ORIGINAL ARTICLE

11-Ketotestosterone Is a Major Androgen Produced in Human Gonads

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Context: 11-ketotestosterone (11-KT) is a novel class of active androgen. However, the detail of its synthesis remains unknown for humans.

Objective: The objective of this study was to clarify the production and properties of 11-KT in human.

Design, Participants, and Methods: Expression of cytochrome P450 and 11β-hydroxysteroid dehydrogenase types 1 and 2 (key enzymes involved in the synthesis of 11-KT) were investigated in human gonads. The production of 11-KT was investigated in Leydig cells. Plasma concentrations of testosterone and 11-KT were measured in 10 women and 10 men of reproductive age. Investigation of its properties was performed using breast cancer-derived MCF-7 cells.

Results: Cytochrome P450 and 11β-hydroxysteroid dehydrogenase types 1 and 2 were detected in Leydig cells and theca cells. Leydig cells produced 11-KT, and relatively high levels of plasma 11-KT were measured in both men and women. There was no sexual dimorphism in the plasma levels of 11-KT, even though testosterone levels were more than 20 times higher in men than in women. It is noteworthy that the levels of testosterone and 11-KT were similar in women. In a luciferase reporter system, 11-KT activated human androgen receptor-mediated transactivation. Conversely, 11-KT did not activate estrogen receptor-mediated transactivation in aromatase-expressed MCF-7 cells, whereas testosterone did following conversion to estrogen. 11-KT did not affect the estrogen/estrogen receptor -mediated cell proliferation of MCF-7 cells. Furthermore, it significantly inhibited cell proliferation when androgen receptor was transfected into MCF-7 cells.

Conclusions: The current study indicates that 11-KT is produced in the gonads and represents a major androgen in human. It can potentially serve as a nonaromatizable androgen. ***(J Clin Endocrinol Metab* 101: 3582–3591, 2016)**

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Abbreviations: AR, androgen receptor; CYP, cytochrome P450; DHT, 5α-dihydrotestosterone; EIA, enzyme immunoassay; ER, estrogen receptor; ERE, estrogen-response element; FBS, fetal bovine serum; FF, follicular fluid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HSD11B2, 11β-hydroxysteroid dehydrogenase type 2; IRB, Institutional Review Board; 11-KT, 11-ketotestosterone; LC-MS/MS, liquid chromatography-tandem mass spectrometry; 11-OHA, 11β-hydoxyandrostenedione; 11-OHT, 11β-hydroxytestosterone; Q, quantitative; SF-1, steroidogenic factor-1.

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Androgens are sex steroid hormones that play a role in various physiological processes via pathways involving the androgen receptor (AR) (1). Testosterone is the most important androgen and is produced from cholesterol in the gonads in a series of steps catalyzed by steroid P450 hydroxylases and hydroxysteroid dehydrogenase (2). Although testosterone itself strongly activates AR-mediated transactivation, it is also converted to a more potent androgen, 5α-dihydrotestosterone (DHT), by 5α-reductase in male peripheral tissues. The androgen/AR pathway is essential for male physiology, reproduction, and the development of sexual characteristics (3). Although androgens are traditionally viewed as male hormones, androgen/AR signaling is also important for optimal female reproduction and physiology (4, 5). Because androgen levels reduce with aging before menopause, postmenopausal women suffer various symptoms by androgen insufficiency (5, 6). On the other hand, androgen excess in women results in a variety of pathological conditions, including polycystic ovary syndrome and idiopathic hirsutism (7). Therefore, proper androgen signaling is important for the health of women. In addition to androgenic actions, testosterone is also a precursor for estrogen. Aromatase (CYP19A1) converts testosterone into the most potent estrogen, 17β-estradiol (E_2). It activates 2 subtypes of estrogen receptors (ERs), ERα and ERβ, the expression patterns of which are tissue and cell specific. Adrenal gland also produces testosterone and high concentrations of weak androgens, such as dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, 11β-hydoxyandrostenedione (11-OHA), and 11β-hydroxytestosterone (11-OHT), which are also precursors for stronger androgens in target tissues (8–10). In previous studies, we and others have reported that 11-ketotestosterone (11-KT) (International Union of Pure and Applied Chemistry name is 17-hydroxyandrost-4-ene-3,11-dione) is another class of active androgen that can be converted from testosterone and other weaker precursors (9–11).

Cytochrome P450 and 11β-hydroxysteroid dehydrogenase types 1 and 2 (CYP11B1 and HSD11B2) play important roles in the 11-KT synthesis from testosterone (10, 11). It is well known that these enzymes are involved in glucocorticoid synthesis and metabolism. CYP11B1 catalyzes the final step of glucocorticoid production in the adrenal gland (12, 13), whereas HSD11B2 converts active glucocorticoids into inactive 11-ketosteroid forms, which are abundantly expressed in the kidney and placenta (14). We have demonstrated that Cyp11b1 and Hsd11b2 are expressed in murine gonads and are involved in gonadotropin-induced 11-KT production (11). 11-KT can strongly activate mammalian AR-mediated transactivation. It was also reported that 11-KT can be detectable in human blood samples (9). Thus, it is conceivable that 11-KT is common androgen in mammals, although the detail of its synthesis remains unknown. In the present study, we evaluated the production and properties of 11-KT in humans.

Materials and Methods

Cell culture and transfection

Human ovarian granulosa cell tumor-derived KGN cells (kindly donated by Dr Toshihiko Yanase, University of Fukuoka, Fukuoka, Japan) and MCF-7 cells were cultured in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS). Human adrenocortical tumor-derived H295R cells were cultured in Opti-MEM supplemented with 2% Nuserum IV (BD Biosciences). Human Leydig cells were purchased from ScienCell Research Laboratories, which are sourced within the United States under protocols that have obtained Institutional Review Board (IRB) approval. They were cultured in Leydig Cell Meidum (ScienCell Research Laboratories). The research protocol using human materials was approved at the ethical committee of Asahikawa Medical University. H295R cells were transfected using an Amaxa Nucleofector Technology system (Lonza) as described (15). KGN and MCF-7 cells were transfected using Lipofectamine LTX reagent (Life Technologies, Inc) and HilyMax (Dojindo Laboratories). One day before transfection, the cells were seeded on 24-well plates and cultured with phenol red-free medium supplemented with 10% charcoal/dextran-stripped-FBS. After 24 hours of transfection, the cells were treated with vehicle (ethanol) or steroid hormones for 24 hours. Luciferase assays were performed as described previously (15, 16). Each data point represents the mean of at least 4 independent experiments.

