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EFFECT OF INSULIN AND TESTOSTERONE ON ANDROGEN PRODUCTION AND TRANSCRIPTION OF *SULT2A1* IN THE NCI-H295R ADRENOCORTICAL CELL LINE

Ashim Kumar, M.D.^{a,c}, Denis Magoffin, PhD.^{a,b,c}, Iqbal Munir, M.D., PhD.^{a,b}, and Ricardo Azziz, M.D., M.P.H., M.B.A.^{a,b,c,d}

^aDepartment of Obstetrics and Gynecology, Cedars-Sinai Medical Center, Los Angeles, CA 90048

^bCenter for Androgen Related Disorders, Cedars-Sinai Medical Center, Los Angeles, CA 90048

^cCenter for Department of Obstetrics and Gynecology, The David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

^dCenter for Department of Medicine, The David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

Abstract

Objective—To determine changes in AA production, and transcription of DHEA sulfotransferase (*SULT2A1*) in the NCI-H295R human adrenocortical cell line in response to insulin and testosterone, an environment mimicking the PCOS state.

Design—*In vitro* experiment using NCI-H295R adrenocortical cell lines.

Setting—Academic medical center.

Patients—NCI-H295R human adrenocortical cell line

Interventions—The transcriptional activity of *SULT2A1* and adrenal steroid production was quantified after exposure to various treatments (e.g. forskolin, insulin, testosterone, and combinations thereof).

Main Outcome Measures—Quantification of mRNA for DHEA sulfotransferase (*SULT2A1*) by real-time reverse transcription-polymerase chain reaction and measurement of steroid production by radioimmunoassay.

Results—Testosterone decreased DHEAS and cortisol, and increased DHEA, secretion by H295R cells; the inhibitory effects of testosterone on DHEAS and cortisol production were augmented by insulin. There was a trend towards an increase in the transcription of *SULT2A1* by insulin and testosterone.

Conclusions—Testosterone and insulin appear to be modulators of AA production in this human adrenocortical cell model. These results suggest that testosterone may augment DHEA secretion in

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Corresponding Author: Ricardo Azziz, M.D., M.P.H., M.B.A. 8700 Beverly Blvd., Suite 160W, Los Angeles, CA 90048. Phone: 310-423-7433; fax: 310-423-3470, azzizr@cshs.org.

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the human adrenal, although they do not support the role of this sex steroid or insulin on the elevated DHEAS levels frequently observed in PCOS.

Keywords

insulin; testosterone; sulfotransferase; NCI-H295R; adrenal; steroidogenesis

Introduction

Polycystic ovary syndrome (PCOS) is a hyperandrogenic disorder, affecting at least ~7% of unselected reproductive-aged women, and exacts a high financial cost to society (1–5). In addition to ovarian hyperandrogenism, Adrenal androgen (AA) secretion appears to be upregulated in PCOS. Principal AAs are dehydroepiandrosterone (DHEA) and its sulfated form, DHEAS; approximately 25% of patients demonstrate basal levels of DHEAS above the upper normal age-adjusted limit (6).

Regulation of AA production and its role in the pathophysiology of PCOS remain elusive (4, 7). The lack of a feedback loop between AAs and their main secretagogue, adrenocorticotrophic hormone (ACTH), implies loose central regulatory control. Thus, extra-adrenal factors have been implicated in the modulation of AA production (8). These could include testosterone, as well as insulin, which are elevated in ~65% of PCOS subjects (9).

Previous studies have provided conflicting data regarding the *in vivo* effects of insulin and testosterone on AA synthesis (10–18). Study populations have ranged from normal males and/or females to women diagnosed with PCOS. The association between insulin or testosterone and AA production was studied in the basal state and in response to alterations in insulin and testosterone levels. The conclusions are discordant across the various stratagem used in the *in vivo* studies.

