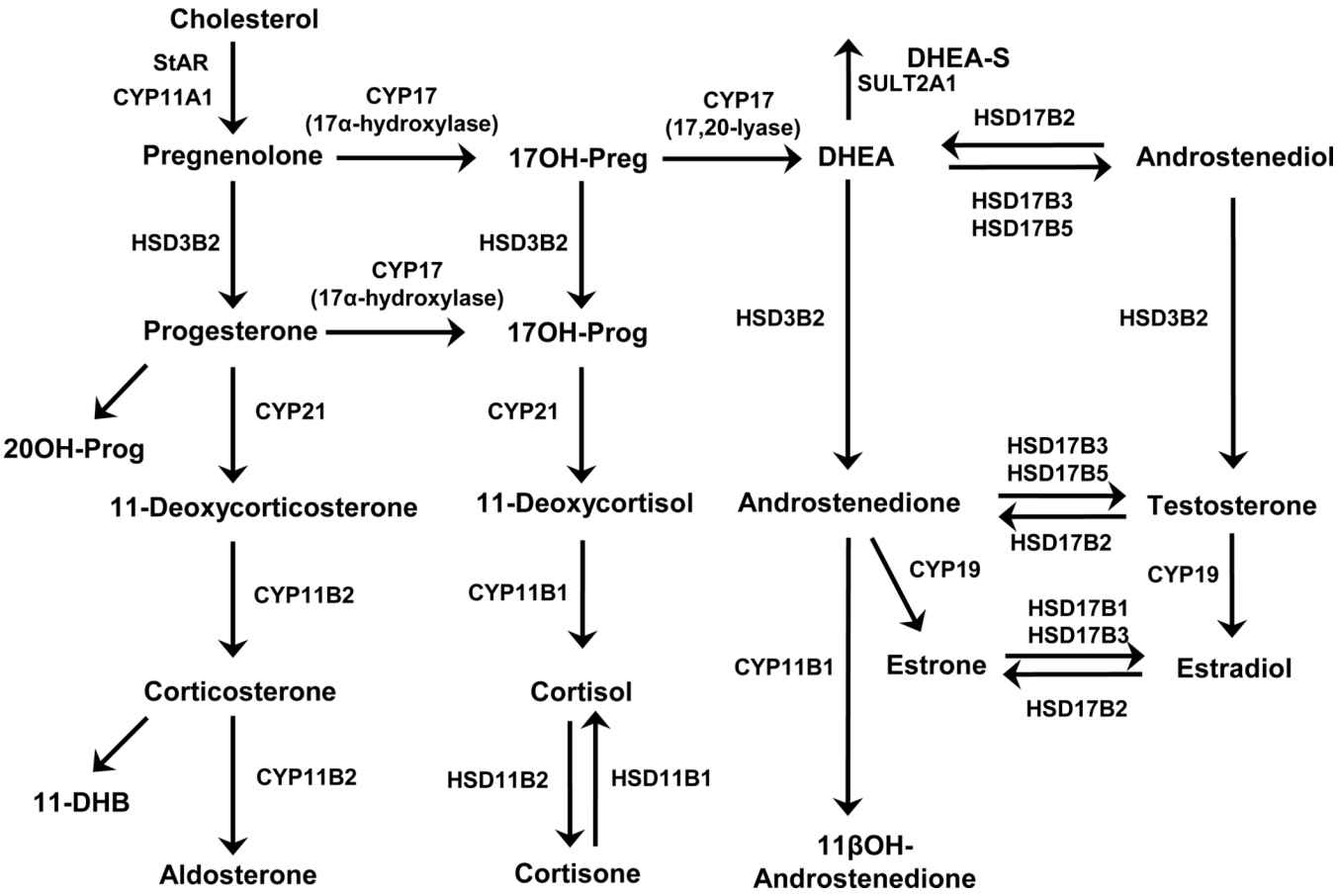

Fig. 1. Steroid biosynthesis pathways

Summary of the steroidogenic pathways leading to synthesis of glucocorticoids, mineralocorticoids, androgens and estrogens.

