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EFFECT OF SEX STEROIDS AND INSULIN ON DEHYDROEPIANDROSTERONE SULFATE (DHEAS) PRODUCTION BY HEPG2 CELLS

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Abstract

OBJECTIVE—To test the hypothesis that DHEAS production from DHEA occurs in hepatic cells, and that this production is augmented by the presence of sex steroids or insulin (INS).

DESIGN—In-vitro prospective experiment

SETTING—Academic medical center.

INTERVENTIONS—Hepatoma (Hep) G2 cells cultured in media supplemented with: i) DHEA (10^{-5} M) only, ii) DHEA (10^{-5} M) + testosterone (T, 10^{-6} M), iii) DHEA (10^{-5} M) + estradiol (E2, 10^{-6} M), iv) DHEA (10^{-5} M) + dihydrotestosterone (DHT 10^{-6} M), v) DHEA (10^{-5} M) + insulin (INS 10 ng/mL), or vi) DHEA (10^{-5} M) + insulin 100 ng/mL,

OUTCOME MEASURES—DHEAS levels in the media were measured at 0, 2, 4, 6, 8, 12, 24, 48, and 72 h after adding treatments at time-point 0.

RESULTS—DHEAS was first detected in the HepG2 cell culture media at 12h incubation. The cumulative production rate of DHEAS increased linearly until 72h incubation. When compared to the effect of treatment with DHEA only, treatment with DHEA plus T, DHT, or E2 delayed the cumulative DHEAS production; alternatively, the addition of INS did not alter DHEAS production.

CONCLUSION—These data suggest that while hepatic cells have the ability of converting DHEA to DHEAS, neither sex steroids nor INS result in the increased hepatic production of DHEAS.

Keywords

Adrenal androgen excess; DHEA sulphotransferase; HepG2 cells; dehydroepiandrosterone; DHEAS; *SULT2A1*

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INTRODUCTION

While most women with polycystic ovary syndrome (PCOS) demonstrate hyperandrogenemia of ovarian origin, approximately 25% of these patients also have overt adrenal androgen (AA) excess, reflected principally in elevated circulating levels of the AA metabolite dehydroepiandrosterone (DHEA) sulfate (DHEAS) (1,2). Both DHEA and DHEAS can act as substrates for steroidogenic enzymes, and are converted into potent androgens and/or estrogens in peripheral tissues that possess the necessary enzymatic machinery (3,4). In vitro studies have demonstrated that DHEAS increases the expression of the androgen receptor (5) and DHEA has been observed to activate not only the androgen receptor but also aromatase, 3 β -hydroxysteroid dehydrogenase, and cyclooxygenase-2 receptors (6).

The development of PCOS may, in part, be related to the presence of AA excess (7). Recent studies suggest that excessive prenatal androgen exposure may be associated with the development of PCOS-like phenotype, and in these experiments a temporal relationship was seen between DHEAS levels and the presence of the hyperandrogenic phenotype (8). Premature adrenarche is also associated with an increase in the risk of PCOS (9). Finally, congenital non-classic adrenal hyperplasia (NCAH) due to 21-hydroxylase deficiency results in PCOS-like features (10).

The mechanisms underlying AA, and in particular DHEAS, excess in PCOS remain unclear and were recently reviewed (11). Principally, many patients with PCOS, and most notably those with DHEAS excess, demonstrate a generalized hyper-responsiveness of AA production to ACTH stimulation (12). However, it is also clear that the production of DHEAS may reflect extra-adrenal factors. For example, short-term treatment of oophorectomized women with testosterone (T) increases the circulating DHEAS to DHEA ratio, suggesting altered DHEA sulfotransferase (DHEA-ST, also called *SULT2A1*) activity (13). The inherited abnormality of *CYP21* in patients with NCAH results in AA, mainly androstenedione, excess; however, over 50% of NCAH patients do not demonstrate DHEAS excess (10).

Changes in DHEAS concentration in vivo are dependant on both adrenocortical biosynthesis and changes in *SULT2A1* activity (14–16). The enzyme *SULT2A1* is cytosolic and is expressed at very high levels in liver, intestine, and adrenal tissue. It plays an important role in the regulation of steroid hormone action, forming steroid sulphate esters and sulfating 3 α - and 3 β -hydroxysteroids, estrogens, and T (17–22). There is genetic variability in *SULT2A1* and its sulfate donor, the co-substrate, 3'-phosphoadenosine 5'-phosphosulfate (*PAPS*) (15). Recent data from our laboratory suggests that polymorphisms of *SULT2A1* may have a potential role in the AA excess in PCOS (23). DHEAS can also be metabolized by the intracellular microsomal enzyme steroid sulfatase (*STS*) to yield DHEA in human leukocytes, placental, ovarian, and breast tissues (24–27).