Adenovirus production and infection

Adenovirus vectors for β-galactosidase, green fluorescent protein (GFP), and steroidogenic factor-1 (SF-1) were prepared using the Adeno-X Expression System 1 (Takara), following the manufacturer's instructions as described (16). Using these vectors, replication-defective recombinant adenoviruses were propagated and titered in HEK 293 cells. Then, they were used to infect KGN cells at multiplicity of infection 10. GFP-expressing adenovirus used as a transduction control during infection. Transduction efficiency and optimal concentrations of virus were determined by investigating GFP expression using fluorescent microscopy. At 48 hours after infection, cells were processed for RNA or protein extraction.

DNA microarray

Expression analysis by DNA microarray has been described elsewhere (17). Briefly, labeled cRNA was prepared from KGN cells with adenovirus-mediated expression of β-galactosidase, GFP, or SF-1. After fragmentation of cRNA, hybridization was performed with a human U133 Plus 2.0 Affymetrix GeneChip (Affymetrix). The arrays were scanned using a Gene-Array scanner, and the data were generated by Affymetrix Microarray Suite 4.0. Data were analyzed using Subio Platform software.

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RT-PCR and quantitative (Q)-PCR

Total RNA from the cultured cells was extracted using TriPure Isolation Reagent (Roche). Total RNA from human testis, ovary, adrenal, liver, and kidney was purchased from Takara Bio, Inc and Biochain Institute, Inc. RT-PCR and Q-PCR was performed as described (16, 18). The RT-PCR products were electrophoresed on a 1.5% (wt/vol) agarose gel, and the resulting bands were visualized by staining with ethidium bromide. In Q-PCR, each gene expression was measured by real-time PCR and normalized with β-actin expression. The primers used for PCR are described in Supplemental Table 1. The primers used for other genes were as described (16, 18).

Western blot analysis

Extraction of total proteins from cultured cells and subsequent quantification were conducted as described previously (16). Protein extract samples from each human tissue were purchased from BioChain Institute, Inc. Ovarian tissue donors were able to donate healthy organs and were of reproductive age (aged 30–34 y). Each tissue sample was collected under strict IRB ethical consenting practices. Equal amounts of protein (20 μg) were resolved using 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blot analyses of HSD11B2, CYP11B1, AR, β-actin, and GAPDH were performed with antibodies directed against HSD11B2 (H-145; Santa Cruz Biotechnology, Inc), CYP11B1 (13), AR (N-20; Santa Cruz Biotechnology, Inc), β-actin (C4; Santa Cruz Biotechnology, Inc), and GAPDH (6C5; Santa Cruz Biotechnology, Inc), respectively. Enhanced chemiluminescence Western blotting reagents (Bio-Rad Laboratories, Inc) were used for detection.

Plasmids

The pcDNA3 expressing HSD11B2 was generated by cloning the open reading frame of HSD11B2 into a pcDNA3 vector (Invitrogen). A Slp-ARU/Luc reporter and pQCXIP/human AR were prepared as described (11, 19). The estrogen-response element (ERE)/Luc was constructed by inserting the oligonucleotides into a pGL4.24 plasmid (Promega Corp).

Immunohistochemistry

Immunohistochemistry was performed as described previously (11, 19). Sections of human testis (from men aged 23 and 50 y) and ovary (from woman aged 19 and 34 y) were purchased from US Biomax, Inc and Biochain Institute, Inc. Sections were