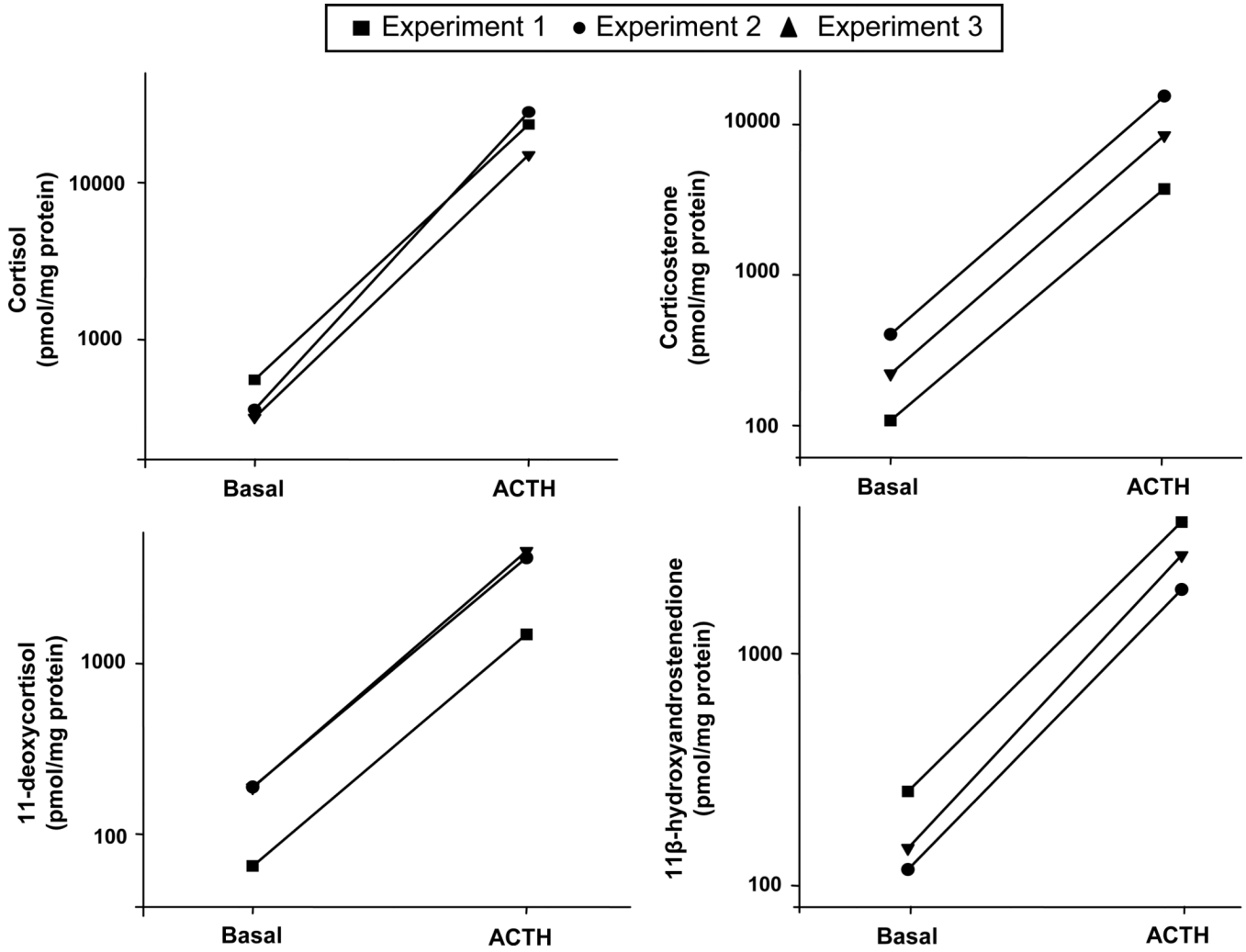

Fig. 2. Major steroids produced by AA cells with/without ACTH treatment

Primary human adrenal culture cells were isolated as described in Materials and Methods, and plated at a density of 300,000 cells/well in 24-well dishes. Cells were treated with/without ACTH (10 nM) for 48 h before harvest. The concentration of steroids in the medium was measured by LC-MS/MS and normalized to the amount of protein. The amount of four major steroids produced by AA cells, cortisol, corticosterone, 11-deoxycortisol and 11OHA are shown in the graphs with each line representing an individual, independent experiment.