Previous *in vitro* experiments focusing on the effect of insulin and testosterone on human adrenocortical tissue suggested a regulatory role in AA production, although the end effects were variable from one adrenal to another (19). Published reports on studies conducted using human adrenals have generally disregarded the inherent adrenal to adrenal variability (20). The constancy of the NCI-H295R adrenocortical cell line facilitates a close examination of the steroidogenic pathway to determine the effect of insulin, testosterone and the combination thereof on androgen biosynthesis. By decreasing 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity, testosterone shifts steroidogenesis towards the delta-5 pathway (21). An increase in *CYP17* expression induced by insulin further directs steroidogenesis toward the delta-5 pathway (22–23). The end result of the combined effect would be increase in DHEA production. DHEAS levels, however, depend upon the activity of *SULT2A1*.

Abnormal insulin signaling and excessive ovarian steroidogenesis are the leading etiologic hypotheses in PCOS. The resulting elevated testosterone and insulin levels may be the primary mediators of a wide array of sequelae, including elevated AAs, associated with PCOS. To test this hypothesis we evaluated the effects of insulin and testosterone on AA production and on *SULT2A1* transcription using the NCI-H295R adrenocortical cell line.

MATERIALS & METHODS

Experimental Model

The NCI-H295 adrenocortical cell line was established by Gazdar and colleagues, and has been used extensively to study human adrenocortical function (24–28). The cells have been documented to express the panel of steroidogenic enzymes of interest in androgen biosynthesis

(24,27). However, it is a transformed cell line and thus does not exactly mimic the adrenocortical cells as evidenced by the NCI-H295R cells' decreased expression of ACTH receptors necessitating forskolin to simulate ACTH stimulation (28). IRB approval was not required because of the use of the NCI-H295R adrenocortical cell line.

Cell Culture

The NCI-H295R cells were obtained from the American Type Culture Collection (ATCC CRL-2128, Manassas, VA), and were propagated and subcultured per ATCC guidelines. The cells were plated in 24-well plates at a density of 1×10^5 cells per well, and cells were grown in the medium outlined by ATCC for 1 to 2 days until sub-confluent. The medium was aspirated and replaced with medium without Nu-serum (BD Biosciences, San Jose, CA) and ITS+ Premix (BD Biosciences), and the various treatments stated below. The cells were then incubated for 24 hours at which time the medium was aspirated from the wells, and both the cells and medium were frozen at -80°C . Our time course experiments (4, 8, 24, 48, 72 hr) revealed near maximal steroidogenesis and mRNA at 24 hours (data not shown) as noted by other investigators (29–30).

Treatments

The treatments included control (medium only), forskolin (15 $\mu\text{mol/L}$), insulin (10 ng/mL), testosterone (1 mmol/L), insulin+testosterone, forskolin+insulin, forskolin+testosterone, and forskolin+insulin+testosterone were performed in triplicate. The dose of forskolin was chosen after evaluation of dose-response incubations and is similar to published reports (data not shown) (31). Previous experiments indicated that 10 ng/mL is the physiologic dose of insulin consistent with upper physiologic post-prandial levels (22). A supra-physiologic testosterone concentration was used to mimic the hyperandrogenic adrenal environment. Forskolin was used in lieu of ACTH as the NCI-H295R cells have been reported to have an attenuated response to ACTH, perhaps due to a decrease in ACTH receptors (28).

Radioimmunoassay (RIA)

The aspirated medium was stored at -80°C until thawed for evaluation by RIA for DHEA, DHEAS, and cortisol. Double antibody RIA kits were used for the assays (Diagnostic Systems Laboratories, Webster, TX). The medium was diluted as necessary so that the unknowns fell within the linear portion of the standard curve. The results were normalized to the controls.

Real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR)

The mRNA for *SULT2A1* was measured by real time RT-PCR. The cells were homogenized using a QiaShredder (Qiagen, Valencia, CA) and total RNA was extracted using the RNeasy Mini Kit (Qiagen). First strand cDNA was generated using the Omniscript reverse transcription kit (Qiagen) with oligo(dT)_{12–18} primer (Invitrogen, Carlsbad, California). PCR was performed using MyiQ Single-Color Real-Time PCR Detection System coupled to the iCycler (Bio-Rad Laboratories, Hercules, CA) using QuantiTect SYBR PCR reagents (Qiagen). A 50 μL reaction mix consisted of 25 μL 2X QuantiTect SYBR PCR Master Mix, 5 μL first strand cDNA, and 200 nmol/L of the sense and antisense primers (4 μL), and 16 μL of H_2O .