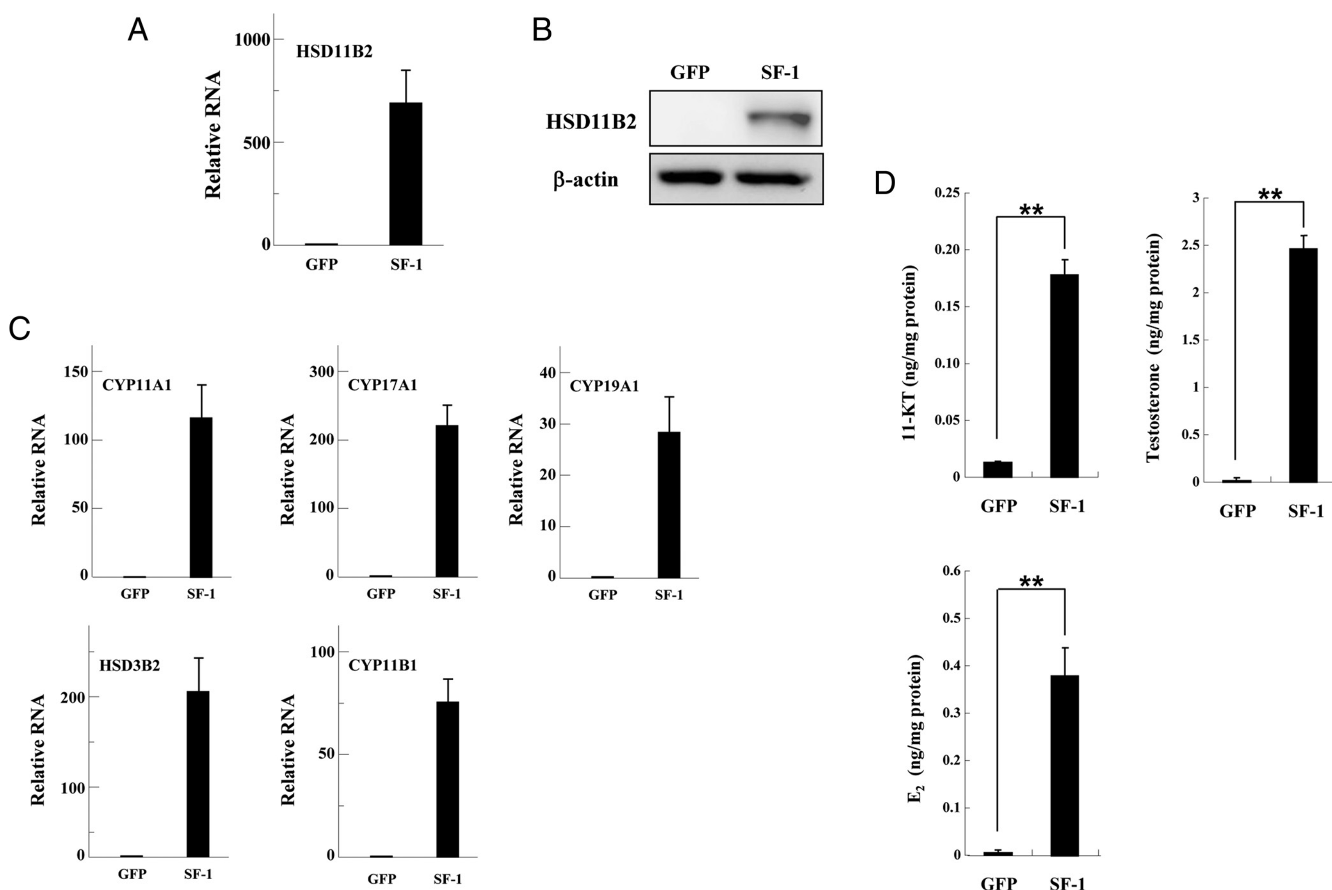

Figure 1. SF-1 induces HSD11B2 expression and 11-KT production in KGN cells. KGN cells were infected with adenoviruses expressing GFP or SF-1. Induction of HSD11B2 mRNA (A) and protein (B) by SF-1. A, mRNA expression of HSD11B2 gene was analyzed by Q-PCR and normalized to β-actin expression. Data represent the mean ± SEM of at least 3 independent experiments. B, Western blot analyses were performed with antibodies against HSD11B2 and GAPDH using the same lysates. C, Induction of the enzymes for the synthesis of testosterone, 11-KT, and E_2 was confirmed by Q-PCR. Data represent the mean ± SEM of at least 3 independent experiments. D, 11-KT, testosterone, and E_2 levels in each group were measured by EIA. Data represent the mean ± SEM of at least 3 independent experiments. Differences between groups are indicated by **, $P < .01$.

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subjected to the antigen retrieval technique with Dako Target Retrieval Solution (pH 9) (Dako Denmark A/S) and treated with anti-CYP11B1 (13) and anti-HSD11B2 (20). They were then developed using a Vectastain Elite ABC kit (Vector Laboratories).

Human blood samples

Blood samples were collected in collection tubes containing heparin from the median cubital vein of 2 healthy women volunteers at University of Fukui Hospital in 2007. Plasma was separated by centrifugation at 1000*g* for 5 minutes. Other plasma samples were purchased from AllCells and ProMedDX. All plasma samples were collected under IRB-approved collection protocols and subject informed consent. The donors were 10 men (aged 32.1 ± 7.8 y) and 10 women (aged 31.7 ± 6.0 y). Plasma samples were stored at −80°C until assays. The research protocol for using human materials was approved by the Ethics Committee of the University of Fukui.

Measurements by enzyme immunoassays (EIAs) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Concentrations of testosterone, 11-KT, and E_2 in culture media of KGN, H295R, and human Leydig cells were determined by competitive EIA (11, 19). Each sample was diluted with EIA buffer and analyzed using progesterone, testosterone, 11-KT, and E_2 EIA kit (Cayman Chemical Co) following the manufacturer's instructions in a microplate reader (Molecular Device SpectraMax M5; Molecular Device, LLC). On the other hand, concentrations of steroid hormones in human plasma were measured by LC-MS/MS for optimal quantification of the clinical samples. Processing of human plasma samples and quantification of testosterone, 11-KT, and E_2 by LC-MS/MS are based on methods as described previously (9).

Retrovirus preparation and infection

The Phoenix packaging cell line was transiently transfected with the retroviral plasmids using the FuGENE 6 reagent (Promega). The supernatant was concentrated by centrifugation. The virus solution was stored at −80°C until use. MCF-7 cells were infected with the retrovirus in the presence of 8-μg/mL Polybrene (Sigma-Aldrich) for 48 hours. The cells were then replated and selected using puromycin.

Proliferation assay

MCF-7 cells or AR-introduced cells were seeded with DMEM/F-12 supplemented with 10% or 2% charcoal/dextran-stripped-FBS at 1×10^3 cells/well in 96-well plates. At 24 hours after seeding, the cells were treated with the media containing various concentrations of DHT, testosterone, 11-KT, or E_2. Six days (parental cells) or 9 days (AR-introduced cells) after incubation, cell proliferation was evaluated using a CellTiter 96 Aqueous One Solution kit (Promega) following the manufacturer's instructions. To evaluate the effect of an aromatase inhibitor, fadrozole (Sigma) or an ER antagonist, fulvestrant (Sigma), on the growth of MCF-7 cells, a proliferation assay was performed with or without these agents.

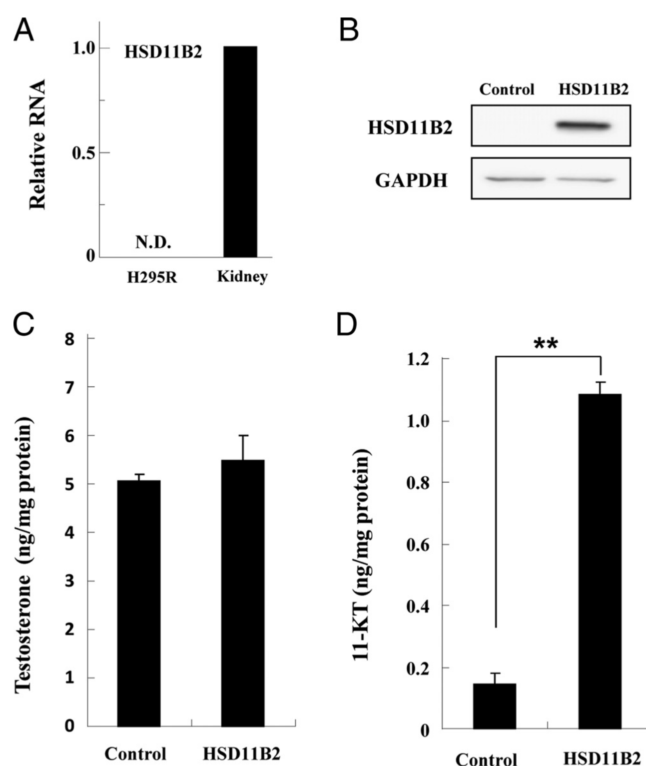

Figure 2. 11-KT production was induced by the ectopic expression of HSD11B2 in H295R cells. A, Expression of HSD11B2 in H295R cells. Expression of HSD11B2 was analyzed by Q-PCR and normalized to β-actin expression. The kidney was used as positive controls. Q-PCR data represent the mean ± SEM of at least 3 independent experiments. B–D, H295R cells were transfected with the empty vector or the expression vector for HSD11B2. B, Protein extracts from cells in each treatment were subjected to SDS-PAGE, and Western blotting was performed using each antibody. C and D, Testosterone (C) and 11-KT (D) levels in each group were measured by EIA. Data represent the mean ± SEM of at least 3 independent experiments. Differences between groups are indicated by **, $P < .01$.