While the assumption has been that the adrenal cortex is the principal site of DHEAS production in PCOS, it is also possible that the excess circulating levels of this metabolite observed in PCOS are secondary to increased sulfation of circulating DHEA by extra-adrenal tissues, principally the liver. The regulatory mechanisms determining the ratio of DHEA to DHEAS are complex and the role of the liver is not clear. An exaggerated hepatic sulfation of DHEA could be the result of PCOS-associated factors, such as hyperinsulinemia, hyperestrogenemia, or hyperandrogenemia. As the half-life of DHEAS is considerably longer than that of DHEA, 7–10 hr for DHEAS compared to 15–30 minutes for DHEA, respectively (28), small increases in hepatic sulfation of DHEA could result in more significant cumulative elevations in circulating DHEAS levels.

We have hypothesized that DHEAS production from DHEA occurs in the liver, and that this production is augmented by the presence of sex steroids or insulin (INS). To begin testing these hypotheses, we determined i) whether DHEAS production could be detected in a human hepatoma (Hep) G2 cell line, and ii) whether co-incubation of DHEA with T, dihydrotestosterone (DHT), estradiol (E2), or INS would modulate DHEAS production.

MATERIALS AND METHODS

Cell cultures

HepG2 cells were generously provided by the Women's Cancer Research Institute at the Samuel Oschin Cancer Institute, Cedars-Sinai Medical Center. The original source was American Type Culture Collection (ATCC). The cells were grown in culture flasks at 37°C in 95% air and 5% CO₂. The culture medium used was Minimum Essential Medium (MEM) (1X), with Earle's salts, and without L-glutamine (Invitrogen Corporation, Carlsbad, CA) and was supplemented with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA), 1% L-glutamine 200mM 100X (Invitrogen Corporation, Carlsbad, CA), 10% MEM non-essential amino acids solution, 10 mM 100X (Invitrogen Corporation, Carlsbad, CA), 1% sodium pyruvate 100mM 100X (Invitrogen Corporation, Carlsbad, CA), and 10% penicillin-streptomycin (Invitrogen Corporation, Carlsbad, CA).

At ~60–70% confluency cell viability was determined with trypan blue exclusion. Cells were washed with phosphate-buffered saline (PBS) and then seeded into 24-well culture plates at a density of 2.5×10^5 cells per well in 500 µL of serum-containing media and grown for 24hrs. Thereafter, the cells were washed with PBS, and the serum-containing media was replaced with fresh serum-free media for 24 hours prior to adding the test treatments.

After incubation of the cells with serum-free media for 24hrs, the cell culture media was replaced with serum-free media, supplemented with the following treatments at time-point 0 hours only: i) DHEA (10^{-5} M) only, ii) DHEA (10^{-5} M) + T (10^{-6} M), iii) DHEA (10^{-5} M) + E2 (10^{-6} M), iv) DHEA (10^{-5} M) + DHT (10^{-6} M), v) DHEA (10^{-5} M) + INS (10 ng/mL), or vi) DHEA (10^{-5} M) + INS (100 ng/mL). The cumulative production of DHEAS levels in the media was determined at 0, 2, 4, 6, 8, 12, 24, 48 and 72 hours of incubation. There were separate cell culture plates for each incubation time-point. At each time point, the media was removed from the plate and frozen until at -80°C until analyzed. At the conclusion of the experiment, the plates containing the cells were removed from the incubator and stored at -80°C . These cells were later used for estimation of total cellular protein, in order to normalize the measured hormone results. DHEAS was only detected in the media supplemented with DHEA (data not shown), and only data using this supplement will be reported. Cell cultures supplemented with serum-free media only were used as controls.

In each of the experiments, the six treatment types were performed in triplicate. Each experiment used the same number of cell culture passes, and hence the same number of days in culture prior to treatment. When cells reached 70% confluency experiments were started at the same time point during the day in all three experiments.

Radioimmunoassay

Both DHEAS and DHEA levels in the media were measured by a direct radioimmunoassay (DSL-2700 DHEA-S-7, Diagnostic Systems Laboratories, Inc., Webster, TX) using a Cobra II gamma counter (Packard Instrument Company Meriden, CT). The inter-assay and intra-assay coefficients of variation for this test were 5.3% and 1.8% respectively.

