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Human adrenal cells that express both 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) and cytochrome b5 (CYB5A) contribute to adrenal androstenedione production

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ABSTRACT

Androstenedione is one of several weak androgens produced in the human adrenal gland. 3β-Hydroxysteroid dehydrogenase type 2 (HSD3B2) and cytochrome b5 (CYB5A) are both required for androstenedione production. However, previous studies demonstrated the expression of HSD3B2 within the zona glomerulosa (ZG) and fasciculata (ZF) but low levels in the zona reticularis. In contrast, CYB5A expression increases in the zona reticularis (ZR) in human adrenal glands. Although their colocalization has been reported in gonadal theca and Leydig cells this has not been studied in the human adrenal. Therefore, we immonolocalized HSD3B2 and CYB5A in normal human adrenal glands and first demonstrated their co-expression in the cortical cells located at the border between the ZF and ZR in normal human adrenal. Results of *in vitro* studies using the human adrenal H295R cells treated with the HSD3B2 inhibitor, trilostane, also demonstrated a markedly decreased androstenedione production. Decreasing CYB5A mRNA using its corresponding siRNA also resulted in significant inhibition of androstenedione production in the H295R cells. These findings together indicate that there are a group of cells co-expressing HSD3B2 and CYB5A with hybrid features of both ZF and ZR in human adrenal cortex, and these hybrid cortical cells may play an important role in androstenedione production in human adrenal gland.
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1. Introduction

The major adrenal androgens are dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS) and androstenedione [1,2]. Androstenedione is the common precursor of sex hormones in human. Ronde et al. also previously reported that adrenals are the main sources of circulating androstenedione in postmenopausal women [3]. Bidlingmaier et al. also demonstrated that adrenal glands are the main source of androstenedione in infant boys [4]. Therefore, it becomes very important to clarify the regulatory mechanisms of androstenedione production in human adrenal glands.

P450c17 (CYP17) catalyzes both 17α-hydroxylation and 17,20-lyase conversion of 21-carbon steroids to 19-carbon precursors of sex steroids [5]. CYP17 can mediate androstenedione biosynthesis via the conversion of pregnenolone to DHEA (the Δ^5 pathway) or via conversion of progesterone to 17α-hydroxyprogesterone (the Δ^4 pathway) [5] (Fig. 1). However, it is also true that CYP17 in the human adrenal has high 17,20-lyase activity only in the Δ^5 pathway [5]. In addition, conversion of DHEA to androstenedione also requires 3β-hydroxysteroid dehydrogenase (HSD3B2) [5] (Fig. 1). The 17,20-lyase activity of CYP17 only occurs through its association with cytochrome b_5 (CYB5A) [6]. Human CYB5A has been postulated to act principally as an allosteric effector which interacts primarily with the CYP17/P450 oxidoreductase complex to stimulate 17,20-lyase activity [2,7]. In human adrenal, 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) catalyzes the oxidation and isomerization of 3β-hydroxy-5-ene (Δ^5) steroids into 3-keto-4-ene (Δ^4) steroids leading directly to the production of progesterone and 17α-hydroxyprogesterone from their corresponding precursors, pregnenolone and 17α-hydroxypregnenolone, respectively [7,8] (Fig. 1). HSD3B2 also plays a pivotal role in the pathway leading to production of androgens through its conversion of DHEA to androstenedione [5,9] (Fig. 1).

Both HSD3B2 and CYB5A are co-expressed in the Leydig cells of the testis and theca cells of the ovary, which contributes greatly to efficient gonadal androgen production [10,11]. In human adrenal glands, however, HSD3B2 is distinctively expressed in the zona glomerulosa (ZG) and the zona fasciculata (ZF) but its expression in the zona reticularis (ZR) is quite low, whereas ZR specifically

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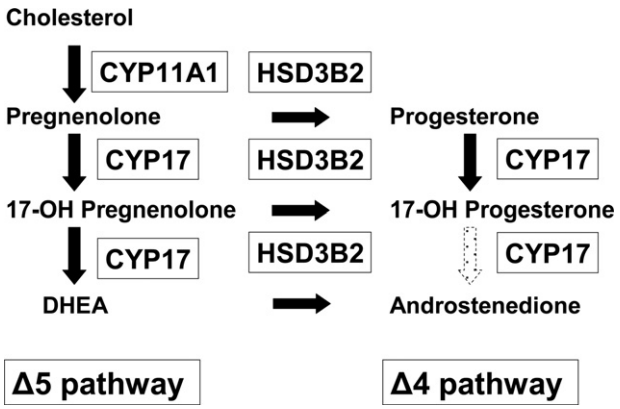