Statistics

Data are presented as the mean ± SEM. Differences between groups ($P < .05$) were assessed by the Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison tests using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (21), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

Results

HSD11B2 is induced by SF-1 and involved in the 11-KT production

Human ovarian granulosa cell tumor-derived KGN cells have low steroidogenic capacity under basal conditions. However, they can be transformed to produce a range of steroid hormones by infection with an adenovirus expressing SF-1 and its coactivator (16). To examine changes in gene expression in this transformation, we used a DNA microarray with GFP- or SF-1-introduced KGN

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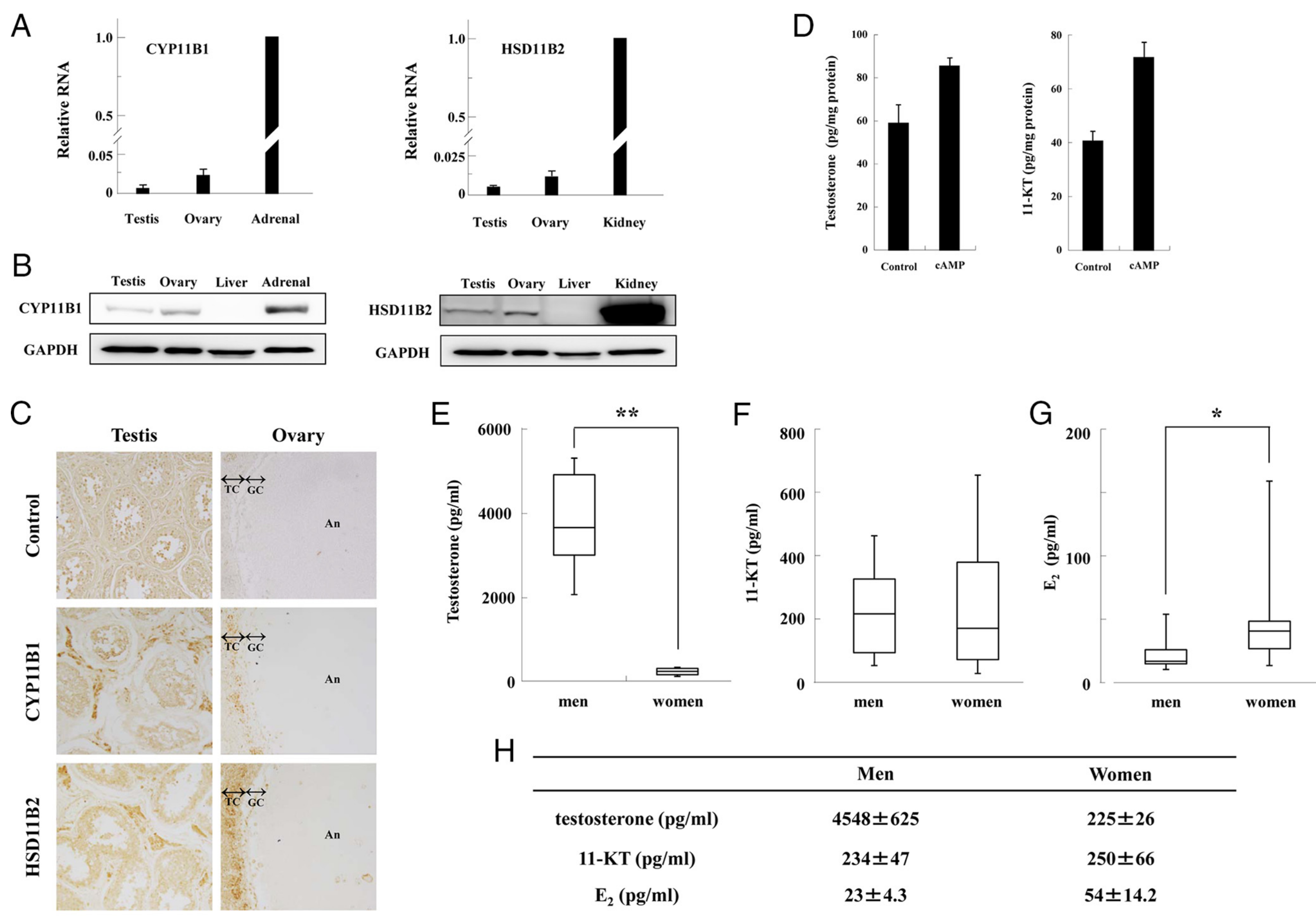

	Men	Women
testosterone (pg/ml)	4548±625	225±26
11-KT (pg/ml)	234±47	250±66
E_2 (pg/ml)	23±4.3	54±14.2

Figure 3. Expression of 11-KT synthetic enzymes in human gonads and plasma concentrations of 11-KT. A and B, Expression of CYP11B1 and HSD11B2 in human testis and ovary. A, mRNA levels of each gene analyzed by Q-PCR and normalized to β-actin expression. The adrenal gland and kidney were used as a positive control for each analysis. Q-PCR data represent the mean ± SEM of at least 3 independent samples. B, Western blot analyses were performed with antibodies against CYP11B1, HSD11B2, and GAPDH using lysates from each human tissue (20-μg protein). Western blot analysis is representative of the 2 experiments. C, Localization of CYP11B1 and HSD11B2 proteins in human gonads. Positive staining both for CYP11B1 and HSD11B2 was observed in testicular Leydig cells and ovarian theca cells. No staining was observed in control sections incubated with nonimmune serum. An, antrum. D, Production of testosterone and 11-KT by human Leydig cells with or without 8br-cAMP for 48 hours. Each androgen levels in each group were measured by EIA. Data represent the mean ± SEM of at least 3 independent experiments. E–H, Levels of plasma testosterone, 11-KT, and E_2 in humans. Plasma testosterone (E), 11-KT (F), and E_2 (G) levels in men and women were measured by LC-MS/MS. In the box and whisker plots, boxes show 75th and 25th percentiles. Horizontal lines in the boxes represent the medians. Whiskers show the lowest values and the highest values. Differences between groups are indicated by *, $P < .05$ and **, $P < .01$. H, Comparison of plasma testosterone, 11-KT, and E_2 levels within each sex. Data represent the mean ± SEM (n = 10 men, n = 10 women).

cells. SF-1 introduction induced a number of genes, including steroidogenic enzymes such as CYP11A1, HSD3B2, CYP17A1, and CYP19A1 (Supplemental Table 2), which are known SF-1 targets (22). In addition to these genes, HSD11B2 was a strong SF-1-inducible candidate gene (Supplemental Table 2) and was almost undetectable in GFP-introduced cells (Figure 1, A and B). Consistent with DNA microarray data, Q-PCR and immunoblotting analyses revealed that introduction of SF-1 strongly induced HSD11B2 mRNA and proteins (Figure 1, A and B). In previous studies, it was demonstrated that HSD11B2 is involved in 11-KT production from testosterone with CYP11B1 (Supplemental Figure 1) in murine gonads (11, 19) or from adrenal androgens by in vitro analysis (10). The DNA microarray analyses suggest that CYP11B1 is an SF-1-inducible gene, as well as other steroidogenic genes that are involved in testosterone and E_2 synthesis (Figure 1C and Supplemental Table 2, and Supplemental Figure 1). In fact, the introduction of SF-1 induced not only the production of testosterone and E_2 but also the CYP11B1 expression and 11-KT production (Figure 1, C and D).