The PCR protocol consisted of HotStarTaq DNA polymerase activation at 95°C for 15 min and then 40 cycles of amplification (95°C for 15 sec, 56°C for 30 sec, and 72°C for 15 sec). The oligonucleotide primers 5'TCGTGATAAGGGATGAAGATGTAATAA3' (sense) and 5'TGCATCAGGCAGAGAATCTCA (antisense) were used to amplify *SULT2A1* (32). The sequence was then cloned into TA vector (Topo TA Cloning Kit, Invitrogen). The plasmid was linearized. The CT values/crossing points were plotted against the log of a series of known DNA concentrations. The cDNA was diluted as necessary so that the quantitative results were

within the linear range of the standard curve to allow for interpolation of the amount of mRNA in each sample. RT-PCR was performed for 18s RNA for each sample in order to standardize the results.

Statistical Analysis

The Wilcoxon signed-rank test was used to determine significance. All values are expressed as mean $\pm$ standard error. A P -value of <0.05 was used to determine statistical significance.

RESULTS

In order to assess the modulatory effect of insulin and testosterone on AA production, the DHEA, DHEAS, and cortisol levels in the growth medium of the NCI-H295R adrenocortical cells were measured by RIA after 24 hours of exposure to the treatment(s) (Figure 1). Real time RT-PCR was performed for a quantitative analysis of *SULT2A1* RNA.

DHEAS production after 24 hours of incubation with the various modulators is depicted in Figure 1a. The DHEAS levels in the wells treated with insulin+forskolin was similar to the wells treated with forskolin alone. The addition of testosterone resulted in a significant decrease in DHEAS production at 24 hours, compared to forskolin alone ($P<0.05$). The combination of insulin and testosterone suppressed DHEAS further than testosterone alone ($P<0.05$).

The levels of DHEA in response to the various treatments are depicted in Figure 1b. As for DHEAS, the DHEA level in the wells treated with insulin+forskolin was similar to the level in the wells with forskolin alone. In contrast to what was observed with DHEAS, the addition of testosterone (testosterone+forskolin) significantly increased DHEA production ($P<0.05$). The addition of insulin to testosterone did not further augment DHEA production.

Cortisol production behaved similar to that of DHEAS (Figure 1c). The cortisol levels in the wells treated with insulin+forskolin was similar to the wells treated with forskolin alone, the addition of testosterone resulted in a significant decrease in production at 24 hours ($P<0.05$), and the combination of insulin and testosterone suppressed cortisol even further ($P<0.05$).

In order to determine whether the effect of insulin and testosterone on DHEAS and DHEA were due to changes in sulfotransferase activity, we determined the effect of these hormones, alone or in combination on *SULT2A1* (Figure 2). However, neither insulin nor testosterone alone appeared to affect transcription of *SULT2A1*, compared to forskolin alone. Although not statistically significant, there was a trend towards an increase in the transcription *SULT2A1* with the combination of insulin and testosterone, as compared to forskolin alone, contrary to expected ($P=0.11$).

DISCUSSION

Our results indicate that extra-adrenal modulators, specifically insulin and testosterone, may influence adrenocortical steroidogenesis. Testosterone appears to be a stimulator of DHEA production in the NCI-H295R cells. Alternatively, testosterone suppressed DHEAS and cortisol production; an effect augmented by the addition of insulin. These data suggest that extra-adrenal (e.g. ovarian) testosterone may augment DHEA secretion in the human adrenal, as is observed in PCOS. Alternatively, they do not support the role of this sex steroid or insulin on the elevated DHEAS levels frequently observed in PCOS.