Fig. 1. Potential pathways of androstenedione synthesis in the human adrenal gland. Cholesterol is converted to pregnenolone by CYP11A1. Conversion of pregnenolone to androstenedione might take place by either of two pathways. In the Δ^5 pathway, pregnenolone undergoes 17Δ-hydroxylation to 17Δ-hydroxypregnenolone and scission of the C17–C20 bond to yield DHEA, both catalyzed by CYP17. HSD3B2 can also convert each Δ^5 steroid to its corresponding Δ^4 steroid, including androstenedione. For progesterone to be converted to testosterone, it must follow a Δ^4 pathway through 17Δ-hydroxyprogesterone and to androstenedione. However, CYP17 in human adrenal gland have high 17, 20-lyase activity only in the Δ^5 pathway ($). This figure was reproduced based on a previous study [5].

expressed CYB5A [10,11]. Therefore, it is important to determine whether the adrenal androstenedione production results from a unique set of adrenal cells that co-express HSD3B2 and CYB5A. In our present study, the layers of adrenocortical parenchymal cells between the ZF and ZR were firstly demonstrated to co-express both CYB5A and HSD3B2 as is seen in the testicular Leydig cells and theca interna cells of the ovarian follicle. In addition, adrenocortical cultured cells were shown to decrease androstenedione production following either the inhibition of CYB5A expression or blockage of HSD3B2. These findings all suggest that one potential source of androstenedione in human adrenal glands are those cortical cells which co-express CYB5A and HSD3B2.

2. Materials and methods

2.1. Human tissue preparation

Whole human normal adult adrenal glands were retrieved from surgical or autopsy files of Department of Pathology, Tohoku University Hospital (Sendai, Japan). The use of these tissues was approved by the Institutional Review Boards of Tohoku University School of Medicine (2004-355).

2.2. Double immunofluorescence analysis

We performed double immunofluorescence analysis for CYB5A using a monoclonal antibody purchased from Abcam (Cambridge, MA) and HSD3B2 using a polyclonal antibody kindly provided by Dr. Mason (University of Edinburgh, Edinburgh, U.K.), respectively. Fluorescence labeled ALEXA-488 anti-rabbit and ALEXA-647 anti-mouse secondary antibodies (Invitrogen, San Diego, CA) were applied in double staining experiment according to the manufacturer's instructions.

2.3. Transfection of CYB5A siRNA in H295R cells

The human adrenocortical cell line (H295R) was used for all transfection experiments and was routinely cultured in Dulbecco's Modified Eagles/Ham F12 (DME/F12) medium (Gibco, Carlsbad, CA) supplemented with 2.5% Ultroser G (Life Sciences, Cergy, France) and antibiotics, including 1% penicillin/streptomycin solution (Gibco), 0.1% gentamicin solution (Sigma–Aldrich), and 1% ITS plus Universal Culture Supplement Premix (BD Biosciences, Bedford, MA) [12]. Electrical transfection assays were performed using the Nucleofector System (AMAXA, Gaithersburg, MD).

Silencing-cell electronucleation was performed using AMAXA reagents (Lonza group Ltd., Switzerland). Briefly, H295R cells were cultured until 80% confluence in normal growth media before usage. Cells were tyrosinized and pelleted at the speed of 1000 rpm for 5 min. Supernatant was discarded and cell pellet was resuspended in supplemented buffer R (Lonza) with the ratio of 3,000,000 cells/100 μl. For CYB5A siRNA silencing experiments, Mission predesigned siRNA reagents was ordered from Sigma–Aldrich and dissolved in nuclear-free water with a stock concentration of 50 μM. As a negative control, Stealth™ RNAi Negative Control Duplexes were also used (Invitrogen, Carlsbad, CA). 2 μl of siRNA was used per 1,000,000 cells and electronucleation was performed under program T-20 in AMAXA system. Cells were allowed to recover in growth media for 10 min before plated into 75 mm culture flask. The cells were cultured for another 48 h and a second electronucleation with the procedure was performed. Incubation was conducted 48 h later. And then, the medium was also collected for steroid measurement as below. Both RNA and protein were isolated from the cells for quantitative RT-PCR (qPCR) and protein assay. The protocol of qPCR analysis has been described in detail [13].