To verify the role of HSD11B2 during 11-KT production in human steroidogenic cells, it was ectopically expressed in human adrenocortical H295R cells. H295R cells expressed CYP11B1 and other steroidogenic enzymes for testosterone synthesis (Supplemental Figure 2), whereas endogenous HSD11B2 was undetectable (Figure 2A). Even though H295R cells produce testosterone at relatively high levels, its conversion to 11-KT was marginal (Figure 2, C and D). Transient transfection of the

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HSD11B2 expression vector (Figure 2B) markedly increased 11-KT production compared with the control group (Figure 2D), whereas testosterone concentrations were similar in both groups (Figure 2C). These results indicate that the expression of human HSD11B2 could be an important factor for the production of 11-KT in steroidogenic cells.

11-KT is produced in gonads and is one of the major androgens in human

To elucidate the 11-KT synthesis pathway, we investigated the expression of CYP11B1 and HSD11B2 in human gonads (Figure 3, A and B). Q-PCR and Western blot analyses showed that both genes were detectable in the testis and ovary at the mRNA and protein levels (Figure 3, A and B). Immunohistochemical analysis showed that both proteins are localized on testicular Leydig cells and ovarian theca cells, even though HSD11B2 is also detectable in some populations of ovarian granulosa cells (Figure 3C). These results strongly suggest that 11-KT is produced in testicular Leydig cells and ovarian theca cells. To confirm this hypothesis, we investigated the production of 11-KT in human Leydig cells. In support of the immnohistochemical analyses, Leydig cells expressed CYP11B1 and HSD11B2 genes (Supplemental Figure 3A). They can produce progesterone, testosterone, and 11-KT under basal conditions (Figure 3D and Supplemental Figure 3B). cAMP treatment moderately increased the production of these steroid hormones.

Then, we measured plasma concentrations of 11-KT, testosterone, and E_2 in both sexes (Figure 3, E–G). Testosterone levels in men were about 22-fold higher than those in women, whereas 11-KT levels were similar between the sexes (Figure 3, E and F). It is noteworthy in women that 11-KT concentrations were similar to testosterone concentrations and about 5-fold higher than E_2 concentrations (Figure 3H). Human AR-mediated transactivation was significantly increased by 11-KT at concentrations more than 10^{-9}M in KGN cells (Figure 4). This level is lower than that for the induction by other androgens, although transactivation was increased to similar levels by DHT and testosterone at 10^{-8}M and 10^{-7}M, respectively. These results suggest that 11-KT may play some roles in human as one of the major androgens.

11-KT is not convertible to estrogenic hormones

In female individuals, testosterone acts as both an androgen and a precursor for estrogen. To determine whether 11-KT is a precursor for estrogen, we performed a luciferase assay using a reporter plasmid containing the ERE in human breast cancer-derived MCF-7 cells, which endogenously express aromatase and ERα. In contrast to

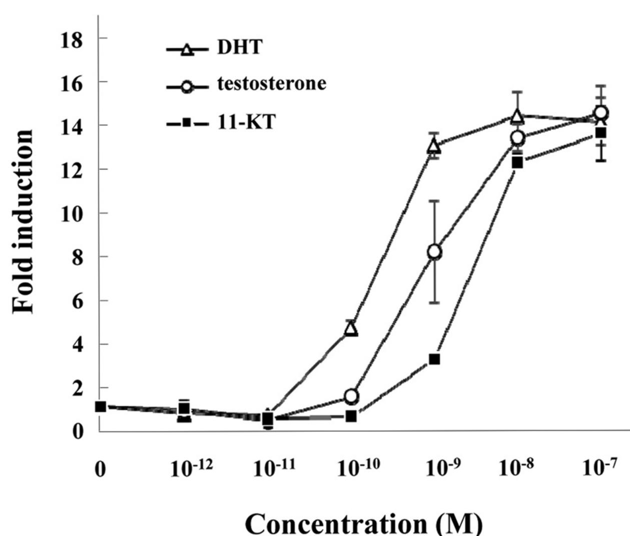

Figure 4. Activation of human AR-mediated transcription by DHT, testosterone, and 11-KT. KGN cells was transfected with the ARE/Luc vector and the human AR-expression vector. At 24 hours after transfection, cells were incubated with or without increasing concentrations of each androgen for 24 hours. Data represent the mean ± SEM of at least 4 independent experiments.

E_2, androgens had no effect on luciferase activity at lower concentrations (10^{-11}M and 10^{-10}M) (Figure 5A). However, testosterone activated ER-dependent transcription at high concentrations and at 10^{-7}M testosterone was effective as E_2. This activity was completely suppressed by an aromatase inhibitor, fadrozole (Figure 5B). DHT weakly activated ER-mediated transactivation in an aromatase-independent manner at 10^{-7}M. In contrast, 11-KT had no effect on ER-mediated transactivation, even at 10^{-6}M (Figure 5A; data not shown). These results indicate that 11-KT is a nonaromatizable androgen, and does not convert to a compound that activates ER-mediated transactivation.

Then, we assessed the effect of 11-KT on cell proliferation in MCF-7 cells. The proliferation of MCF-7 cells is highly dependent on estrogens. Consistent with previous studies, MCF-7 cells were increased by more than 10^{-11}M E_2 (Figure 6A). In support of the reporter assays result, testosterone significantly stimulated cell proliferation at higher concentrations (Figure 6A), and fadrozole and the ER antagonist fulvestrant completely inhibited testosterone-induced cell proliferation (Figure 6, B and C). Conversely, 11-KT had no effect on cell growth at any concentration, similar to DHT. These results strongly suggest that 11-KT acts as a stable androgen in both AR- and aromatase-positive cells. To further test this hypothesis, AR was stably transfected into MCF-7 cells (Figure 6D) and we measured the AR-mediated growth inhibition of each androgen. Consistent with previous reports (23), DHT strongly suppressed cell proliferation in AR-introduced MCF-7 cells (Figure 6E). Similarly, 11-KT also in-