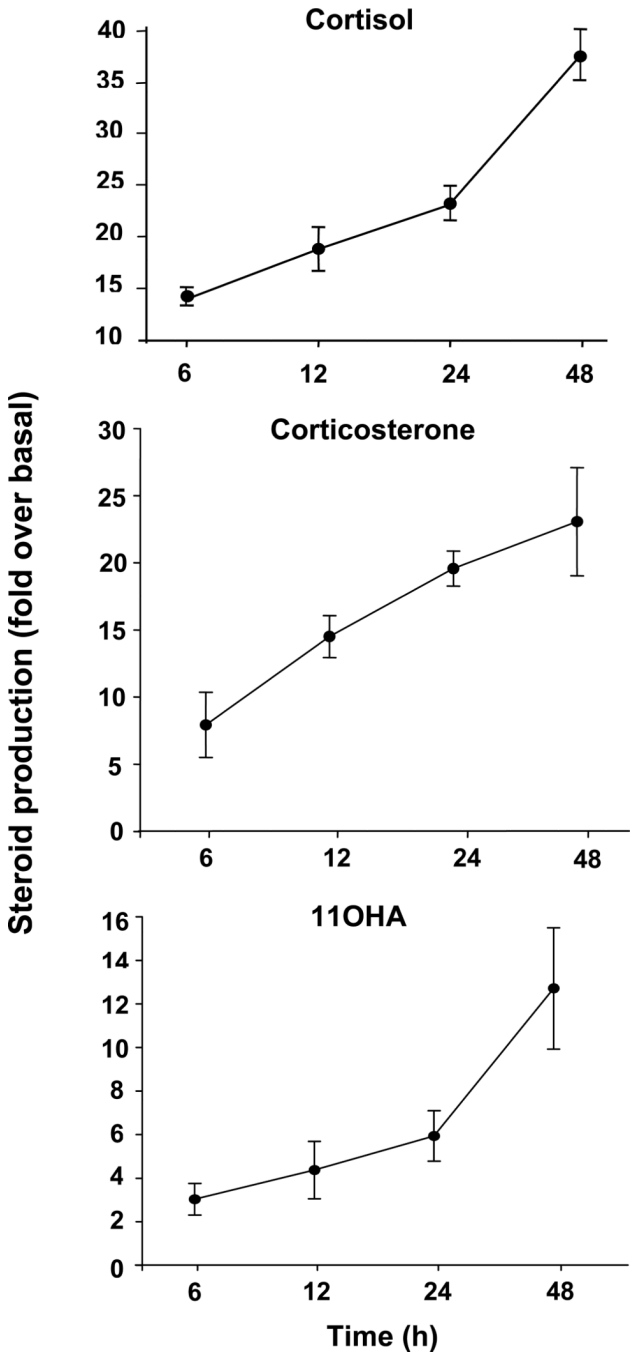

Fig. 3. Time-dependent stimulation of three major steroids produced in AA cells by ACTH treatment

Primary human adrenal culture cells were isolated as described in Materials and Methods, and plated at a density of 300,000 cells/well in 24-well dishes. Cells were treated with/without ACTH (10 nM) for indicated times. The concentration of steroids (cortisol, corticosterone and 11OHA) in the medium was measured by immunoassay and expressed as fold changes over basal level. Results represent means ± S.E.M of data from at least three experiments using cells isolated from different adrenal glands.

A.

Steroid	Basal (% total)	ACTH (% total)
21 Carbon Steroids		
Pregnenolone	0.5±0.2	0.2±0.1
17α-Hydroxypregnenolone	1.0±0.6	0.7±0.4
Progesterone	0.6±0.2	0.1±0.0
17α-Hydroxyprogesterone	4.9±1.7	2.1±1.1
20α-Hydroxyprogesterone	ND	ND
Deoxycorticosterone	3.1±1.5	1.0±0.5
Corticosterone (B)	13.3±4.0	15.7±4.2
Aldosterone	ND	ND
11-Deoxycortisol (S)	8.2±2.3	6.2±1.8
Cortisol	30.5±6.1	59.6±8.3
Cortisone (E)	14.7±3.7	1.8±0.5
11-Dehydrocorticosterone (11-DHB)	6.1±2.7	0.9±0.4
19 Carbon Steroids		
DHEA	0.4±0.1	0.3±0.2
DHEA-S	ND	0.9±0.1
7α-DHEA	ND	ND
16α-DHEA	ND	ND
Androstenediol	ND	ND
Androstenedione (A4)	4.1±2.3	3.3±1.8
11OHA	8.7±2.3	4.8±1.3
Testosterone	ND	ND
Dihydrotestosterone	ND	ND
18 Carbon Steroids		
Estrone	ND	ND
Estradiol	ND	ND