2.4. Treatment of HSD3B2 inhibitor in H295R cells

H295R cells were incubated for 48 h in a low serum medium containing 0.1% CCS (cosmic calf serum) with or without the HSD3B2 inhibitor, trilostane (10 μM). And then, the medium was also collected for steroid measurement. Lysates were prepared from the cells for protein assay [13].

2.5. The measurement of steroid levels

The medium collected above was followed by cortisol and DHEA measurement using ELISA (ALPCO Diagnostics, Salem, NH), androstenedione and DHEA-S measurement using radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX), respectively.

2.6. Data analysis and statistical methods

Results are given as mean ± SEM where appropriate. Statistical analyses were done by unpaired *t*-test or one-way ANOVA, followed by post hoc test for comparisons between two groups dependent on the data types. Significance was accepted at the 0–0.05 level of probability ($P < 0.05$).

3. Results

3.1. Double immunofluorescence analysis

In the adrenal glands, HSD3B2 immunoreactivity was predominantly detected in the cytoplasm of the ZF, while CYB5A immunoreactivity was mainly detected in cytoplasm of the ZR in human adrenal glands (Fig. 2). We also confirmed the presence of double positive cortical cells in the border between the ZF and ZR (Fig. 2).

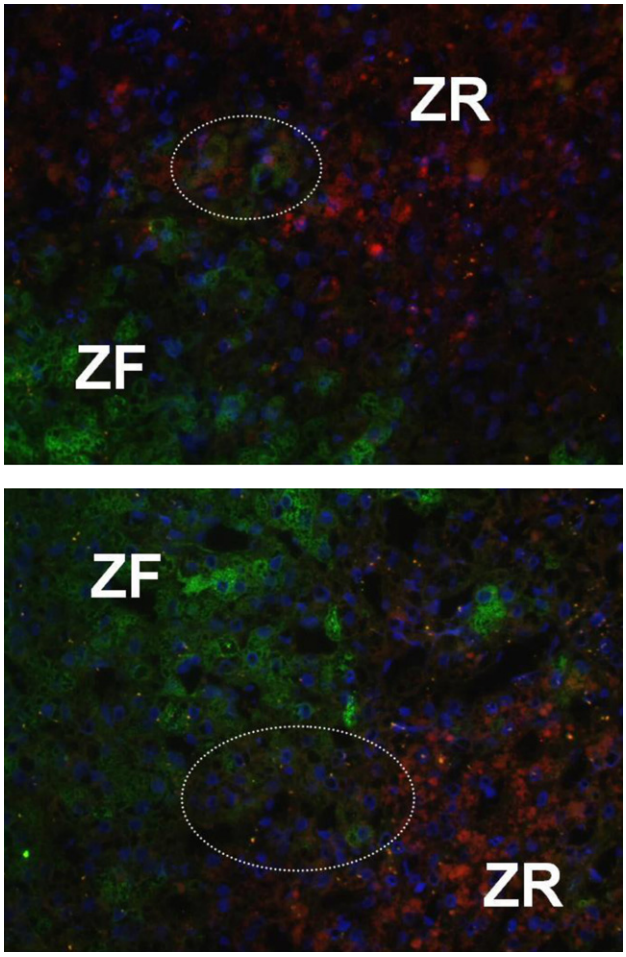

Fig. 2. The localization of CYB5A and HSD3B2 in the human adult adrenal gland. Immunopositive cells for CYB5A appear *red*, and immunopositive cells for HSD3B2 appear *green*. Double-immunopositive cells are detected in the border between the ZF and ZR (circles). The nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). We performed these double immunofluorescence analysis at least three sets of the human adrenal glands (*ZF*: zona fasciculata; *ZR*: zona reticularis).