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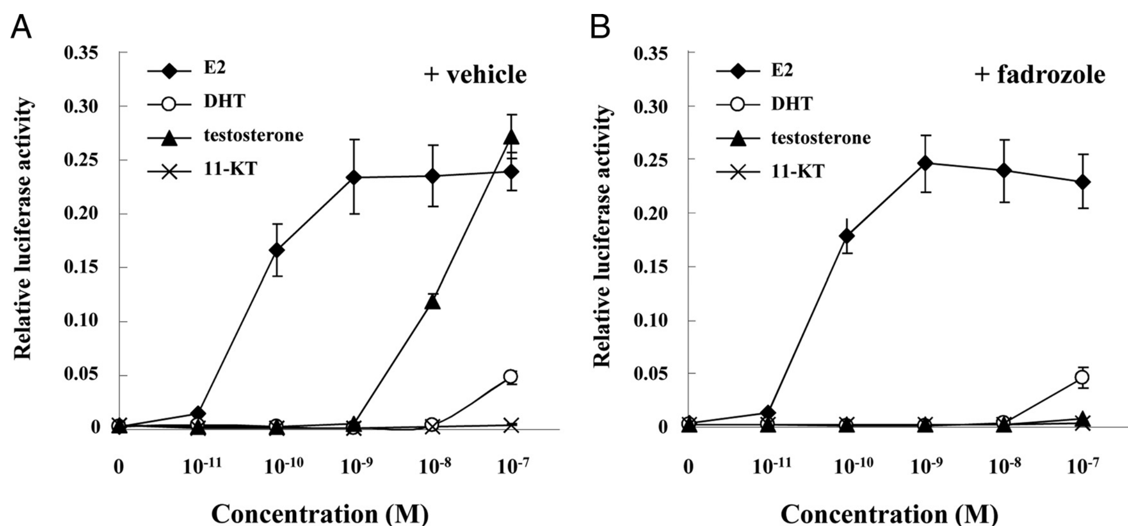

Figure 5. Comparison of ER-mediated transactivation by E_2, DHT, testosterone, and 11-KT in MCF-7 cells. MCF-7 cells were transfected with the ERE/Luc reporter vector. At 24 hours after transfection, increasing concentrations of each steroid hormones were added with (A) or without (B) the aromatase inhibitor fadrozole for 24 hours. Data represent the mean ± SEM of at least 4 independent experiments.

hibited proliferation, although to a lesser extent than DHT. In contrast, testosterone did not significantly inhibit cell growth.

Discussion

11-KT is one of the active androgens, which was originally characterized as a teleost-specific hormone. However, we and others have since noted that it is also present in mammals (9–11). Here, we found evidence that 11-KT is produced in human gonads and is one of the major androgens. It can potentially act as a nonaromatizable androgen. Testicular Leydig cells and ovarian theca cells expressed the enzymes for producing 11-KT from testosterone, CYP11B1 and HSD11B2. In fact, Leydig cells produced 11-KT, autonomously.

HSD11B2 protects mineralocorticoid receptor from glucocorticoid by inactivating cortisol to corticosterone in aldosterone-sensitive tissues (14). The gonads are target organs for mineralocorticoids, which express mineralocorticoid receptor (24, 25). Mineralocorticoids can stimulate the production of testosterone and progesterone in testicular Leydig cells (24) and ovarian granulosa cells (26), respectively. Conversely, glucocorticoids inhibit testicular and ovarian steroidogenesis (27). Because HSD11B2 proteins are expressed in these cells, it is reasonable to assume that gonadal HSD11B2 plays a role in the protection of steroidogenic cells from the adverse effects of glucocorticoids. In addition to this classical role, the involvement of HSD11B2 in 11-KT production in Leydig cells and theca cells may represent another important role (10, 11). Our results strongly suggest that the gonads are main organs for 11-KT production in humans by the expression of HSD11B2.

In mammals, CYP11B1 is thought to be an adrenal-specific enzyme that is essential for the final step of glucocorticoid synthesis (12). However, it is also expressed in the testicular Leydig cells and ovarian theca cells to a lesser extent. There is also evidence that CYP21 is expressed in the gonads (25, 28). Thus, a contribution of gonadal CYP11B1 to local glucocorticoid synthesis cannot be ruled out, although there are no studies documenting high levels of cortisol in the gonads. Rather, it is often reported that cortisol levels are lower in ovarian follicular fluid (FF) than in the blood (29, 30). Therefore, it is conceivable that the production of precursors for 11-KT could be one of the most important functions of CYP11B1 in the gonads. Indeed, testosterone and androstenedione are efficiently converted to the 11-KT precursors (11-OHT and 11-OHA) by ectopic expression of human CYP11B1 with ferredoxin in COS-1 cells (10). Consistent with this in vitro study, high levels of these steroids are produced in the CYP11B1-abundant adrenal gland, even though they are rarely converted to 11-keto products by low-level expression of HSD11B2 in the adrenal. However, it is probable that adrenal-derived 11-OHT and 11-OHA could be another sources of human 11-KT by the conversion in HSD11B2-expressing organs, including gonads.

There was no sexual dimorphism of human plasma 11-KT levels, even though testosterone levels were much higher in men. Similar phenomena were also observed in rodents (11). This indicates that the dominance of 11-KT production in female is conserved between rodents and humans. In mice, the abundant expression of ovarian HSD11B2 is likely responsible for this dominance (11). In

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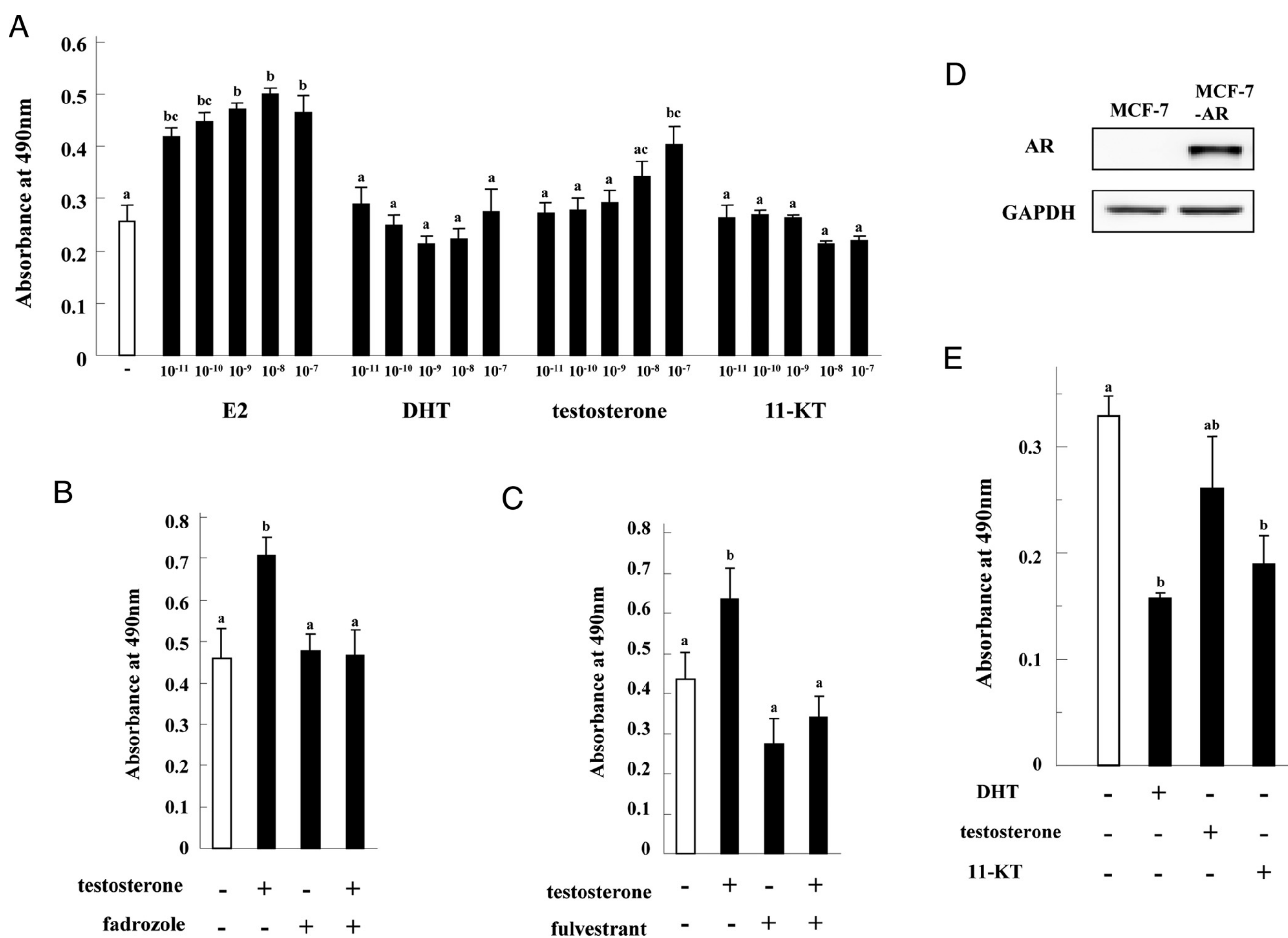