B.

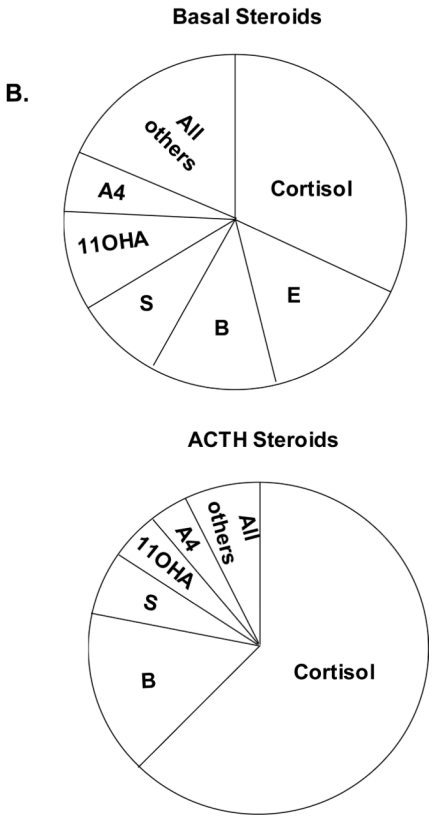

Figure 4. Percentage of major steroids produced by AA cells with/ without ACTH treatment

Primary human adrenal cells were isolated as described in Materials and Methods, and plated at a density of 300,000 cells/well in 24-well dishes. Cells were treated with/without ACTH (10nM) in 0.1% experimental medium for 48 h before harvest. The concentration of steroids in the medium was measured by LC-MS/MS and normalized to the amount of protein. Percentage of each steroid was calculated by dividing the amount of individual steroid with total steroid. **A**. Table summarizing the percentage of individual steroid compared to total steroid produced by AA cells. **B**. Pie chart summarizing the relative percentage of the major steroids produced in AA cells. Data represent results from three independent experiments and shown as means ± S.E.M; ND, not detectable.

A.

Steroid	Basal (% total)	Forskolin (% total)
21 Carbon Steroids		
Pregnenolone	0.6±0.2	3.7±0.1
17α-pregnenolone	1.9±1.0	2.0±1.1
Progesterone	0.2±0.1	0.1±0.0
17α-Hydroxyprogesterone (17OHP)	6.1±1.6	1.6±0.1
20α-Hydroxyprogesterone	ND	ND
Deoxycorticosterone	2.1±0.2	4.1±1.0
Corticosterone (B)	3.0±1.3	7.9±1.4
Aldosterone	ND	ND
11-Deoxycortisol (S)	42.37±1.1	29.8±2.9
Cortisol (F)	9.6±3.4	25.7±5.6
19 Carbon Steroids		
DHEA	1.3±0.8	1.2±0.7
7α-DHEA	ND	ND
16α-DHEA	ND	ND
Androstenediol	ND	ND
Androstenedione (A4)	28.6±4.5	21.0±2.8
11OHA	3.0±1.1	3.5±0.1
Testosterone	0.5±0.1	0.3±0.0
Dihydrotestosterone	ND	ND
18 Carbon Steroids		
Estradiol	0.01±0.0	0.07±0.0
Estrone	0.3±0.1	0.2±0.03

B.