Total cellular protein assay

In order to normalize the results of the radioimmunoassay, total cellular protein was estimated by the dye-binding method of Bradford (29), using the Bio-RAD kit with bovine serum albumin as the standard. For the protein assay, the cells were harvested with a cell scraper in 67–75 μ L of lysis buffer composed of 20mM Tris-HCl, 145 mM NaCl, 10% Glycerol, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40 (Sigma-Aldrich St. Louis, MO) plus fresh inhibitors (200 mM PMSF, 200 mM NaVO₄, 1 mM leupeptin, 10 mg/ml aprotinin, Sigma-Aldrich St. Louis, MO). The cells were stored at -80°C until homogenized and assayed. Homogenization was carried out by centrifugation at 3000 rpm for 5 minutes. Ratios of steroid to protein concentrations were calculated. Cumulative DHEAS production in the cells after exposure to the hormonal treatments (T, INS, DHT, E) was compared to cells exposed to substrate only (i.e. DHEA only) and to controls (serum-free media only).

Statistics

The data are reported as the mean $\pm$ standard error. The Student-t test for two samples was used and $p \leq 0.05$ was considered statistically significant. As previously stated, the experiments were performed in triplicate to ensure the reproducibility of the results.

RESULTS

Time course of cumulative DHEAS expression

DHEAS was first detected at 12h and the absolute concentration of DHEAS at the various time-points is shown in Figure 1. The level of measured DHEAS increased significantly at the 12 hr as compared to the 24 hr time-point ($p=0.0006$). Thereafter, the levels of DHEAS measured, remained fairly constant until 72 hr.

Substrate (DHEA) availability during the time-course of the experiment was evaluated. In a separate experiment, frozen media from the various time-points, was thawed and analyzed for DHEA (Figure 2). When compared to levels of DHEA at 12 hr, there was a non-significant 18% decrease of DHEA at 24 hr but a significant 85% decrease of DHEA at 48 hr ($p=0.03$). No DHEA was detected at 72 hr. For this reason, only data up to 48 hrs is presented.

Effect of T, DHT, E2, and INS on cumulative DHEAS expression

To determine whether extra-hepatic factors modulate hepatic production of DHEAS the following treatments were added at time-point 0 hours: i) DHEA (10^{-5}M) only, ii) DHEA (10^{-5}M) + T (10^{-6}M), iii) DHEA (10^{-5}M) + E2 (10^{-6}M), iv) DHEA (10^{-5}M) + DHT (10^{-6}M), v) DHEA (10^{-5}M) + INS (10 ng/mL), or vi) DHEA (10^{-5}M) + INS (100 ng/mL). Two parameters were examined: a) the cumulative DHEAS production up to a given time-point, i.e. the absolute levels of DHEAS in the media at 12, 24, 48 or 72 h; and b) the calculated DHEAS production rate per hour between two time points, i.e. the sum of levels of hormone in the media at two time-points, divided by the number of hours of incubation.

The addition of sex steroids to the media was associated with significantly lower cumulative DHEAS levels at 24 hr as compared to DHEAS alone (Figure 3). Likewise, a decrease in the calculated DHEAS production rate between 12 and 24h incubation after treatment with T, DHT and E2, as compared to DHEA alone, was also observed ($p<0.05$, data not shown). Alternatively, there was no difference in DHEAS production or the production rate between the cells incubated with DHEA only and those incubated with the different treatments at 48 and 72 hrs. The addition of INS to the media had no effect on DHEAS production or production rate, as compared with addition of DHEA alone, at any time point (Figure 3).

To ensure that the added steroids or INS were not metabolized and hence removed by the HEPG2 cells during the time course of the experiment, these substances were measured in the removed media. The concentrations of T, DHT, E2 and INS measured throughout the experiment did not differ significantly (data not shown).

DISCUSSION

In this study, we aimed to determine i) if DHEAS production, as an indicator of DHEA-ST activity, could be detected in a human HepG2 cell line, when DHEA alone was used as substrate, and ii) if T, DHT, E or INS, when added together with DHEA, would affect DHEAS production. Our data indicates that the addition of DHEA resulted in a steady increase in DHEAS production from 12 up to 72 hr. These results suggest that the liver may be an important site for DHEAS production in normal and PCOS individuals, and that excess DHEA secretion by the adrenal gland may result in enhanced DHEAS production by the liver. Hammer et al. also have demonstrated that *SULT21A*, and STS, is abundantly expressed in both normal human liver and HepG2 cells (30). In their study there was no evidence of STS activity as incubation with DHEAS up to 24h did not result in measurable conversion to DHEA or other steroids. Our study suggests a similar finding with full depletion of DHEA in the culture media during the time-course of the experiment, indicating no conversion of DHEAS to DHEA. A recent study by Hammer et al. also found that intravenous administration of DHEAS to healthy young men did not increase serum DHEAS levels (30). These studies suggest that hepatic STS plays a minor role in the regulation of DHEA and DHEAS production in the liver.