Figure 6. The effects of each androgen on the proliferation in parental and AR-introduced MCF-7 cells. A–C, The effects of each steroid hormone on the proliferation of parental MCF-7 cells. A, Cells were cultured with or without each steroid hormones at various concentrations for 6 days. Data represent the mean ± SEM of 3 independent experiments. Values marked by different letters are significantly different ($P < .05$). B and C, Effects of the aromatase inhibitor fadrozole (B) and the ER-antagonist fulvestrant (C) at 1μM on testosterone-induced (10^{-7}M) cell proliferation. Data represent the mean ± SEM of 3 independent experiments. Values marked by different letters are significantly different ($P < .05$). D and E, Cell growth inhibition by androgens in AR-introduced MCF-7 cells. D, Western blot analyses were performed with the antibodies against AR and GAPDH using lysates derived from parental and AR-introduced MCF-7 cells. E, The effects of each androgen on the inhibition of cell growth in AR-introduced MCF-7 cells. Cells were cultured with or without each androgen at 10^{-7}M for 9 days. Data represent the mean ± SEM of 5 independent experiments. Values marked by different letters are significantly different ($P < .05$).

addition, the testicular expression of Hsd11b1 was much higher than in the ovary (11). Because this 11β-HSD isoform preferentially catalyzes the reduction of 11-ketosteroid (31), the difference in its expression between sexes also results in ovarian dominance of 11-KT production. Such sexual differences in the expression of both HSD11B1 (Y. Imamichi and T. Yazawa, unpublished data) and HSD11B2 were unclear in human gonads. In addition to the level of expression, the activity of 11β-HSD enzymes is regulated by a number of factors (32, 33). It is interesting that human ovarian FF contains the selective inhibitor for HSD11B1 (34). Additionally, it was also reported that ovarian FF contains much higher concentrations of 11-OHA than plasma levels, which is converted from cortisol in granulosa cells (35). This might be another pathway for ovarian 11-KT production. Further studies are needed to evaluate the sexual dimorphism of 11-KT production in humans.

DHT, testosterone, and 11-KT can strongly activate AR-mediated transactivation, even though there are some differences, especially at lower levels. Testosterone acts not only as an androgen but also as a precursor for estrogens. It can be converted to E_2 in aromatase-expressing cells and induces estrogen-dependent phenomena. Conversely, it is probable that 11-KT is not converted to estrogen by aromatase. Although DHT is also a nonaromatizable androgen, it can be converted to 3β-diol by HSD17B7, which can bind to ERs (36). Therefore, 11-KT likely represents extremely difficult androgen to convert to an estrogenic steroid. In addition, plasma 11-KT levels are similar to testosterone levels in women at reproductive age

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and in menopausal women (9). Thus, it is possible that 11-KT plays important roles in female, especially in AR- and aromatase-expressing tissues such as ovary and breast. In a previous study, we demonstrated using mice that testosterone and 11-KT are elevated at ovulatory LH/human chorionic gonadotropin stimulation and are involved in the expression of ovulation-related genes, such as cyclooxygenase-2 and amphiregulin, in granulosa cells (19). This issue could be a subject of further investigation in human, because preovulatory follicles express CYP19A1 gene at high levels (37).

In summary, we demonstrated that 11-KT is a major androgen and produced in gonads. Because androgens are essential for reproduction and physiology, their excess and deficiency often induce pathogenesis. Then, it is possible that 11-KT could be responsible for, and the novel target of therapies against, such diseases. In addition, it might provide novel insights for elucidating ambiguous AR-mediated phenomena.