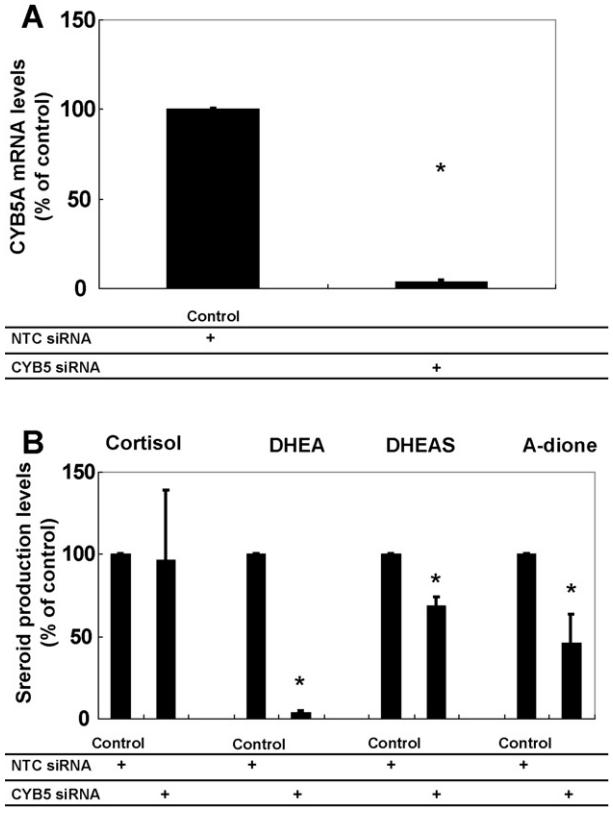

Fig. 3. Effects of siRNA depletion of CYB5A on adrenal cell production of adrenal steroids. (Panel A) H295R adrenal cells were transfected with or without siRNA against CYB5A (*CYB5 siRNA*) or Stealth™ RNAi Negative Control Duplexes (*Control*). After 48 h, mRNA for CYB5A was detected by qPCR. 18s rRNA expression were used for normalization. Data are presented as mean ± standard error (*$P < 0.05$). (Panel B) The level of cortisol, DHEA, DHEAS, and androstenedione in the media with H295R cells at 48 h after double transfection of either CYB5A (*CYB5 siRNA*) or Stealth™ RNAi Negative Control Duplexes (*NTC*). Data are presented as mean ± standard error (*$P < 0.05$). We performed three independent experiments.

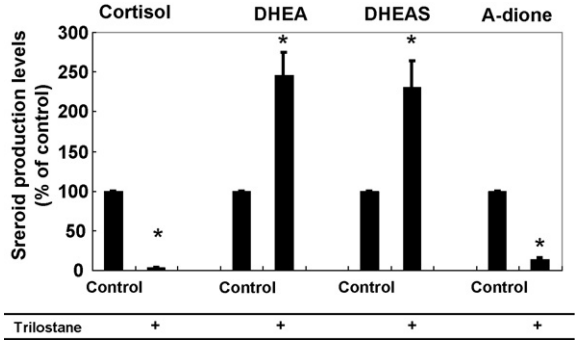

Fig. 4. Effects of trilostane on adrenal cell production of steroids. The amount of cortisol, DHEA, DHEAS, and androstenedione in the media with H295R cells was determined after 48 h incubation with or without trilostane (10 μM). Data are presented as mean ± standard error (*$P < 0.05$). We performed three independent experiments.

3.2. Adrenal steroid production in H295R cells after CYB5A siRNA transfection or HSD3B2 inhibition

The H295R adrenal cell line has been reported to produce a variety of steroids, including cortisol, DHEA, DHEAS, and androstenedione [14]. In particular, CYB5A and HSD3B2 are expressed in H295R cells, and therefore these cells can act as a model to define the relative contribution of these enzymes in adrenal androstenedione production [15,16].

Firstly, we repressed the expression of CYB5A in H295R cells and examined the effect of CYB5A on *in vitro* adrenocortical steroid production. After transfection of H295R cells with CYB5A specific siRNA, the levels of CYB5A mRNA dropped by 93% as compared to control cells (Fig. 3A). The production of DHEA, DHEAS, and androstenedione was significantly lower in CYB5A siRNA-transfected cells when compared to control cells (Fig. 3B) (by 96%, 31%, and 54%, respectively). Cortisol production did not change in CYB5A siRNA-transfected cells when compared to control cells (Fig. 3B).

Secondly, we inhibited HSD3B2 in H295R cells and examined its effects of CYB5A on *in vitro* adrenal steroid production. The production of cortisol and androstenedione was significantly lower when these cells were treated with the HSD3B2 inhibitor trilostane when compared to control cells (Fig. 4) (by 97% and 87%, respectively). DHEA and DHEAS production was significantly higher in trilostane treated cells when compared to control cells (Fig. 4) (2.5- and 2.3-fold, respectively).