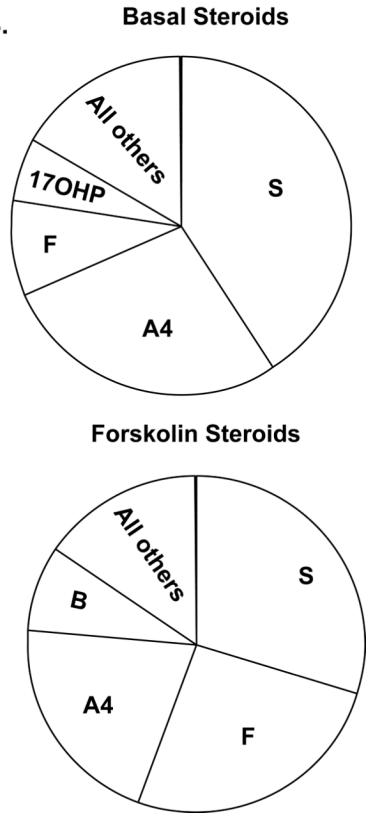

Figure 5. Percentage of major steroids produced by H295R cells with/ without forskolin treatment

Three independent strains of the H295R adrenal cell model were cultured as described in Materials and Methods, and plated at a density of 400,000 cells/well in 12-well dishes. Cells were treated with/without forskolin (10 μM) in 0.1% experimental medium for 48 h before harvest. The concentration of steroids in the medium was measured by LC-MS/MS. Percentage of each steroid was calculated by dividing the amount of individual steroid with total steroid. **A**. Table summarizing the percentage of individual steroid compared to total steroid produced by H295R cells. **B**. Pie chart summarizing the relative percentage of four major steroids produced in H295R cells. Data represent results from three independent experiments and shown as means ± S.E.M; ND, not detectable.

Table 1
Steroids produced under basal and ACTH treatments

AA cells were incubated with/without ACTH (10 nM) for 48 h. Steroids were grouped according to carbon number and property. Concentrations of steroids were measured using LC-MS/MS method and normalized to protein amount. Data represent results from three independent experiments and shown as means ± S.E.M. *P* values were calculated using the paired t-test with 95% confidence intervals.

Steroid	Pre-ACTH pmole /mg protein	Post ACTH pmole /mg protein	Fold Change	*p*-value
21 Carbon Steroids				
Pregnenolone	9.6±4.1	136.6±74.6	13.4±3.6	0.07
17α-Hydroxypregnenolone	16.9±10.0	392.0±211.1	32.2±14.0	0.15
Progesterone	12.1±4.0	73.0±31.3	5.3±1.4	0.09
17α-Hydroxyprogesterone	88.7±27.3	1167.4±512.0	15.6±6.2	0.14
20α-Hydroxyprogesterone	ND	ND	-	-
Deoxycorticosterone	60.5±30.9	613.1±272.3	12.8±2.7*	0.05
Corticosterone	243.8±85.8	9183.9±3392.5	37.0±1.3*	0.001
Aldosterone[a]	21.8±1.4	201.3±18.2	9.3±1.1*	0.02
11-Deoxycortisol	147.2±41.0	3406.6±968.2	23.1±0.7*	0.001
Cortisol	514.6±94.4	31464.4±5609.2	63.4±13.3*	0.04
Cortisone	259.3±81.5	988.0±340.2	3.7±0.5*	0.04
11-Dehydrocorticosterone	111.5±81.5	535.7±239.7	4.9±0.7	0.03
19 Carbon Steroids				
DHEA	8.5±2.7	188.3±142.3	17.6±9.2	0.21
DHEA-S	ND	582.8±83.4	-	-
7α-DHEA	ND	ND	-	-
16α-DHEA	ND	ND	-	-
Androstenediol	ND	ND	-	-
Androstenedione	84.1±44.6	2059.6±999.1	25.7±1.2*	0.002
11OHA	174.7±40.4	2844.0±545.5	16.6±1.1*	0.005
Testosterone	ND	11.43 + 6.08	-	-
Dihydrotestosterone	ND	ND	-	-
18 Carbon Steroids				
Estrone	ND	23.6+12.3	-	-
Estradiol	ND	ND	-	-

*$P<0.05$; ND, not detectable.

Note

[a]Aldosterone is shown as RIA data due to the low concentration present in experiment medium. As a conjugated steroid, DHEA-S was also measured by immunoassay. The detection limit for DHEA-S is 140.