There are no reports in the literature evaluating AA production in the NCI-H295R adrenocortical cells in response to testosterone. In one study, expression of the androgen receptor was observed in the NCI-H295R cell line and in human adrenal tissue (30). In NCI-

H295R cells dihydrotestosterone resulted in a significant reduction in cellular proliferation; however, these investigators did not evaluate the change in AA production in response to testosterone itself (30). In mouse adrenal cells, Stalvey found that testosterone resulted in decreased transcription of 3 β -HSD (21). This effect could potentially decrease cortisol and increase delta-5 steroid (e.g. DHEA) production as observed in our data, although we did not assess transcription of the 3 β -HSD gene (i.e. *HSD3B*). Our *in vivo* studies, using exogenous testosterone administered for one month to oophorectomized healthy women (12), or ovarian suppression with a long-acting GnRH-a in hyperandrogenic premenopausal women (32), indicate that while ovarian factors, primarily androgens, may affect the rate of DHEA sulfation (and consequently the circulating levels of DHEAS), the adrenocortical response to ACTH remains relatively unchanged. Although, both our *in vivo* and *in vitro* data suggest a potential modulatory role for testosterone in the regulation of AA production and DHEAS levels, the mechanisms are variant and the end-effects observed inconsistent.

Kelly and colleagues evaluated the cortisol:androstendione ratio of the NCI-H295R adrenocortical cells in response to insulin and reported no change in this ratio with insulin + forskolin as compared to forskolin alone (23). We found similar results with insulin alone. Alternatively, the addition of insulin to H295R cells decreased cortisol and DHEAS production only in the presence of testosterone (insulin + testosterone + forskolin). These data do not support our hypothesis of a stimulatory effect of insulin on DHEAS, with an apparent negative relationship between insulin and DHEAS production, at least in this model of the adrenal cortex. *In vivo* data also does not support an active role of insulin on AA production. We were unable to detect a significant association between circulating DHEAS and fasting insulin levels in PCOS women and age and race-matched healthy eumenorrheic controls (6), and Kauffman and colleagues failed to detect an association between DHEAS and insulin in White and Mexican- American women with PCOS (33).

Preliminarily, our data does not suggest that changes in *SULT2A1* accounts for the decrease in DHEAS observed with testosterone or insulin + testosterone, as the combination of testosterone and insulin actually appeared to increase the transcription of *SULT2A1*. While this observation remains to be confirmed, other mechanisms may be responsible for this discrepancy including increase degradation of DHEAS and post-translational modification (e.g. glycosylation) of the *SULT2A1* mRNA. Although the adrenocortical cell line does provide a modality for isolating the effects of the potential secretagogues, the use of a transformed cell line limits the interpretability of the data to the *in vivo* setting. Another limitation of the study includes the use of a supra-physiologic dose of testosterone.

The data from this study suggests that insulin and testosterone may play a role, albeit limited, in the regulation of AA production. While testosterone did result in an increase in DHEA production, contrary to our initial hypothesis testosterone alone or insulin and testosterone acted to decrease cortisol and DHEAS production by adrenocortical cells. These data imply that the adrenal hyperandrogenemia or the excess DHEAS found in some PCOS patients is not a result of their hormonal milieu, but may represent an intrinsic (e.g. inherited) abnormality. Finally, we should note that genetic association studies may point towards areas for future exploration.

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Figure 1a.

Figure 1b.

Figure 1c.

Figure 1.

DHEA (Figure 1a), DHEAS (Figure 1b), and cortisol (Figure 1c) levels after 24 hours of incubation with forskolin alone, insulin + forskolin, testosterone + forskolin, and insulin + testosterone + forskolin. The y-axis is in relative units. The Y-bar indicates standard error of the mean. a, b, and c signify significant differences from each other, $P < 0.05$.

Figure 2.
SULT2A1 mRNA after 24 hours of incubation with forskolin alone, insulin + forskolin, testosterone + forskolin, and insulin + testosterone + forskolin. The y-axis is in relative units. The Y-bar indicates standard error of the mean.