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References

1. **Mooradian AD, Morley JE, Korenman SG.** Biological actions of androgens. *Endocr Rev*. 1987;8:1–28.
2. **Miller WL, Auchus RJ.** The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. 2011;32:81–151.
3. **Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS.** Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev*. 1995;16:271–321.
4. **Lebbe M, Woodruff TK.** Involvement of androgens in ovarian health and disease. *Mol Hum Reprod*. 2013;19:828–837.
5. **Rivera-Woll LM, Papalia M, Davis SR, Burger HG.** Androgen insufficiency in women: diagnostic and therapeutic implications. *Hum Reprod Update*. 2004;10:421–432.
6. **Bachmann G, Bancroft J, Braunstein G, et al.** Female androgen insufficiency: the Princeton consensus statement on definition, classification, and assessment. *Fertil Steril*. 2002;77:660–665.
7. **Azziz R, Sanchez LA, Knochenhauer ES, et al.** Androgen excess in women: experience with over 1000 consecutive patients. *J Clin Endocrinol Metab*. 2004;89:453–462.
8. **Cohn GL, Mulrow PJ.** Androgen release and synthesis in vitro by human adult adrenal glands. *J Clin Invest*. 1963;42:64–78.
9. **Rege J, Nakamura Y, Satoh F, et al.** Liquid chromatography-tandem mass spectrometry analysis of human adrenal vein 19-carbon steroids before and after ACTH stimulation. *J Clin Endocrinol Metab*. 2013;98:1182–1188.
10. **Swart AC, Schloms L, Storbeck KH, et al.** 11β-hydroxyandrostenedione, the product of androstenedione metabolism in the adrenal, is metabolized in LNCaP cells by 5α-reductase yielding 11β-hydroxy-5α-androstanedione. *J Steroid Biochem Mol Biol*. 2013;138:132–142.
11. **Yazawa T, Uesaka M, Inaoka Y, et al.** Cyp11b1 is induced in the murine gonad by luteinizing hormone/human chorionic gonadotropin and involved in the production of 11-ketotestosterone, a major fish androgen: conservation and evolution of the androgen metabolic pathway. *Endocrinology*. 2008;149:1786–1792.
12. **Kawamoto T, Mitsuuchi Y, Toda K, et al.** Role of steroid 11 β-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. *Proc Natl Acad Sci USA*. 1992;89:1458–1462.
13. **Nishimoto K, Nakagawa K, Li D, et al.** Adrenocortical zonation in humans under normal and pathological conditions. *J Clin Endocrinol Metab*. 2010;95:2296–2305.
14. **White PC, Mune T, Agarwal AK.** 11 beta-Hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess. *Endocr Rev*. 1997;18:135–156.
15. **Imamichi Y, Mizutani T, Ju Y, et al.** Transcriptional regulation of human ferredoxin reductase through an intronic enhancer in steroidogenic cells. *Biochim Biophys Acta*. 2014;1839:33–42.
16. **Yazawa T, Inaoka Y, Okada R, et al.** PPAR-γ coactivator-1α regulates progesterone production in ovarian granulosa cells with SF-1 and LRH-1. *Mol Endocrinol*. 2010;24:485–496.
17. **Ju Y, Mizutani T, Imamichi Y, et al.** Nuclear receptor 5A (NR5A) family regulates 5-aminolevulinic acid synthase 1 (ALAS1) gene expression in steroidogenic cells. *Endocrinology*. 2012;153:5522–5534.
18. **Yazawa T, Mizutani T, Yamada K, et al.** Differentiation of adult stem cells derived from bone marrow stroma into Leydig or adrenocortical cells. *Endocrinology*. 2006;147:4104–4111.
19. **Yazawa T, Kawabe S, Kanno M, et al.** Androgen/androgen receptor pathway regulates expression of the genes for cyclooxygenase-2 and amphiregulin in periovulatory granulosa cells. *Mol Cell Endocrinol*. 2013;369:42–51.
20. **Nishimoto K, Rigsby CS, Wang T, et al.** Transcriptome analysis reveals differentially expressed transcripts in rat adrenal zona glomerulosa and zona fasciculata. *Endocrinology*. 2012;153:1755–1763.
21. **Kanda Y.** Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant*. 2013;48:452–458.
22. **Yazawa T, Imamichi Y, Miyamoto K, et al.** Regulation of steroidogenesis, development and cell differentiation by steroidogenic factor-1 and liver receptor homolog-1. *Zoological Science*. 2015;32:323–330.
23. **Andò S, De Amicis F, Rago V, et al.** Breast cancer: from estrogen to androgen receptor. *Mol Cell Endocrinol*. 2002;193:121–128.
24. **Ge RS, Dong Q, Sottas CM, Latif SA, Morris DJ, Hardy MP.** Stimulation of testosterone production in rat Leydig cells by aldosterone is mineralocorticoid receptor mediated. *Mol Cell Endocrinol*. 2005;243:35–42.
25. **Amin M, Simerman A, Cho M, et al.** 21-Hydroxylase-derived steroids in follicles of nonobese women undergoing ovarian stimulation for in vitro fertilization (IVF) positively correlate with lipid content of luteinized granulosa cells (LGCs) as a source of cholesterol for steroid synthesis. *J Clin Endocrinol Metab*. 2014;99:1299–1306.

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26. **Fru KN, VandeVoort CA, Chaffin CL.** Mineralocorticoid synthesis during the periovulatory interval in macaques. *Biol Reprod*. 2006; 75:568–574.
27. **Whirledge S, Cidlowski JA.** Glucocorticoids, stress, and fertility. *Minerva Endocrinol*. 2010;35:109–125.
28. **Hu L, Monteiro A, Johnston H, King P, O'Shaughnessy PJ.** Expression of Cyp21a1 and Cyp11b1 in the fetal mouse testis. *Reproduction*. 2007;134:585–591.
29. **Lewicka S, von Hagens C, Hettinger U, et al.** Cortisol and cortisone in human follicular fluid and serum and the outcome of IVF treatment. *Hum Reprod*. 2003;18:1613–1617.
30. **von Wolff M, Schneider S, Kollmann Z, Weiss B, Bersinger NA.** Exogenous gonadotropins do not increase the blood-follicular transportation capacity of extra-ovarian hormones such as prolactin and cortisol. *Reprod Biol Endocrinol*. 2013;11:87.
31. **Gathercole LL, Lavery GG, Morgan SA, et al.** 11β-hydroxysteroid dehydrogenase 1: translational and therapeutic aspects. *Endocr Rev*. 2013;34:525–555.
32. **Quinkler M, Johanssen S, Grossmann C, et al.** Progesterone metabolism in the human kidney and inhibition of 11β-hydroxysteroid dehydrogenase type 2 by progesterone and its metabolites. *J Clin Endocrinol Metab*. 1999;84:4165–4171.
33. **Kossintseva I, Wong S, Johnstone E, Guilbert L, Olson DM, Mitchell BF.** Proinflammatory cytokines inhibit human placental 11β-hydroxysteroid dehydrogenase type 2 activity through Ca2+ and cAMP pathways. *Am J Physiol Endocrinol Metab*. 2006;290:E282–E288.
34. **Thurston LM, Norgate DP, Jonas KC, et al.** Ovarian modulators of 11β-hydroxysteroid dehydrogenase (11βHSD) activity in follicular fluid from gonadotrophin-stimulated assisted conception cycles. *Reproduction*. 2002;124:801–812.
35. **Holownia P, Owen EJ, Conway GS, Round J, Honour JW.** Studies to confirm the source of 11 β-hydroxyandrostenedione. *J Steroid Biochem Mol Biol*. 1992;41:875–880.
36. **Törn S, Nokelainen P, Kurkela R, et al.** Production, purification, and functional analysis of recombinant human and mouse 17β-hydroxysteroid dehydrogenase type 7. *Biochem Biophys Res Commun*. 2003;305:37–45.
37. **Jeppesen JV, Kristensen SG, Nielsen ME, et al.** LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. *J Clin Endocrinol Metab*. 2012;97:E1524–E1531.

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