4. Discussion

Androstenedione is known to be produced in both gonads and adrenal cortex. It is postulated that half of androstenedione is derived from testis in male [17]. In reproductive age women

the ovary contributes approximately 25–30% of the circulating levels of androstenedione and the adrenal gland are generally considered the primary source of androstenedione [18,19]. It has been demonstrated that intra-ovarian androstenedione levels were negligible in the postmenopausal ovary [20]. Therefore, it is reasonably postulated that adrenal gland is a key organ for production of androstenedione, and it becomes very important to examine the detailed expression patterns of enzymes associated with adrenal androstenedione synthesis in human adrenals using immunohistochemistry, and its regulatory mechanisms of adrenal androstenedione production using *in vitro* models.

The co-localization of CYB5A and HSD3B2 in Leydig cells in human testis and theca cells in human ovary suggest a role for intratesticular or intra-ovarian androstenedione production. However, subsequent conversion of androstenedione to testosterone appears to be more biologically important due to the expression of 17beta-hydroxysteroid dehydrogenase type 3 (HSD17B3) in Leydig cells, and subsequent production of estrogen from androstenedione more important by 17beta-hydroxysteroid dehydrogenase type 1 (HSD17B1) and aromatase in granulosa cells [25,26]. In humans, androstenedione is a critical precursor of active sex steroids in gonads. In contrast, results of our present double immunofluorescence study demonstrated that both HSD3B2 and CYB5A are co-expressed mainly in the border between the ZF and ZR cortical cells in human adrenal glands, suggesting the presence of hybrid cells of ZF and ZR which may be a key source for adrenal androstenedione. Immunohistochemical localization of CYB5A was reported in human adult adrenal gland, demonstrating that CYB5A immunoreactivity is absent in the ZF and exclusively positive in the ZR in human adult adrenal gland [21,22]. However, the expression of HSD3B2 has been reported to be detectable only in the ZG and ZF of the human adrenal gland [10,23]. Wang et al. subsequently demonstrated that HSD3B2 mRNA levels were 5-fold higher in the ZF than those in the ZR [24]. To the best of our knowledge, previous studies above, however, did not employ double immunostaining and did not demonstrate the presence of the hybrid cells positive for both HSD3B2 and CYB5A in human adult adrenal gland. In addition, we confirmed the presence of hybrid cells in both male and female adult adrenal gland but not in those of young children (data not shown). It awaits further investigations to clarify the regulatory mechanisms of their expression in these human cortical cells.

We previously reported that the H295R cell line is a good model to study adrenal androgen production including androstenedione [27]. We therefore used H295R cells to define the roles of HSD3B2 and CYB5 in the production of androstenedione in human adrenal glands. Adrenal androstenedione has been postulated to be synthesized predominantly via the conversion of pregnenolone to DHEA (the Δ^5 pathway), and subsequently to androstenedione via HSD3B2 [5]. CYB5A is known to enhance the 17,20-lyase activity of CYP17, essential for the conversion from pregnenolone to DHEA [28–32]. Results of our present study did demonstrate that repression of CYB5A inhibited the production of DHEA and androstenedione. This finding is consistent with results of a previous study demonstrating that inhibitor of C17-20 lyase activity reduces the synthesis of adrenal androgens in NCI-H295 cells [33]. HSD3B2 is known to influence the production of aldosterone, cortisol, and DHEA by competing with CYP17 for the metabolism of pregnenolone and 17α-hydroxypregnenolon in human adrenal [7,34]. Abundant levels of HSD3B2 expression combined with low CYP17 activity tend to lead to aldosterone synthesis and oppose cortisol and adrenal androgens synthesis [7]. Conversely, low abundance of HSD3B2 expression associated with high CYP17 activity tends to result in the production of Δ^5 adrenal androgens [7]. In this study, we reported that inhibition of HSD3B2 induced a marked increase of DHEA production and concomitant decrement of cortisol and androstenedione production in H295R cells. We also confirmed that an inhibition of both HSD3B2 and CYB5A resulted in marked repression of androstenedione production in H295R cells.

In summary, results of our present study demonstrate that the human adrenal has patches of hybrid ZF/ZR cells that co-express HSD3B2 and CYB5A and that this likely contributes to the ability of the adrenal to produce androstenedione.

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