The addition of T, DHT and E had only a modest negative effect on DHEAS production, delaying full production by only 24 hours. This was also reflected in the calculated DHEAS production rate. This delay in production could not be attributed to depletion of substrate, as a significant decrease in DHEA in the media was seen only at 48 hr incubation, the same time point at which the inhibitory effect of the steroids no longer was detected. In other reports, the effect of exogenous sex steroids on hepatic DHEA-ST activity is unclear. In vivo studies in rats have demonstrated a pituitary-dependant antagonistic regulation of hepatic DHEA-ST mRNA levels by E and DHT (31). Dihydrotestosterone suppressed *SULT2A1* activity, but when E and DHT were both present, no such suppression was seen. Estradiol alone did not have a marked effect on DHEA-ST activity in this model. Exogenous sex steroid administration in fertile women has also been associated in decreased DHEAS levels (32). Our data suggests that the effect of sex steroids on hepatic DHEA-ST activity, if present, is modest at best.

In our study INS did not have an apparent effect on hepatic DHEAS production. Recent human studies on the effect of hyperinsulinemia on AA production and metabolism have demonstrated divergent results (33–36). These results may be due to the variability of the populations studied. Studies in women with PCOS and insulin resistance have shown an association between insulin resistance and DHEAS levels in PCOS (36). We have recently demonstrated a negative association between DHEAS and fasting INS levels in Caucasian women with PCOS, an association not seen in healthy women (2). No effect of INS on DHEAS production was seen suggesting that hyperinsulinemia may not affect hepatic DHEA-ST activity. Overall, these data suggest that neither sex steroids nor INS significantly result in the increased hepatic production of DHEAS in PCOS.

There are a few limitations to the present study. Firstly, while HepG2 cells have been well characterized (37–40), they may not be representative of normal hepatic tissue (41–44) or hepatic tissue in PCOS patients. It is also possible that alternative effects of the factors added to the media may have affected our results. For example, increased substrate (e.g. DHEA) exposure to the hepatoma cells may induce liver enzymes capable of metabolizing the substrate or treatments, including proteolytic enzymes which could degrade INS, eliminating any effect

of this treatment. In addition, the added treatments could have induced secretion of some unknown hepatic factor or factors, altering *SULT2A1* function.

In summary, the production of DHEAS after the addition of DHEA was present in HepG2 cell cultures, suggesting that *SULT2A1* activity and the capacity to generate DHEAS are present in the liver. Contrary to our hypothesis, we noted that addition of sex steroids had only a modest effect, delaying full DHEAS production by 24 hours; INS had no effect. These data suggest that neither sex steroids nor INS result in the increased hepatic production of DHEAS in PCOS, although whether the hepatic response to sex steroids or INS differs in PCOS compared to that observed in HepG2 cells remains to be determined. Additional studies are also required to determine whether these or other factors have an effect on adrenocortical *SULT2A1* activity.

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Figure 1.

Cumulative DHEAS production after addition of DHEA substrate to HepG2 cell cultures.

* $p=0.0006$, ** $p=0.07$ and *** $p=0.02$ as compared with 12 hr

Figure 2.
DHEA in HepG2 cell culture media after incubation for 12–72 hr.
* $p < 0.05$ as compared to 12 and 24 hr

Figure 3.

Cumulative DHEAS production after incubation of HepG2 cells with DHEA only ($10^{-5}M$), co-incubation of DHEA ($10^{-5}M$) with testosterone ($10^{-6}M$), DHEA ($10^{-5}M$) with DHT ($10^{-6}M$) and DHEA ($10^{-5}M$) with estradiol ($10^{-6}M$).

* $p=0.01$ and ** $p=0.08$ as compared to DHEA only at 24 hr

Figure 4. Cumulative DHEAS production after incubation of HepG2 cells with DHEA only ($10^{-5}M$), co-incubation of DHEA ($10^{-5}M$) with insulin 100ng/mL and DHEA ($10^{-5}M$) with insulin 10ng/mL.